Prevention of Fungal Growth on Rubberwood for Toy Production

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ชื่อวิทยานิพนธ์ การยับยั้งการเจริญของเชื้อราบนไม้ยางพาราสำหรับผลิตของเล่น

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บทคัดย่อ

ไม้ยางพารา (*Hevea brasiliensis*) ใช้เป็นวัตถุดิบสำหรับการผลิตของเล่นไม้ การเจริญของเชื้อราบนผิวหน้าของเล่นไม้ยางพาราทำให้ความสวยงามของของเล่นลดลงและอาจเป็นอันตรายต่อเด็ก จึงเป็นปัญหาสำคัญสำหรับผู้ประกอบการ ในการศึกษานี้ได้แยกเชื้อราจากไม้ยางพาราและศึกษาผลของความชื้นสัมพัทธ์ต่อการเจริญของเชื้อราบนไม้ นอกจากนี้ยังได้ศึกษาประสิทธิภาพในการป้องกันการเจริญของเชื้อราบนไม้ยางพาราโดยใช้น้ำมันหอมระเหย ไคโตซาน ไซเลนและมาเลอิกแอนไฮไดรด์

ศึกษาการแยกเชื้อราจากตัวอย่างไม้ยางพาราที่มีการปนเปื้อนที่เก็บมาจากโรงงานผลิตของเล่น จัดจำแนกชนิดของเชื้อราโดยการวิเคราะห์สัณฐานวิทยาและทางพันธุกรรม พบว่าเชื้อราที่พบได้มากคือ *Aspergillus niger* PSU1 (LC127085), *Aspergillus flavus* PSU2 (LC127086) และ *Penicllium citrinum* PSU3 (LC127087) ทำการศึกษาผลของความชื้นสัมพัทธ์ต่อการเจริญของเชื้อรา *A. niger* PSU1 บนไม้ยางพารา ค่าความชื้นสมดุลของตัวอย่างไม้ที่ความชื้นสัมพัทธ์ 75, 80, 85, 92 และ 97 เปอร์เซ็นต์ หลังจากบ่มเป็นเวลา 10 วัน เท่ากับ 9.67, 10.42, 10.76, 13.69 และ 15.61 เปอร์เซ็นต์ ตามลำดับ เชื้อ *A. niger* PSU1 เจริญได้ดีที่ความชื้นสัมพัทธ์ 97 เปอร์เซ็นต์ พบการเจริญเล็กน้อยที่ความชื้นสัมพัทธ์ 92 เปอร์เซ็นต์ และไม่พบการเจริญของเชื้อราดังกล่าวที่ความชื้นสัมพัทธ์ต่ำกว่า 92 เปอร์เซ็นต์ หลังจากบ่มเป็นเวลา 8 สัปดาห์

การศึกหาค่าความเข้มข้นต่ำสุดในการยับยั้งเชื้อรา (minimal inhibitory concentration; MIC) ของน้ำมันหอมระเหยอบเชย กานพลู ยูคาลิปตัส เปปเปอร์มินท์ และ ตะไคร้หอม ต่อเชื้อราที่แยกจากไม้ยางพาราได้แก่ *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3 พบว่าน้ำมันหอมระเหยกานพลูและอบเชยมีฤทธิ์ยับยั้งเชื้อราได้ดีที่สุด ค่า MIC ต่อเชื้อราสามสายพันธุ์คือ 5 และ 2.5-10 ไมโครลิตรต่อมิลลิลิตร ตามลำดับ ทำการทดลองจุ่มตัวอย่างไม้ยางพาราในสารละลายน้ำมันหอมระเหยกานพลูและอบเชย ที่ความเข้มข้นเท่ากับค่า MIC, 2 และ 4 เท่าของ MIC และเพาะเชื้อรา *A. niger* PSU1 บนไม้ พบว่าน้ำมันหอมระเหยอบเชยที่ความเข้มข้น 2 และ 4 เท่าของ MIC ยับยั้งการเจริญของเชื้อราบนไม้ยางพาราได้นาน 12 สัปดาห์ ขณะที่น้ำมันหอมระเหยกานพลูไม่มีฤทธิ์ยับยั้งการเจริญของเชื้อราดังกล่าวบนไม้

การศึกษากิจกรรมการยับยั้งการเจริญของเชื้อรา 2 ชนิดคือ *Aspergillus niger* BAM 4 และ *Penicillium decumbens* CBS 121928 โดยไคโตซานสามชนิด ได้แก่ C1 (น้ำหนักโมเลกุล 37 กิโลดาลตัน), C2 (น้ำหนักโมเลกุล 5.4 กิโลดาลตัน) และ C3 (น้ำหนักโมเลกุล 3.5 กิโลดาลตัน) ที่ความเข้มข้น 0.063-0.5 เปอร์เซ็นต์ พบว่าไคโตซาน C1 และ C3 ความเข้มข้น 0.5 เปอร์เซ็นต์ สามารถยับยั้งเชื้อราทั้งสองชนิดได้ดีที่สุด เตรียมสารละลายไคโตซาน C1 และ C3 ที่ความเข้มข้น 1 และ 2 เปอร์เซ็นต์ นำตัวอย่างไม้มาจุ่มในสารละลายไคโตซาน 15 วินาที หรือ แช่ในสภาวะสุญญากาศที่ 60 มิลลิบาร์ 2 ชั่วโมง ทดสอบการชะละลายและวิเคราะห์ความเข้มข้นของไคโตซานในไม้ พบว่าไคโตซานยึดติดบนไม้ได้ดีทั้งในตัวอย่างไม้ที่จุ่มและแช่ในสภาวะสุญญากาศ ตัวอย่างไม้ที่แช่ในสภาวะสุญญากาศในสารละลายไคโตซาน C1 และ C3 ความเข้มข้น 1% สามารถต้านทานการเจริญของ *A. niger* BAM 4 ได้ดี อย่างไรก็ตาม ตัวอย่างไม้ที่จุ่มมีความต้านทานการเจริญของ *A. niger* BAM 4 ได้ต่ำกว่า ตัวอย่างไม้ที่จุ่มหรือแช่ในสภาวะสุญญากาศในสารละลายไคโตซานไม่สามารถต้านทานการเจริญของ *P. decumbens* CBS 121928 ได้ นอกจากนี้ยังได้ศึกษาการยับยั้งเชื้อราบนไม้โดยใช้สารละลายไซเลนความเข้มข้น 0.125 และ 0.25 โมลาร์ พบว่าตัวอย่างไม้ที่จุ่มหรือแช่ในสภาวะสุญญากาศในสารละลายไซเลนไม่มีความสามารถในการต้านทานการเจริญของเชื้อรา

นำตัวอย่างไม้ยางพารามาจุ่มในสารละลายมาเลอิกแอนไฮไดรด์ที่ความเข้มข้น 0.5-10 เปอร์เซ็นต์ พบว่าการจุ่มไม้ยางพาราในสารละลายมาเลอิกแอนไฮไดรด์เข้มข้น 2.5 เปอร์เซ็นต์ เพียงพอที่จะยับยั้งเชื้อราบนไม้ได้นานกว่า 52 สัปดาห์ การนับจำนวนเชื้อรา *A. niger* PSU1 ที่มีชีวิตบนตัวอย่างไม้ที่จุ่มในสารละลายมาเลอิกแอนไฮไดรด์พบว่าสปอร์ของเชื้อราไม่ถูกทำลาย ความชื้นของตัวอย่างไม้ที่จุ่มในสารละลายมาเลอิกแอนไฮไดรด์และตัวอย่างไม้ที่ไม่จุ่มไม่มีความแตกต่างกัน ทดสอบการชะละลายนาน 60 นาที พบว่าความเข้มข้นของมาเลอิกแอนไฮไดรด์ในตัวอย่างน้ำชะละลายเท่ากับ 0.02 มิลลิกรัมต่อลูกบาศก์มิลลิเมตรและการทดสอบยับยั้งเชื้อราพบว่าตัวอย่างน้ำชะละลายดังกล่าวไม่มีฤทธิ์ยับยั้งเชื้อรา อย่างไรก็ตามตัวอย่างไม้หลังจากการชะละลายยังต้านทานเชื้อราได้ดี เมื่อทดสอบ diffusion test พบว่าตัวอย่างไม้ที่จุ่มในสารละลายมาเลอิกแอนไฮไดรด์ไม่มีฤทธิ์ยับยั้งเชื้อรา การทดสอบความเป็นพิษต่อเซลล์เพาะเลี้ยงพบว่าน้ำชะละลายจากตัวอย่างไม้ที่จุ่มและไม่จุ่มในสารละลายมาเลอิกแอนไฮไดรด์มีค่าความเป็นพิษต่อเซลล์เท่ากัน การศึกษาโดยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดพบว่าผิวหน้าของไม้ที่จุ่มในสารละลายมาเลอิกแอนไฮไดรด์มีความเรียบมากกว่าของตัวอย่างไม้ที่ไม่จุ่ม นำตัวอย่างไม้ไปทดสอบความคงทนต่อสภาพอากาศ เมื่อทดสอบการต้านทานเชื้อราพบว่าตัวอย่างไม้ไม่มีความทนต่อเชื้อรา ศึกษาการใช้วิธีจุ่มตัวอย่างไม้ในสารละลายมาเลอิกแอนไฮไดรด์เทียบกับวิธีสเปรย์ ลดอุณหภูมิการอบตัวอย่างไม้หลังจากจุ่มหรือสเปรย์จาก 90 เป็น 70 และ 50 องศาเซลเซียส เปรียบเทียบการแช่กับไม่แช่ตัวอย่างในน้ำหลังจากอบ

พบว่าตัวอย่างไม้ที่จุ่มที่อุณหภูมิ 70 และ 50 องศาเซลเซียสและไม่แช่ในน้ำหลังจากอบ สามารถทนต่อการเจริญของเชื้อราได้ดีกว่าตัวอย่างที่สเปรย์และมีการแช่

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ABSTRACT

Rubberwood (*Hevea brasiliensis*) is used as a material for production of toys. Growth of mold on the surface of wooden toys decreases their aesthetic value and might be harmful for children. This is a major concern for wooden toy manufacturers. In this work, isolation of molds from rubberwood and the effect of relative humidity on mold growth on wood were studied. Moreover, the effectiveness of essential oils, chitosan, silane and maleic anhydride (MA) agasint fungal growth on rubberwood were investigated.

Molds were isolated from contaminated rubberwood collected from the wooden toy factory. Isolated molds were identified by examining their morphological and molecular traits. Three dominant molds which colonized rubberwood were *Aspergillus niger* PSU1 (LC127085), *Aspergillus flavus* PSU2 (LC127086) and *Penicllium citrinum* PSU3 (LC127087). Effect of relative humidity (RH) on the colonization of *A. niger* PSU1 on wood was studied. The equilibrium moisture contents (EMCs) of rubberwood at 75, 80, 85, 92 and 97% RH, 25 °C after 10 days were 9.67, 10.42, 10.76, 13.69 and 15.61%, respectively. The heavy growth of *A. niger* PSU1 on wood was observed at 97% RH and the light growth was found at 92% RH but this fungus could not grow when RH was lower than 92% after 8 weeks incubation.

The minimal inhibitory concentration (MIC) of five commercial essential oils (cinnamon, clove, eucalyptus, peppermint and lemongrass oils) was investigated against molds isolated from rubberwood (*A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3). Clove and cinnamon oils showed strong antifungal activity against all these fungi with the MIC value of 5 µl ml^{-1} and 2.5-10 µl ml^{-1}, respectively. Rubberwood samples were dipped in clove or cinnamon oil solutions at MIC, 2 and 4-MIC and inoculated with *A. niger* PSU1 spores. Only cinnamon oil at 2 and 4-MIC showed strong inhibition against growth of *A. niger* PSU1 on rubberwood for at least 12 weeks while clove oil did not inhibit the fungus on rubberwood.

The antifungal activities of chitosan samples, C1 (Mw 37 kDa), C2 (Mw 5.4 kDa) and C3 (Mw 3.5 kDa), were tested against *Aspergillus niger* BAM 4 and *Penicillium decumbens* CBS 121928 at concentrations ranging from 0.063 to 0.5% w/v. Chitosan C1 and C3 at 0.5% exhibited strong antifungal activity against both molds. The solutions of chitosan C1 and C3 were made at 1 and 2%. Rubberwood were either dipped in each chitosan solution for 15 s or vacuum treated at 60 mbar, 2 h. After the leaching test, the content of chitosan in wood was well retained in both dipped and vacuum treated wood. The vacuum treated wood with chitosan C1 and C3 at 1% w/v had strong resistance against *A. niger* BAM 4. However, dipped rubberwood with chitosan solutions showed lower resistance against *A. niger* BAM 4. On the other hand, both dipped and vacuum treated rubberwood with chitosan had no resistance against *P. decumbens* CBS 121928. Additionally, treatment of rubberwood with silane was studied at 0.125-0.25 M silane solution. Both dipped and vacuum treated wood with silane showed no resistance to fungal growth.

Rubberwood samples were treated with 0.5-10% MA solutions. Treatment of rubberwood with 2.5% MA was adequate to prevent growth of molds on wood for up to 12 weeks. The viable count of *A. niger* PSU1 on the MA treated wood indicated that fungal spores were not killed. The moisture contents of MA treated and untreated wood samples were not significantly different. The concentration of MA released from treated wood in the leachate was 0.02 mg mm^{-3} after 60 min leaching in water. However, after leaching MA treated wood still had high resistance to mold growth. Agar well diffusion showed that the leachate had no antifungal activity. The diffusion test showed that MA treated wood slice showed no fungal growth inhibition. The leachates from both MA treated and untreated wood samples had similar cytotoxic effect. The SEM study showed that the surface of MA treated wood had almost smooth surface while the untreated wood showed rough surface. The weathering test was conducted and weathered samples were seriously infected. Treatment of rubberwood with MA by dip and spray treatment together with decreasing the reaction temperature from 90°C to 70°C and 50 °C were studied. Additionally, with and without soaking stage after the heat treatment were compared. The results showed that dip treated samples reacted with MA at 70 and 50 °C and without soaking stage had strong resistance against molds compared to the sprayed samples.

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Kittiya Oldertrøen

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LIST OF ABBREVIATIONS AND SYMBOLS

AEAPTMEOS	[3-(2-Aminoethylamino)propyl]trimethoxysilane
DNA	Deoxyribonucleic Acid
DP	Degree of polymerization
EMC	Equilibrium moisture content
F_A	Fraction of acetylation
GC–MS	Gas chromatography–mass spectroscopy
H-NMR	Nuclear magnetic resonance spectrometry
HPLC	High performance liquid chromatography
ICP	Inductively Coupled Plasma
MA	Maleic anhydride
MC	Moisture content
MEA	Malt extract agar
MFC	Minimal fungicidal concentration
MIC	Minimal inhibitory concentration
Mw	Molecular weight
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RH	Relative humidity

LIST OF PAPERS AND PROCEEDINGS

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SUMMARY OF CONTENTS

CHAPTER I

Introduction and review of literature

Introduction

Rubberwood is obtained from rubber trees (*Hevea brasiliensis*) and available from agricultural plantations. After planting, latex can be gathered economically from rubber trees for 25-30 years before the trees are cut and the fields are replanted (Teoh *et al.*, 2011). Rubberwood lumber is a by-product of rubber tree and nowadays, is a useful raw material for value-added wood products manufacturing, particularly furniture, toys and packing materials (FAO, 2001; Royal Forest Department of Thailand, 2005; Teoh *et al.*, 2011). The attractive properties of rubberwood is its creamy color, good machining and finishing properties together with its environmentally-friendly status (Ratnasingam and Grohmann, 2014).

Wooden toys made from rubberwood are successful use of by-product of the rubber tree. However, rubberwood is extremely susceptible to the colonization of mold on the surface because of its high starch and low wood extractive content (Peters *et al.*, 2002; Akhter, 2005; Wong *et al.*, 2005). Common molds *Aspergillus* spp. and *Penicillium* spp. have been reported to grow rubberwood (Matan and Matan, 2008; Matan *et al.*, 2009). These fungi consume the carbohydrates (e.g., sugar, starch) in the wood as a source of nutrients. The mold causes discoloration of the wood surface by their pigmented spores and/or mycelia, but they generally do not damage the wood structure (Hihara *et al.*, 2013). Non-preservative treated rubberwood is used as raw material for wooden toy manufacturing. Moreover, rubberwood toys are not treated with any preservative so they are very susceptible to mold attack. Mold growth on wooden toys can affect children's health. Traditional wood protection methods employ chemicals are potential health risks and toxic to the environment. Therefore, finding a less toxic and cost effective rubberwood protecting method is urgently needed.

Utilizing natural active compounds to suppress fungal growth has proved to be a powerful alternative. Essential oil is an interesting plant bioactive compound, possessing important antimicrobial, antifungal, antiviral and antioxidant activity (Kremer *et al.*, 2014). Many studies have reported on antifungal activities of essential oils in food and pharmaceutical applications and in other areas (Daferera *et al.*, 2000; Ahmad and Beg, 2001; Wang *et al.*, 2005; Inouye *et al.*, 2007; Turgis *et al.*, 2012). Moreover, essential oils were reported to be highly inhibitory to wood decay

fungi on nutrient medium (Wang *et al.*, 2005) and also inhibited mold growth on wood (Yang and Clausen, 2007; Matan and Matan, 2008; Matan *et al.*, 2009; Singh and Chittenden, 2010). Besides, essential oils are generally recognized as safe (GRAS) (Prakash *et al.*, 2015). Therefore, they are one of the most assuring groups of natural compounds for the development of safer antifungal agents on wood protection applications.

Chitosan is a natural preservative and has had useful applications in many areas important for human well-being and health, such as pharmaceutical, food, crop protection and a range of other processes (Simionato *et al.*, 2006; Chantarasataporn *et al.*, 2014; Plainsirichai *et al.*, 2014; Sarvaiya and Agrawal, 2015). Several studies have shown chitosan and its derivatives to be an effective antibacterial (Kittur *et al.*, 2005; Feng and Xia, 2011) and antifungal agent (Chittenden *et al.*, 2004; Qiu *et al.*, 2014). One of the earliest applications of chitosan was in wood protection (Alfredsen *et al.*, 2004; Eikenes *et al.*, 2005a). Chitosan is biodegradable and has excellent biocompatibility and almost no toxicity to humans and animals (Rabea *et al.*, 2003). For these reasons, chitosan and its derivatives are one of interesting agent for using as a low toxic wood protectant.

Chemical wood modification based on the hydroxyl groups is the approach to make wood hydrophobic and the access to water is prevented or reduced thus growth of microorganisms is decreased (Rowell, 2005). At least three mechanisms have been presented to explain the protection provided by wood modification: (1) Changes of the cell wall polymers that become unrecognizable for fungal enzymes; (2) a reduction of the moisture content; and (3) a lower micro-pore size in the wood cell wall (Li *et al.*, 2011). Anhydrides and silanes are an example of the chemicals that are usually used for chemical wood modification (Hill, 2006). Previous research works have reported that wood modified with anhydride and silanes showed improved durability against decay fungi (Fujimoto, 1992; 1995; Hill *et al.*, 2004; Panov and Terziev, 2009).

In this study, the antifungal activities of essential oils and chitosan on rubberwood were reported. The resistance of fungal growth on wood modified with maleic anhydride or silane were also studied. Moreover, the leaching test was conducted to evaluate the leaching of preservative from wood and to determine the

leaching effects that would occur. The simple treatment methods—dip, spray and vacuum treatment were applied for wood protection and the results were compared. The results obtained from the laboratory scale were applied to the industrial scale.

Review of literature

1. Chemistry of wood

Wood is a complex material of plant origin and the primary constituent of trees. After cutting and drying, wood is used for many different utilities. Wood has been a primary construction material since humans began building and remains in fruitful use today. In addition to building, wood has found common use in applications including furniture, weapons, musical instruments, domestic tools and many others (Bulian and Graystone, 2003).

The main constituent of wood is organic compounds. Wood is composed of the macromolecular components particularly the polysaccharides, cellulose and hemicelluloses as well as lignin. It also contains the low molecular weight substances consist of aromatic phenolic compounds, aliphatic compounds and terpenes (Windeisen and Wegener, 2009). The chemical composition of hardwood and softwood are shown in Table 1.1.

Table 1.1 General chemical composition of wood

Constituent	Hardwood (%)	Softwood (%)
Cellulose	40-50	40-50
Hemicelluloses	25-35	20-30
Lignin	20-30	25-30
Extractives	0.5-10	0.5-10
Inorganics (ash)	0.1-1	0.1-1

Source: Windeisen and Wegener (2009).

2. Cellulose

Cellulose is an organic polymer belonging to the polysaccharides family. The monomer is a simple sugar, namely glucose. The structure of cellulose is displayed in Figure 1.1, is linear being composed, for plant-derived cellulose, of between 7500 and 15,000 glucose units (Eichhorn *et al.*, 2009). The glucose unit is in the β-form connected to each other by β-1, 4 glycosidic bonds (Figure 1.1). Cellulose

is the major constituent of the cell walls of wood fibers. It is a highly polar substance due to the presence of three hydroxyl groups for every structural unit (Bulian and Graystone, 2009).

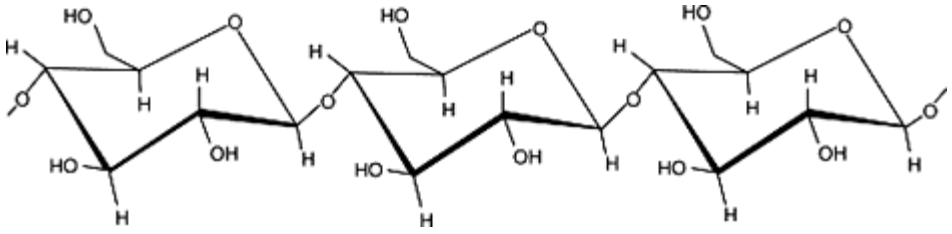


Figure 1.1 The structure of cellulose.
Source: Bulian and Graystone (2009).

3. Hemicellulose

Hemicellulose is a branched carbohydrate copolymer in which the monomers are different sugars. Xylan, glucomannan and galactoglucomannan are predominant types (Figure 1.2). The hemicelluloses found in wood are linear polysaccharides of moderate size with an average degree of polymerization ranged from 150-200 or greater (Rivers and Umney, 2003).

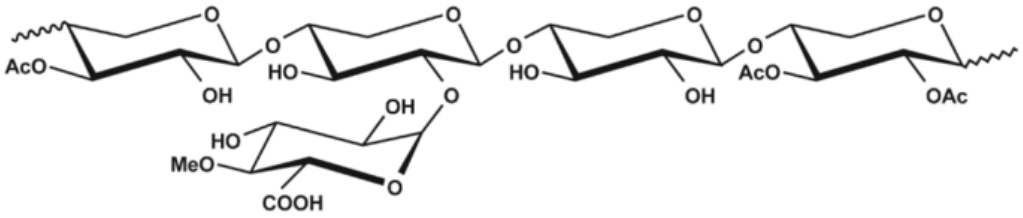


Figure 1.2 The structure of xylan.
Source: Windeisen and Wegener (2009).

4. Lignin

Lignin is the third most plentiful natural polymer present in nature after cellulose and hemicellulose (Buranov and Mazza, 2008). It is an amorphous polymer consisting of phenylpropane units and their precursors are three aromatic alcohols (monolignols) namely p-coumaryl, coniferyl and sinapyl alcohols (Figure 1.3). Lignin

is mainly found within the secondary cell wall and is chiefly responsible for the stiffness of dry wood (Bulian and Graystone, 2009).

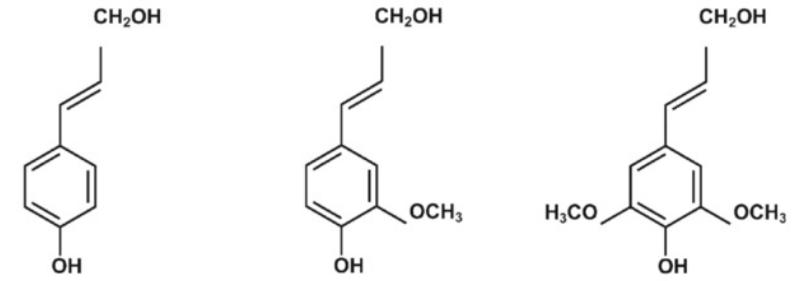


Figure 1.3 Basic units of lignin.
Source: Windeisen and Wegener (2009).

5. Rubberwood

Rubberwood is considered to be a softwood (Thai Royal Forest Department, 2012). The structural elements within rubberwood consist of 61.5% fibers, 9.5% vessels and 29.0% parenchyma cells (Mohd Nor, 1999). Fresh, sawn rubberwood is white to creamy in color, sometimes with a pinkish tinge and has a fairly straight grain. It turns yellowish after seasoning. Heartwood and sapwood are not distinguishable. Pores are large and scattered and show radially and tangentially as brown lines (Killmann, 1992). The density of rubberwood is not uniform and its mechanical properties vary longitudinally, radially and tangentially (Mohd Shukari, 1999). Air-dried rubberwood has a density of 640 kg m^{-3}, with a moisture content of 17.2% and modulus of rupture (MOR) and modulus of elasticity (MOE) values at 66 N mm^{-2} and 9240 N mm^{-2}, respectively (Teoh *et al.*, 2011).

Plantation-grown rubber trees between 25-30 years of ages which the production of latex is unprofitable have been cut for the production of lumber (Balsiger *et al.*, 2000). Sawing of rubberwood is directly correlated to the quality of wood production and profits of enterprises. The sawing is the most important process, the core is to process the lumber in agreement with enterprises standard or meet the needs of consumers (Yongdong *et al.*, 2007). An economical schedule for the industrial-scale treatment of rubberwood using boron compounds in the form of disodium octaborate

tetrahydrate ($Na_2B_8O_{13}\cdot 4H_2O$), disodium tetraborate decahydrate ($Na_2B_4O_7\cdot 10H_2O$) and boric acid (H_3BO_3) has been developed, particularly for indoor applications, to protect from insects borers and fungi (Mohd Dahlan *et al.*, 1999; De Vis *et al.*, 2006).

6. Wooden toys processing

The general step of wooden toy processing of the Plan Creations Co., Ltd. is displayed in Figure 1.4.

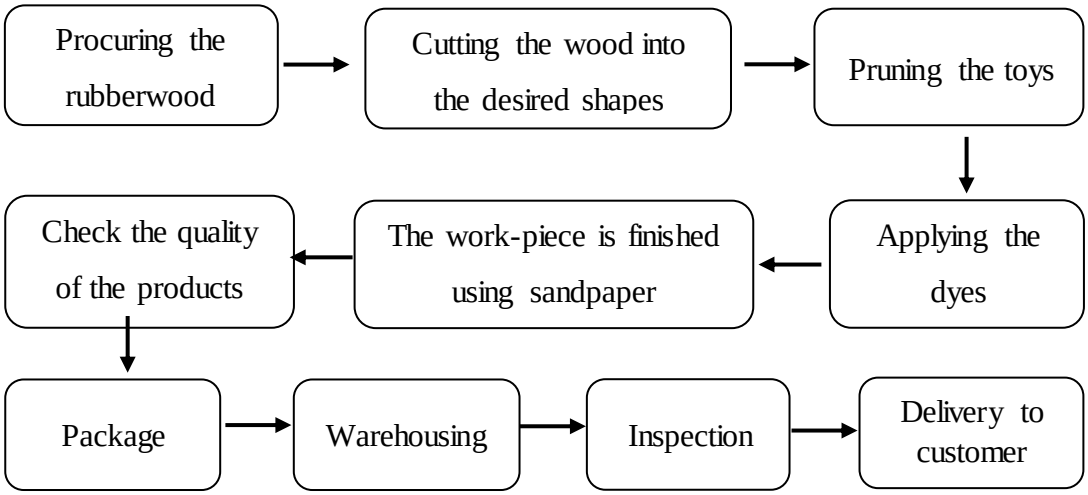


Figure 1.4 Wooden toy production process.

Non-preservative treated rubberwood is used as raw material for wooden toy processing. Wood materials are stored in the warehouse before cutting into the desired shapes according to the designs. Wood pieces are pruned on the lathe. This step makes the surfaces of wood materials smoothened for the process of coloring. Wood pieces are colored by spraying or dipping method using water based dyes and left to dry. The coloring process depends on the design of wooden toys. Some wooden toy products are not pained or colored with dyes. The semi-manufactured products are finished using sandpaper before the products are assembled and joint. Wooden toy assembly technique often uses wooden screws or glue to join the various toy components and to ensure the functionality of the toy. Wooden screws are commonly used in wooden toy assembly because they are fitting for toys with moving parts or fine mechanical components. Non-formaldehyde glue is also used to join any parts of toys

and it gives a flat surface, quick and easy and also reduce swells and shrinks problem of wooden screw. The qualities of wooden toys are checked before packing. Cardboard packing boxes are common packaging materials. These containers used for delivering products are also often labeled or imprinted with company logos. Wooden toys are stored in a warehouse stabilized at 60% RH and 20 °C. Wooden toys are final inspected to ensure the quality of the products before delivering.

6.1 Safety in wooden toy

Toy safety is the practice of assuring that toys, especially those made for children, are safe, usually through the application of set safety standards. In many countries, commercial toys must be able to pass safety tests in order to ensure a high level of protection of children against risks caused by chemical substances in toys and the use of dangerous substances.

- **United States regulations**

In August 2008, the Consumer Product Safety Improvement Act was passed, which limits the amount of lead and phthalates that may be contained in children's toys (ages 12 and under). The legislation reduces the limit of lead allowed in surface coatings or paint to 100 ppm effective on 14 August 2011 and as of 10 February 2009, any children's toy that can be put in a child's mouth or a childcare article that contains the phthalates levels higher than 0.1 percent is prohibited (United States Consumer Product Safety Improvement Act, 2008).

- **European regulations**

In Europe, the comprehensive legislation addressing toy safety is the European Union (EU) Toy Safety Directive (European Commission, 2009). This directive has been used for toys imported into or toys manufactured within the EU since July 2013. The toy safety directive provides for harmonized EU-wide standards on physical and mechanical properties, flammability, chemical properties and electrical properties. The substances and mixtures classified as carcinogenic, mutagenic or toxic for reproduction (CMR) in toys, in components of toys or in micro-structurally distinct parts of toys are not allowed. Additionally, the using of nitrosamines and nitrosatable

substances in toys intended for use by children under 36 months or in other toys intended to be placed in the mouth is prohibited.

7. Molds

Molds are eukaryotic microorganisms. In other word, they have membrane-bound nuclei containing several chromosomes and a range of membrane-bound cytoplasmic organells (mitochondria, vacuoles etc.). Other characteristics of mold include: cytoplasmic streaming, DNA that contains non-coding region called introns, membrane that regularly contains sterols and ribosome of the 80S type in contrast to the 70S ribosome of bacteria (Deacon, 1997). Molds grow best in moist environment, but they are found terrestrially wherever organic material is available. They require moisture to grow and they can acquire water from a humid atmosphere as well as from the medium or which they live (Engelkirk and Engelkirk, 2010).

Molds are multicellular (Campbell and Johnson, 2013). The body composes of long, branched, threadlike filament termed hyphae. Hyphae comprise of tubular cell walls surrounding the plasma membrane of the cells. A typical characteristic of mold cell walls is the presence of chitin (Dube, 2009). In most molds, hyphae are divided by crossed walls, named septa, into individual cells containing one or more nuclei (Figure 1.5). The septa of many molds are punctured by a pore that may be large enough to permit organelles to flow from cell to cell. Some molds called coenocytic mold, lack septa. In this species, nuclear division is not followed by cytoplasmic division. In consequence, a coenocytic mold is one elongated, multinucleated, giant cell (Figure 1.5). As hyphae grow, they form a tangled mass or tissue-like network, called mycelium (Solomon *et al.*, 2011).

7.1 Growth of molds on rubberwood

Molds consume the carbohydrates (e.g., sugar, starch) in the wood as a source of nutrients. They normally grow on wood surface and utilize simple sugars and starch present in cell lumens but they are unable to depolymerize the cellulose and lignin or hemicellulose in the cell walls of wood (Hihara *et al.*, 2013). As containing rich starches and other nutrients, rubberwood is very susceptible to colonization of mold. The development of molds occurs quickly specially under extreme moisture con-

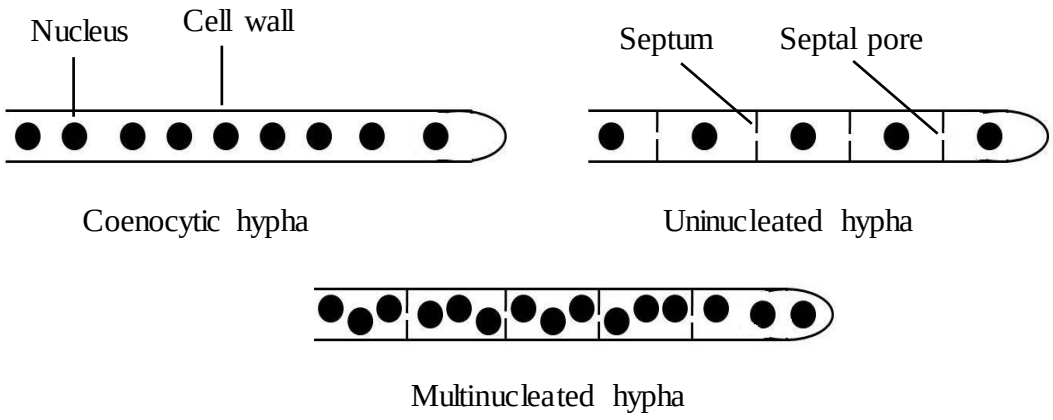


Figure 1.5 Three types of mold hypha.
Source: Modified from Solomon *et al.* (2011).

ditions. Previous studies have demonstrated that the fungal genera detected from rubberwood are predominantly *Penicillium* and *Aspergillus* (Kelin 1999; Matan and Matan, 2008; Matan *et al.*, 2009). Recently, Seephueak *et al.* (2011) identified 461 fungal species from the decaying rubberlogs from Thailand comprising 384 anamorphic taxa, 46 Ascomycota and 31 Basidiomycota. The dominant wood degrading fungi were, for example, *Bactrodesmium rahmii* and *Botryodiplodia* sp.

There are many factors affecting growth of molds on rubberwood. Likes other wood materials, molds can colonize and multiply on rubberwood materials if adequate nutrients and moisture are presented. In addition, many other factors such as temperature, spore level and fungal species also affect growth of molds (Pasanen *et al.*, 2000; Murtoniemi *et al.*, 2003; Nielsen *et al.*, 2004; Yang and Clausen, 2007). Each factor in nature does not work individually but reinforce or weaken themselves mutually (Schmidt, 2006). The relative humidity (RH) also affects growth of molds on rubberwood since moisture content in a piece of wood is related to RH (Jain *et al.*, 2009). Relative humidity of the atmosphere defines how much bound water in wood (Bulian and Graystone, 2003). The humid atmosphere in rainy season makes perfect conditions for mold growth on rubberwood and the number of infected rubberwood increased compared with in dry season. The average RH of southern part of Thailand in rainy season and summer are 87 and 76%, respectively (Thai Metrological Department, 2014). In addition, temperature exerts an influence on molds largely via its effects on enzyme-catalyzed reactions. The favorable temperatures on growth of

molds on a common wood substrate were usually ranging between 10-32 ºC and the optimum was about 21-29 ºC (Meier *et al.*, 2010). Besides, the nutrient content of the substrate on which the mold grows, is the important influence factor for mold development. In wood materials, however, there are smaller amounts of nutrients available to the fungus, compared with the complete medium and the nutrients may be more non-degradable, depending on the substrate. Apart from some mineral nutrients and trace elements, carbonaceous and nitrogenous nutrients are of predominant importance. With the help of their enzymes, molds can decompose substrates and transform them into utilizable matters (Sedlbauer, 2001).

Fortunately, growth of mold on wood surface does not change the strength of wood. Molds produce pigments and color spores on wood surface which reduces the aesthetic quality of wood and shows the appearance of damage on wood (Mansour and Salem, 2015). Mold growth on wooden products such as toys, furniture and kitchen wares are considered to be undesirable phenomenon. This incident causes significant economic loss and is a major concern for manufacturers as well as consumers. Moreover, mold growth can also potentially trigger health related issues.

8. Natural wood preservatives

Some wood species have natural decay resistance of the heartwood but some species are nonresistant. When extra protection is needed to protect wood from biological degradation, chemical preservatives are applied to the wood either by pressure or non-pressure process. The objective of adding wood preservatives is to obtain long-term effectiveness for the wood products (Rowell, 2005). In recent years, important effort has been made to find natural antimicrobials that can inhibit fungi and fungal growth on wood in order to improve quality and shelf-life. Similarly, consumers have become concerned about the safety of synthetic preservatives used in wood. As a result, there is increasing demand for natural products that can serve as preservatives used in wood (Wu *et al.*, 2011; Mohareb *et al.*, 2013; Pánek *et al.*, 2014).

8.1 Essential oils

Essential oils are aromatic oily liquid obtained from various plant parts,

such as bark, wood, leaves, seeds and fruits. The most commonly used method for production of essential oils is steam distillation (Burt, 2004). The two major components of essential oils are terpenoids and phenolics showing lipophilic nature (Prakash *et al.*, 2015). These components are principally responsible for the antimicrobial properties of essential oils (Cosentino *et al.*, 1999). The structural formulae of selected components are presented in Figure 1.6.

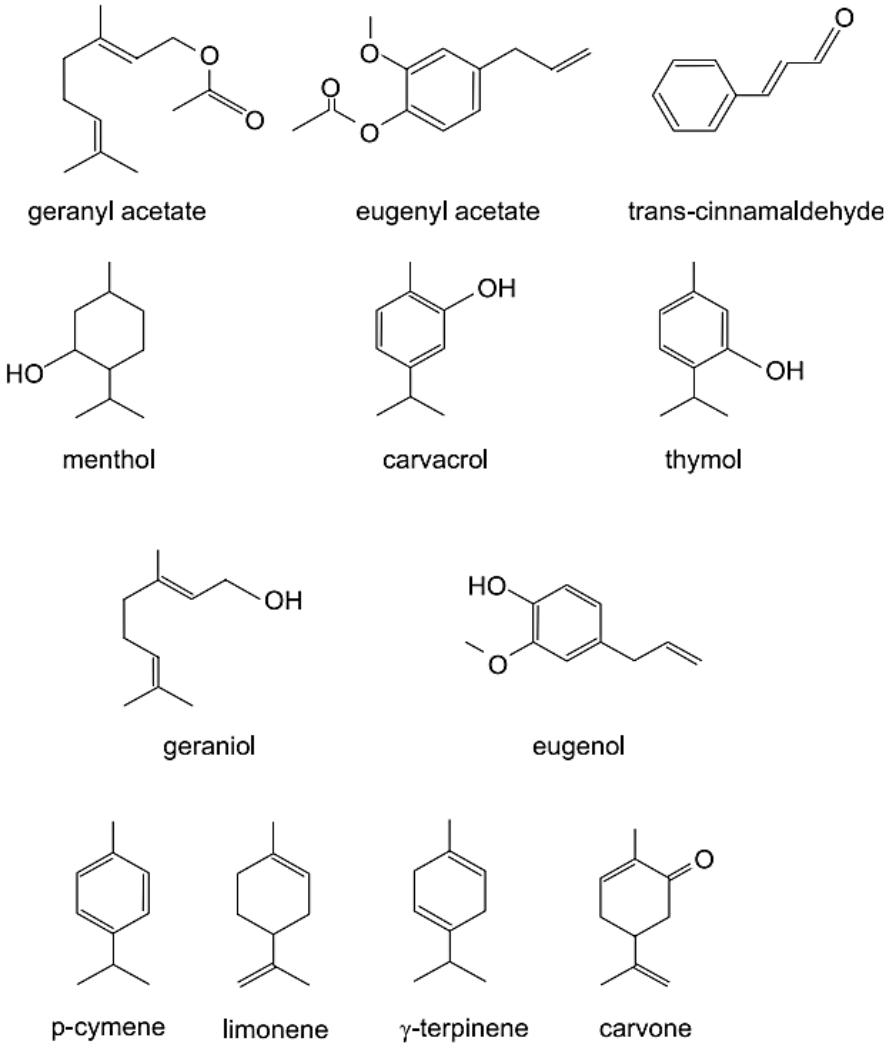


Figure 1.6 Structural formulae of selected components of essential oils.
Source: Burt (2004).

Because of the great number of constituents, essential oils seem to have no specific cellular targets (Carson *et al.*, 2002). As typical lipophiles, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of different layers of

polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage. In eukaryotic cells, essential oils can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential, affect ionic Ca^{++} cycling (Richter and Schlegel, 1993; Novgorodov and Gudz, 1996; Vercesi *et al.*, 1997) and other ionic channels and reduce the pH gradient, affecting the proton pump and the ATP pool. It seems that chain reactions from the cell wall or the outer cell membrane invade the whole cell, through the membranes of different organelles like mitochondria and peroxisomes (Fukumoto and Mazza, 2000; Sakihama *et al.*, 2002; Burt, 2004; Barbehenn *et al.*, 2005).

Essential oils have been used in food, seasonings, cosmetics and medical industries (Zhang *et al.*, 2016). Some essential oils such as cinnamon and eucalyptus have been placed under Generally Recognized as Safe (GRAS) category by Food and Drug Authority of USA (USEPA, 1993; Tzortzakis, 2009). There have been a number of reports of essential oils that inhibit growth of molds. In recent past, the antifungal activities of clove, eucalyptus and peppermint oils were reported against major molds found on rubberwood (Matan *et al.*, 2009; Matan and Matan, 2011).

8.2 Chitosan

Chitosan is the N-deacetylated derivative of chitin and is a polymer of glucosamine (β(1–4)-linked 2-amino-2-deoxy-D-glucose) and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) (Figure 1.7). Chitosan is produced through hydration (i.e. amide hydrolysis under alkaline conditions (concentrated NaOH)) by removing an acetate moiety from chitin or through enzymatic hydrolysis in the presence of chitin deacetylase (Shukla *et al.*, 2013) (Figure 1.8). Chitosan is a positively charged polymer with great potential in broad range industries such as pharmaceutical, medical and agricultural industries (Hernández-Lauzardo *et al.*, 2008). In the food

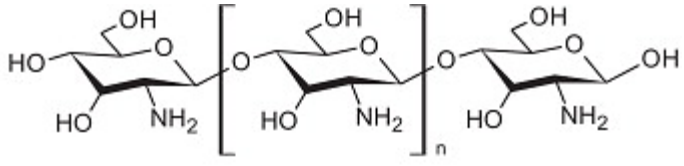


Figure 1.7 Structure of chitosan.
Source: Shukla *et al.* (2013).

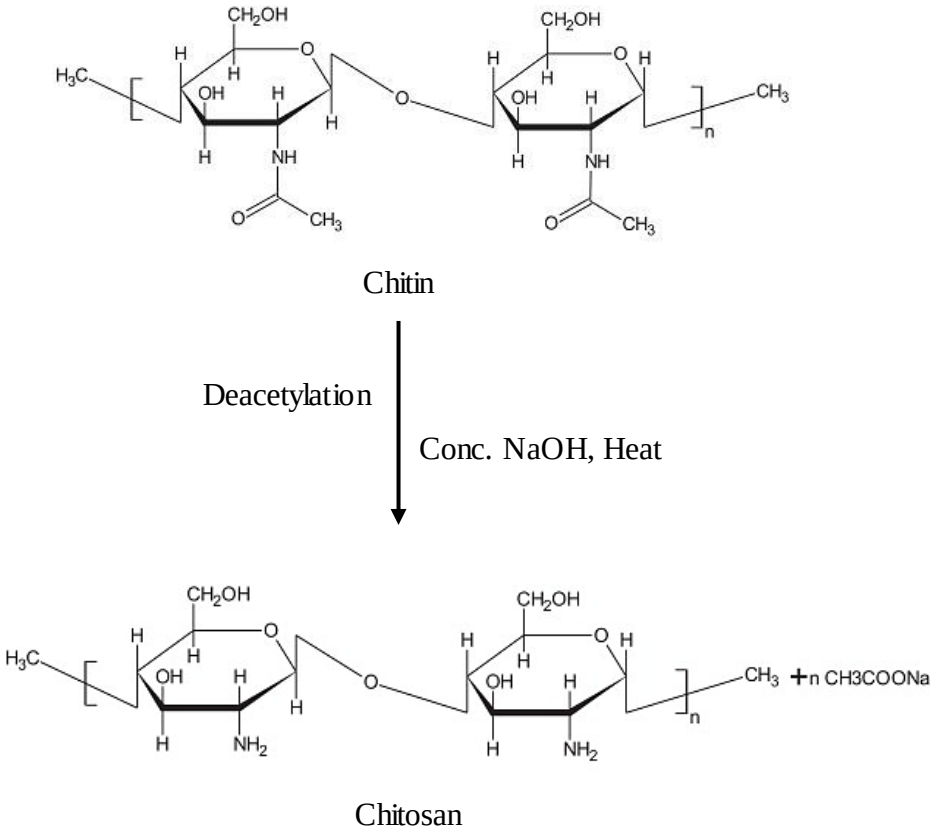


Figure 1.8 Deacetylation of chitin to chitosan.
Source: Shukla *et al*. (2013).

industry, chitosan (ChitoClear product) has been marked as GRAS in the US for use in foods in general (Baldrick, 2010). Besides, chitosan is listed as a food additive in Japan (Tago *et al.*, 2007) as well as Italy and Finland (Illum *et al*., 2001). The antifungal activity of chitosan has been considered in a wide variety of microorganisms. There is strong evidence that the mycelial growth of fungi can be retarded or inhibited when the fungal growth media are amended with chitosan. For example, growth of fungi such as *Fusarium oxysporum*, *Rhizopus stolonifer* and *Penicillium digitatum* was completely inhibited by chitosan as 3% concentration. In addition, effective control of decay fungi on rubberwood was achieved using chitosan (Alfredsen *et al*., 2004; Eikenes *et al*., 2005a). The exact mechanism of the antimicrobial action of chitosan is still unknown, but different mechanisms have been proposed. Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents (Rabea *et al*., 2003). Chitosan also acts as a chelating agent that selectively binds trace metals and thereby

inhibits the production of toxins and microbial growth (Cuero *et al.*, 1991). It also activates several defense processes in the host tissue, acts as a water binding agent and inhibits various enzymes (El Ghaouth *et al.*, 1992). Binding of chitosan with DNA and inhibition of mRNA synthesis occurs through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of mRNA and proteins (Sudarshan *et al.*, 1992).

9. Chemical modification of wood

The physical properties of any material are determined by its chemical structure. Wood contains an abundance of chemical groups called free hydroxyls. Free hydroxyl groups readily absorb and release water according to changes in the climatic conditions to which they are exposed. It is also believed that the digestion of wood by fungal enzymes initiates at the free hydroxyl sites which is one of the reasons why wood is susceptible to biodegradation (Rowell, 2005). An alternative to preservation of wood is chemical modification, which changes the wood structure and wood chemistry so that the wood becomes less susceptible to biodegradation (Lande *et al.*, 2004). Chemical modification of wood is defined as the reaction of a chemical reagent with the wood polymeric constituents, resulting in the formation of a covalent bond between the reagent and the wood substrate (Windeisen and Wegener; 2009). Thus, such a chemical reaction results in a lower capacity in water absorption of the chemically modified wood, with lower equilibrium moisture content at a specified atmospheric relative humidity, compared with untreated wood (Teaca *et al.*, 2014).

9.1 Chemical modification of wood with silanes

Silanes are silicone compounds with general formula Si_nH_{2n+2}. They are applied in plastics, textiles, buildings and papers. They provide the basis, for example, for hydrophobation of ceramics, scratch-resistant surfaces and anti-graffiti coatings or as adhesion promoters between organic and inorganic materials (Donath *et al.*, 2006). Several studies were promised the effectiveness of silanes in protecting wood against fungal decay. Chemical wood modification with silanes results in a change of hygroscopic properties of wood, thus reducing negative effects of exposure to water and as a

consequence it protects against fungal development (Sèbe and Brook, 2001; Hill *et al.*, 2005; Tingaut *et al.*, 2005; Donath *et al.*, 2006; Panov and Terziev, 2009). If silane monomers are used for wood impregnation, they polymerize *in situ* and do not leach out from the wood during outdoor use (Hill *et al.*, 2005).

Silanes are unstable in water. A basic reaction of silanes is called the sol-gel process. Silanes can be hydrolyzed to silanols which subsequently condense to three dimensional structures. Condensation begins while hydrolysis is in process which results in the formation of the colloid oligomeric particles called sols. Further reaction causes cross-linking of sol particles so that highly condensed gels are formed (sol-gel process). The silanol groups of silanes can react with hydroxyl groups of cell wall polymers forming a covalent bond between the silicon compound and cell wall polymers (Donath *et al.*, 2004). Figure 1.9 shows a scheme for the sol-gel deposition of alkoxysilane on the wood surface, causing covalent bonds with the wood surface and resulting in hydrophobication.

9.2 Chemical modification of wood with anhydrides

The chemical modification of wood using di- and tricarboxylic acid anhydrides has been reported (Papadopoulos and Hill, 2002; Iwamoto and Itoh, 2005). Treatment of wood using carboxylic acid anhydrides is accomplished by forming a co covalent bond between the anhydride and cell wall polymers and without leaving toxic residues within the wood (Papadopoulos *et al.*, 2008). Wood modification with linear chain anhydrides produces carboxylic acid which is undesired by-product and this must be removed from the wood following the modification. However, wood modification with cyclic anhydrides such as succinic anhydride (SA), maleic anhydride (MA) or phthalic anhydride (PA) does not yield a by-product (Figure 1.10) (Hill and Mallon, 1998). Fujimoto (1992; 1995) and Iwamoto and Itoh (2005) showed that MA treated wood samples had improved durability against different standard fungal species e.g., *Fomitopsis palustris* and *Trametes versicolor*.

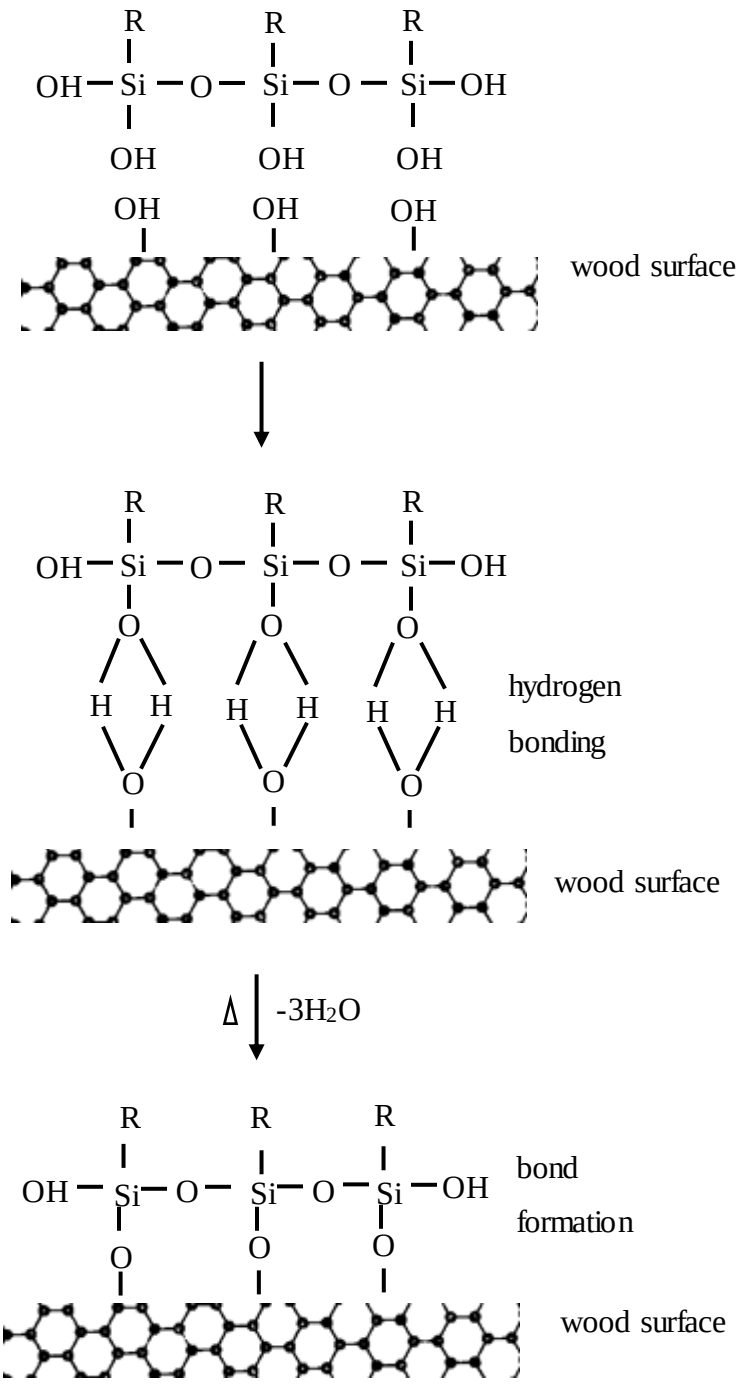


Figure 1.9 Deposition of alkoxysilane on wood and hydrophobication – covalent bond formation in silanol groups of alkoxysilane and hydrophobication on wood surface.

Source: Kartal *et al*. (2009).

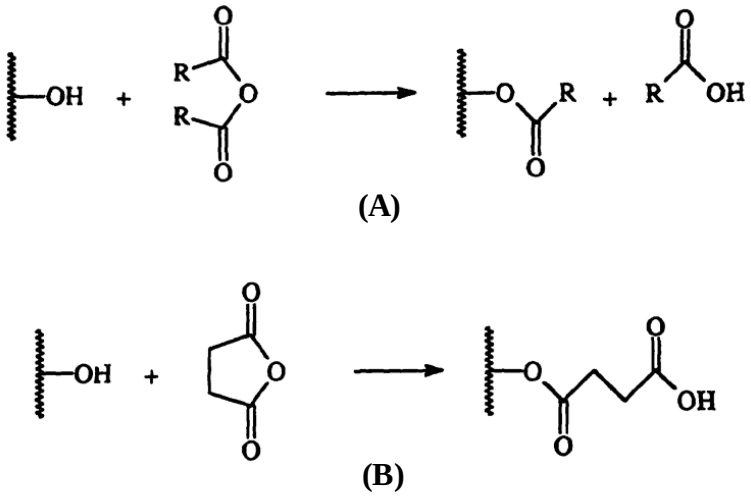


Figure 1.10 Reaction scheme for reaction of linear chain anhydride with wood (A) and cyclic anhydride with wood (B).

Source: Hill and Mallon (1998).

10. Objectives of research work

1. To apply commercial essential oils and chitosan as antifungal agent on rubberwood.
2. To study modification of rubberwood with silane or maleic anhydride with respect to fungal growth inhibition.
3. To study the effect of leaching on the retention of wood preservatives in wood and the resistance of fungal growth of leached samples.
4. To utilize the simple wood treatment such as dip, spray and vacuum treatment and compare their efficacy in wood protection.
5. To select the most effective wood preservative in the current study and study further on its toxicity.
6. To apply the results obtained from the laboratory scale to the wooden toys manufacturing scale.

CHAPTER II

Isolation and identification of molds from contaminated rubberwood and effect of relative humidity on fungal growth on wood

Abstract

Molds were isolated from contaminated rubberwood collected from the wooden toy factory. Isolated molds were identified by examining their morphological and molecular traits. Three dominant molds which colonized rubberwood were *Aspergillus niger* PSU1 (LC127085), *Aspergillus flavus* PSU2 (LC127086) and *Penicllium citrinum* PSU3 (LC127087). Effect of relative humidity (RH) on the colonization of *A. niger* PSU1 on wood was studied. The equilibrium moisture contents (EMCs) of rubberwood at 75, 80, 85, 92 and 97% RH, 25 °C after 10 days were 9.67, 10.42, 10.76, 13.69 and 15.61%, respectively. The heavy growth of *A. niger* PSU1 on wood was observed at 97% RH and the light growth was found at 92% RH but this fungus could not grow when RH was lower than 92% after 8 weeks incubation.

Introduction

In recent years, rubberwood has become an important source of timber, particularly for furniture manufacturing (Findlay, 2013) and today, it is also used for the kitchen wares and wooden toys manufacturing (Hong and Sim, 1994; Jusoh and Kamdem, 2001). Rubberwood has good workability, is easy to season and treat and these properties make it becomes more popular. Its potential to use has been hindered by its high susceptibility to biological attack because of large quantity of starch content (Jusoh and Kamdem, 2001; Findlay, 2013). Rubberwood is readily attacked by molds. This phenomenon effects it appearance and results in reduced market value because of dark pigmented fungal hyphae and spores (Mikluscak and Dawson-Andoh, 2004; Lee *et al.*, 2011). In addition, molds also adversely affect human health (Bush *et al.*, 2006). *Aspergillus* sp. and *Penicillium* sp. are common molds occurring on wood (Findlay, 2013). Frequently, molds are recognizable by their fast growth on the surface of substrate. Wood colonized by molds can make a multicolored surface e.g. black due to *A. niger* or green after *Penicillium* sp. colonization (Schmidt, 2006). Molds obtain food from the sap wood and this neither destroys the wood nor reduces its strength. Thus, wood strength properties are not impaired (Farmer, 2013).

Molds require air, moisture and nutrients in order to colonize on wood. Moisture content (MC) is very important for growth of molds (McKinney, 2004) because hyphae consist of up to 90% of water and fungal enzymes are active in an aqueous environment (Schmidt and Czeschlik, 2006). Additionally, slight condensation of free water on surfaces will support fungal growth (Trechsel, 1994). The MC of wood is related to the relative humidity (RH) of the air. When the RH is raised or decreased so the wood will absorb or release moisture until it is in equilibrium with the air (Simpson and Forest Products Laboratory (U.S.), 1998). When the wood is in equilibrium with the surrounding RH, the wood is defined as being at its equilibrium moisture content (EMC) (Rowell, 2005). The RH requirement for growth depended on the particular material and fungal species involved (Pasanen *et al.*, 2000). Several studies identified the temperature and humidity conditions in which different types of wood begin to mold. The RH has been determined to be 76-96% depending on temperature, time, substrate and fungal species (Pasanen et al., 1992). A study reported

by Nielsen *et al*. (2004) showed that growth of fungi isolated from mold infested building was observed on pine wood at 25 ºC and 78% RH. Thus, moisture management is a critical component for controlling fungal growth on materials. Moisture management requires an understanding of the EMC of a particular material.

In the present research, common molds from rubberwood were isolated and identified. Besides, the EMC of wood samples and colonization of mold on wood surface at various RH levels was investigated whether the results from critical RH levels for growth of mold on rubberwood can predict the safe humidity where rubberwood are kept safely from mold growth.

Materials and Methods

1. Wood materials

For isolation of mold, rubberwood samples with visible mold growth on the surface were collected from Plan Creations Co., Ltd., Trang, Thailand. Wood samples were collected three times from August 2010 to May 2011. The samples were placed in a sterile plastic bag until the isolation process took place.

Kiln-dried rubberwood (20 mm × 20 mm ×10 mm) for wooden toy preparation without wood preservative treated were used for all experiments in the present study. Wood samples were stored in a humidity room stabilized at 60% relative humidity (RH) and 20 ºC.

2. Isolation of molds

The contaminated rubberwood samples were transferred to 0.1% tween-80 solution (LabChem, Zelienople, USA) (four volumes of tween-80 solution to one volume of wood). The resulting solution were diluted with sterile 0.1% tween-80 solution at three dilutions (1:10, 1:100 and 1:1,000). Then, 100 µl of each dilution was spread duplicate onto potato dextrose agar (PDA) plates containing ampicillin (50 mg l^{-1} of PDA). The plates were incubated at 25 °C until the mycelium could be observed and distinguished. Each isolate was picked and transferred onto a new PDA plate. The plates were incubated at 25 °C for pure culture. The pure cultures were kept on PDA slants at at 4 °C for further use and also kept in 60% glycerol kept at −20 °C for long-

term preservation.

3. Fungal identification

Isolated molds were identified based on morphological characteristics and molecular approach. Morphology was examined, e.g. fungal colony growth pattern and spore formation, using keys and methods outlined by Samson *et al*. (2004). The isolated molds were grown on PDA for 3 days and mycelium (100 mg) were transferred to 2 ml 0.1% tween-80 solution to wash them twice. Fungal mycelium were transferred to 1.5 ml tube and kept at −20 °C overnight then were ground with liquid nitrogen using a mortar and pestle and transferred into test tubes. DNA was extracted from each mold using the commercial kits (E.Z.N.A.®Fungal DNA Mini Kit; Omega Bio-tek, Georgia, USA). This extracted DNA was stored at −20 °C until use for PCR amplification. Each PCR consisted of the following components: 25 µl of Taq PCR Master Mix, 5 µl of each 10 µmol l^{-1} primer (forward name: NS1 and reward name: EF3), 12 µl fungal genomic DNA solution and 3 µl distilled water in a final reaction volume of 50 µl. PCR conditions were denaturation at 95 °C for 5 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 m, for 32 cycles followed by final extension at 72 °C for 10 min. The PCR products were sequenced and generated sequences were aligned with BLAST algorithm (GenBank) of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast).

4. The EMC of rubberwood

Wood samples (n=3) were dried at 103 ºC until constant mass was reached and their initial dry weights were recorded. The samples were placed into conditioning chambers where relative RH was regulated with saturated aqueous salt solutions. Five RH ranges were used: 75% (NaC1), 80% (NH_4Cl), 85% (KCl), 92% (NaH_2PO_4) and 97% (K_2SO_4) at 25 °C (Greenspan, 1977). The final weights of the samples were determined periodically until they reached constant mass and recorded. The EMC of the samples was calculated as follows:

$$EMC = (M_{final} - M_{initial})/M_{initial} \times 100$$

where EMC is the equilibrium moisture content (%); $M_{initial}$ is the final weight of wood

material at 103 °C (g) and M_{final} is the final weight of moist wood kept at different RH (g).

5. Humidity requirement for mold growth on rubberwood

The rubberwood samples were chamber-conditioned at 75, 80, 85, 92 and 97% RH, 25 ºC for eight days before inoculation. *Aspergillus niger* PSU1 was isolated from contaminated rubberwood and selected as a model fungus. *A. niger* is the major mold frequently isolated from the rubberwood surface both before and after postharvest and from the viewpoint of public health, this fungus constitutes a serious problem (Matan *et al.*, 2009; Jantamas *et al.*, 2013). The fungus was grown on PDA at 25 ºC, 5 days. Fungal spores were collected by flooding the surface of the plates with sterile tween-80 solution (0.1% v/v) and counted using a haemocytometer (Celeromics, Valencia, Spain). The suspension was standardized to 10^6 spores ml^{-1} before use.

After stabilization, each rubberwood sample was inoculated with 50 µl of *A. niger* PSU1 spore at the center of the sample. The inoculated rubberwood samples were incubated under 75-97% RH, 25 ºC. The fungal growth on wood was examined weekly for at least 8 weeks. Mold growth was assessed following to the rating scale: - = no growth, ++ = light growth and +++ = heavy growth.

6. Statistical analysis

Statistical analysis of the data obtained was carried out using the SPSS version 17.0 software (SPSS Inc, Chicago, USA). One way analysis of variance (ANOVA) was conducted and least significant difference (LSD) tests were used to evaluate the significant difference between results within the confidence interval of 95 %.

Results

1. Isolation and identification of molds from rubberwood

A total of 17 counts were isolated from contaminated rubberwood collected from the wooden toy factory. By observing their morphological characteristics, they belonged to genus *Aspergillus* (14 counts) and *Penicillium* (3

counts). Two isolates from genus *Aspergillus* were strain PSU1 and PSU2. The stain PSU1, grew as black colonies, formed globose-tip conidiophores with the formation of conidial head (Figure 2.1A and B), was identified as *A. niger* PSU1 (99% similarity; DDBJ Acc. No.: LC127085). This strain was the most dominant species (8 counts; 47.1%). The strain PSU2 (6 counts; 35.3%) showed green and fluffy colonies with globose conidia (Figure 2.2 A and B). This strain was identified as *A. flavus* PSU2 (100% similarity; DDBJ Acc. No.: LC 127086). The strain PSU3 (3 counts; 17.6%), belonged to genus *Penicillium*, produced velvety colonies and long-chained spherical conidia (Figure 2.3 A and B). This strain was identified as *P. citrinum* PSU3 (100% of similarity; DDBJ Acc. No.: LC127087) (See appendix C for partial 18S DNA sequence of each fungus).

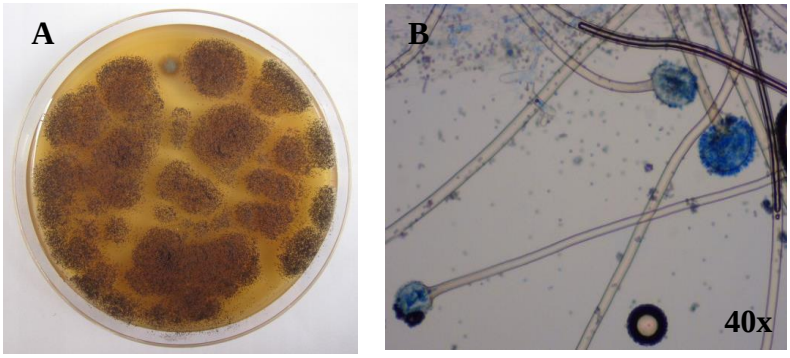


Figure 2.1 *A. niger* PSU1, (A) black colonies cultured on PDA for 7 days and (B) globose-tip conidiophores with the formation of conidial head.

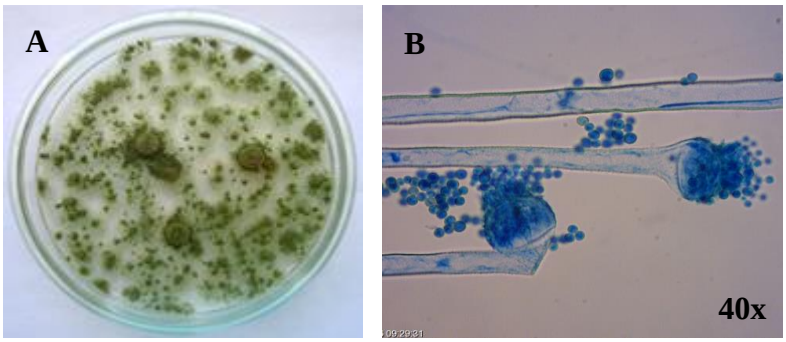


Figure 2.2 *A. flavus* PSU2, (A) green fluffy colonies cultured on PDA for 7 days and (B) globose-tip conidiophores and globose conidia.

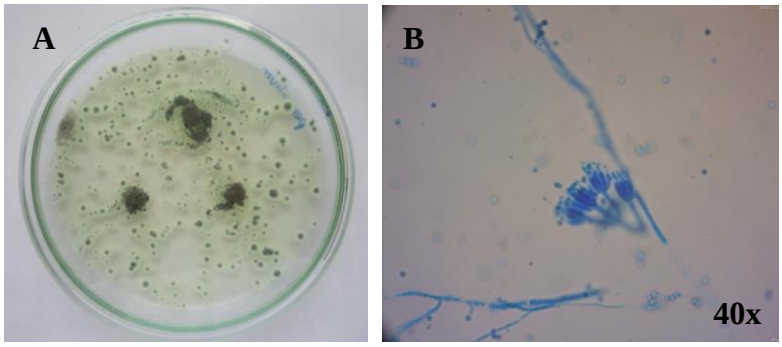


Figure 2.3 *P. citrinum* PSU3, (A) velvety colonies cultured on PDA for 7 days and (B) long-chained spherical conidia.

2. The EMC of rubberwood

The EMC of rubberwood at 75, 80, 85, 92 and 97% RH after 10 days were 9.67, 10.42, 10.76, 13.69 and 15.61%, respectively (Figure 2.4). As shown in Figure 2.4, the moisture content of rubberwood increased when the RH was increased.

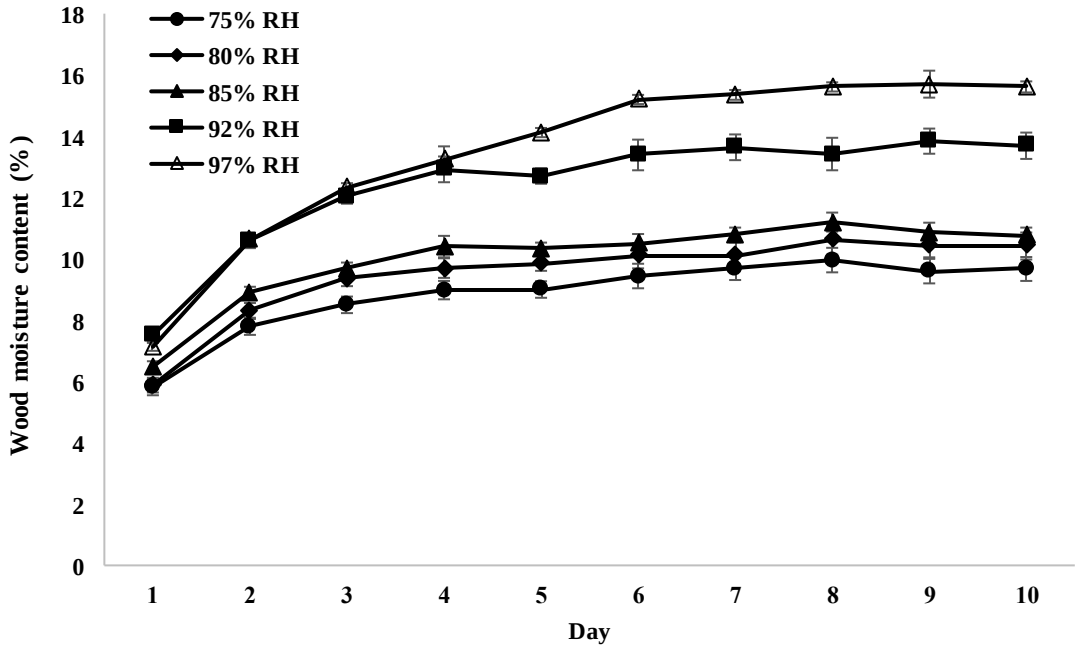


Figure 2.4 Equilibrium moisture content of rubberwood at different relative humidities. Error bars represent the standard deviation of three determinations.

3. Effect of RH on growth of *A. niger* PSU1 on rubberwood

Table 2.1 presents growth of *A. niger* PSU1 on rubberwood under the conditions of 75 to 97% RH at 25 ºC. At the RH below 92%, no growth was observed on the rubberwood samples during 8 weeks but growth of *A. niger* PSU1 at 92 and 97% RH was detected at week 8 and week 2, respectively. Observations on the rate of *A. niger* PSU1 growth showed that at 97% RH supported the most rapid growth and promoted heavy growth in five week.

Table 2.1 Growth of *A. niger* PSU1 on rubberwood at different relative humidities, 25 ºC

RH (%)	Growth of *Aspergillus niger* PSU1							
	1W	2W	3W	4W	5W	6W	7W	8W
75	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-	-
92	-	-	-	-	-	-	-	++
97	-	+	+	+	++	++	++	++

W= week; Visual scale of mold growth: - = no growth; + = light growth; ++ = heavy growth

Discussion

Molds isolated from contaminated rubberwood were the genus *Aspergillus* and *Penicillium*. All three isolates are common molds frequently found on wood. *Penicillium* is a diverse genus occurring worldwide and its species commonly colonize wood surfaces such as conifers, hardwood, round wood, lumber and wood products (Lee, 2012). It is commonly isolated from forest woods (Crawford *et al.*, 1990) and several studies describe it as one of the most frequently encountered genera in sawmills (Halpin *et al.*, 1994; Simeray *et al.*, 1997; Sivrikaya and Kara, 2009). *Penicillium* sp. and *Aspergillus* sp. are common causal agents of mold and sapstain discoloration of lumber (Mikluscak *et al.*, 2004). The related articles have recently been published that *P. implicatum* and *A. versicolor* were the predominant fungal species identified from yellow-poplar lumber while *A. niger* and *P. chrysogenum* were isolated from rubberwood (Mikluscak *et al.*, 2004; Matan and Matan, 2008).

Typically, molds infect or colonize wood either via reproductive spores

carried on air currents or in liquid water (Walker, 2006). Mold spores are also dispersed by animals, insects and man (Schwarze *et al*., 2013). Moreover, if healthy wood is in contact with infected wood, the infection can spread to the sound wood by normal growth of the fungal hyphae from the infected wood, even without the production spores (Kuhad and Singh, 2007). Molds invade wood only where there is a source of water and where environmental conditions favor growth (Walker, 2006).

Foresters and wood technologists define the moisture content of wood as the initial weight of piece of wood and the final weight of wood after oven-drying to constant weight at 103 ºC (Walker, 2006). Equilibrium moisture conten is a moisture content in wood at which the wood is neither gaining nor losing moisture when an equilibrium condition has been reached (U. S. Department of Agriculture, 2007). In an environment with high or low RH, so the wood materials will absorb or release moisture until it is in equilibrium. Periodical measurement of rubberwood samples showed that after approximately eight days, rubberwood reached EMC. Differ from the pine sapwood samples that reached EMC in two weeks after incubation at 20 ºC and 75 to 100% RH with the EMC of approximately 13 to 25% (Viitanen, 1994). The difference in EMC depends on the species (U. S. Department of Agriculture, 2007) and temperature also has effect on EMC (Bulian and Graystone, 2009).

To determine the critical RH level of a material, it is necessary to keep the materials at different humidity levels. The critical RH level will then lies somewhere between the two closest humidity levels tested growth (Johansson *et al*., 2012). In this study, no growth of *A. niger* PSU1 was observed on wood at 85% RH with 8 weeks of testing, but growth could be noticed at 92% RH. Therefore, the critical RH level is between >85% and <92%. The minimum humidity for the occurrence of fungal growth was related to the EMC of each material at each humidity. The EMCs of rubberwood at 85 and 92% RH were 10.76 and 13.69%, respectively (Figure 2.4). In this way, the minimum EMC for the occurrence of *A. niger* PSU1 on rubberwood was around >10.76 to <13.69% at 25 ºC. At levels of relative humidity below 80 to 85%, the fungi cannot use the moisture within the wood for growth and colonization will not occur (American Industrial Hygiene Association, 2008). Additionally, by conditioning the wood to an EMC of 12%, the mold growth problem can be regulated (Zabel and Morrell, 1992). The RH required for growth depended on the particular material and fungal species

involved (Pasanen *et al*., 2000). Growth of molds on wooden materials depends mainly on time, wood properties, moisture and temperature conditions (Viitanen, 1994). The critical RH level for growth of a mixture of fungi on unpainted pine wood at 25 ºC was between 80 to 85% where the EMC of wood was ranged from approximately 12 to 14% (Block, 1953). In contrast, the study by Chang *et al*. (1995) determined that the minimum equilibrium RH for growth of *A. niger* on used ceiling tile blocks was between 94 and 97% and the EMC of used ceiling tile at that condition was between 4.3 and 5.8%. Growth of *A. niger* on gypsum board was observed after 8 weeks at 90-95% RH and 25 ºC where the EMC value for gypsum board was approximately 7% (Hoang *et al*., 2010). The differences in minimum EMC or critical RH level required for fungal growth on wood and other materials reflect the fact that the nature of the substrate materials can have significant impacts on the conditions required for fungal proliferation (Chang *et al*., 1995). This study indicates that fungal growth on rubberwood may be avoided by regulating the RH level and rubberwood must be stored below 92% RH.

Conclusion

This study provides information on the occurrence of mold that colonize dried rubberwood. As a matter of fact, the isolated species are found worldwide and most of these species dominantly cause damage in damp building materials, including logs, lumber and wood products. The EMC of rubberwood ranged from >10.76 to <13.69% was required for the colonization of *A. niger* PSU1 on rubberwood under RH between >85 and <92%. Storing rubberwood under RH below 92% may keep them free of mold growth. However, moisture control is difficult especially outdoor use. With chemical preservation and wood modification, the durability of wood to mold attack is improved.

CHAPTER III

Application of plant essential oils in prevention of mold growth on rubberwood

Abstract

The minimal inhibitory concentration (MIC) of five commercial essential oils (cinnamon, clove, eucalyptus, peppermint and lemongrass oils) was investigated against molds isolated from rubberwood (*A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3). The MIC of clove oil against all these fungi was 5 µl ml^{-1}, while cinnamon oil had MIC from 2.5 to 10 µl ml^{-1}. Rubberwood samples were dipped in clove or cinnamon oil solutions at MIC, 2 and 4-MIC and inoculated with *A. niger* PSU1 spores. Only cinnamon oil at 2 and 4-MIC showed strong inhibition against growth of *A. niger* PSU1 on rubberwood for at least 12 weeks while clove oil did not inhibit the fungus on rubberwood.

Introduction

Non-preservative treated rubberwood is used as raw material for wooden toy manufacturing. Moreover, rubberwooden toys are not treated with any preservative so they are very susceptible to mods attack. Although molds cause little or no significance damage to the structure of the wood, their presence and growth on wood result in a reduction in pleasing quality due to the colonization by their pigmented mycelium (Lee *et al*., 2011; Salem *et al*., 2016). Moreover, they also affect human health (Bush *et al*., 2006). Chemical fungicides are successfully used for preventing fungal attack (Schultz *et al*., 2007). Currently, there is a strong debate about the safety aspects of chemical fungicides since they are considered to have negative effects on human health and environment due to their residual effects (Preston, 2000).

A new approach to prevent growth of mold on wood is the use of essential oils as preservatives. Essential oils are low toxic compounds and have been shown to possess antifungal properties (Naveed *et al*., 2013). The application of essential oils for controlling mold growth on wood has been a subject of interest with a developing trend. These substances are natural products with no adverse effects on the environment (Farzaneh *et al*., 2015). Moreover, essential oils are organic compounds obtained from plants (Bakkali *et al*., 2008) which makes them an alternative to satisfy consumers demands (Aguilar-González *et al*., 2015). The successful uses of combined cinnamon and clove oil (Matan and Matan, 2007) as well as main constituents of oils including eugenol and cinnamaldehyde (Singh and Chittenden, 2010) were reported for prevention of fungal growth on wood. The antimicrobial properties of essential oil are mainly related to the contents and chemical composition of essential oil. Identification of biologically active compounds being potential as bio-controlling natural products for the wood protection would be valuable.

The present study aimed to evaluate the inhibitory effects of essential oils against molds isolated from rubberwood. The effects were measured by determining the minimum inhibitory concentration. Besides, their potential to be utilized as antifungal agents on rubberwood against growth of molds was examined.

Materials and Methods

1. Wood materials

Non preservative treated wood samples were obtained from Plan Creations Co., Ltd. Wood samples were washed with 70% ethanol and oven-dried at 103±2 ºC for 12 h prior to essential oil treatments.

2. Essential oils

Cinnamon, clove, eucalyptus, peppermint and lemongrass oils were purchased from Saiburi Samoonprai Ordinary Partnerships (Hat Yai, Thailand).

3. Fungal cultures

Three strains of fungi, *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3 were isolated from contaminated rubberwood. The fungal spore solution was standardized to 10^6 spores ml^{-1} before use.

4. Determination of minimal inhibitory concentration (MIC) of essential oils

A microdilution broth method (Clinical and Laboratory Standards Institute (CLSI), 2008) was used to determine the MIC. Each well was prepared with 50 µl of double strength sterile potato dextrose broth (PDB). The first well of microplate was prepared by dispensing 50 µl of essential oil solution and removing 50 µl to the next well successively. Then, 50 µl of the spore solutions were added to each well to obtain 100 µl of final volume with final concentrations of essential oils ranging from 40 to 0.31 µl ml^{-1}. The positive growth control was containing 50 µl of PDB and 50 µl of spore suspension without essential oils added. Another well containing ethanol without tested-compound was used as a solvent control. The microplates were incubated at 25 ºC for 72 h. The lowest concentration of essential oil preventing visible growth of tested fungi was designated as the MIC. The minimal fungicidal concentration (MFC) were determined by subculturing 10 µl aliquots of all the wells with no fungal growth onto PDA plates; these plates were subsequently incubated at 25 °C for 48 h. The lowest concentration of essential oil at which negative growth was considered to be the MFC. The MIC and MFC experiment was repeated three times.

5. Chemical analysis of the selected essential oils

Chemical compositions of the selected essential oils were analyzed by a gaschromatograph mass spectrometer (GC-MS) (Trace GC Ultra/ ISQMS, Thermo Scientific Inc., USA) equipped with a TR-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent, Santa Clara, CA, USA) according to the method by Wang *et al*. (2009). Sample (0.2 µl) was injected into the injector with a split ratio of 1:50. Oven temperature was kept at 40 °C for 3 min, increasing to 120 °C at a rate of 5 °C/min and holding for 3 min, then increasing to 180 °C at a rate of 2 °C/min and holding for 3 min, finally increasing to 230 °C at a rate of 5 °C/min and holding for 3 min. Injector temperature was 250 °C, while the detector temperature was 250 °C. The ion source temperature was 250 °C. Helium was used as the carrier gas. Identification of compounds was based on comparisons of their mass spectra with those recorded in the National Institute of Standards and Technology database. Quantitative analysis of each essential oil component (expressed as area percentage) was carried out by peak area normalization measurement.

6. Growth of mold on rubberwood

Essential oils that showed the lowest MIC were selected for dip treatment of rubberwood and mold growth inhibition by these treatments was studied. The concentrations of the selected essential oils were MIC, two and four-fold MIC as determined for *A. niger* PSU1 fungus. Untreated wood sample was served as control and ethanol was used as solvent control. Rubberwood samples (n=3) were dipped for 15 s in each treatment solution, following American Society for Testing and Materials (ASTM) test method D4445-91 (American Society for Testing and Materials (ASTM), 1998). The dip treated rubberwood samples were kept in a closed container overnight at room temperature. The wood samples were then inoculated with 50 µl of *A. niger* PSU1 spore solution and incubated at 25 ºC and 100 % RH with periodic observations of fungal growth for 12 weeks. The fungal growth on each sample was rated on a scale of 0–5, with 0 denoting a clean specimen and 5 representing heavy fungal growth (0=clean, 1=20 %, 2=40 %, 3=60 %, 4=80 %, 5=100 % of fungal growth). The percentage of fungal growth were calculated based on these ratings as (A/B) × 100,

where A = score sum for fungal growth with an actual treatment and B = score sum for for fungal growth over control samples.

Results

1. Determination of MIC

The MIC and MFC of five essential oils against three molds are presented in Table 3.1. The cinnamon oil displayed the strong antifungal effect against *A. niger* PSU1 with the MIC of 10 µl ml^{-1} while the MIC values against *A. flavus* PSU2 and *P. citrinum* were 2.5 µl ml^{-1}. The clove oil was also active against three molds tested, all with MIC value of 5 µl ml^{-1}. Eucalyptus, peppermint and lemongrass oils were less active with more than 40 µl ml^{-1} needed to inhibit growth of *A. niger* PSU1 or *A. flavus* PSU2.

Table 3.1 Determination of MIC and MFC (µl ml^{-1}) of five essential oils against *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3

Essential oils	Fungal species					
	A. niger PSU1		*A. flavus* PSU2		*P. citrinum* PSU3	
	MIC	MFC	MIC	MFC	MIC	MFC
Cinnamon	10	10	2.5	10	2.5	2.5
Clove	5	5	5	5	5	20
Eucalyptus	>40	>40	>40	>40	20	>40
Peppermint	>40	>40	>40	>40	20	>40
Lemongrass	>40	>40	>40	>40	20	>40

2. The chemical composition of the selected essential oils

The results of GC/MS of the two essential oils are presented in Tables 3.2. The major components of clove and cinnamon oil were eugenol (76.35%) and cinnamaldehyde (96.24%), respectively.

Table 3.2 Main components and relative contents (%) of clove and cinnamon oils

No.	Components	Retention time (min)	Relative content (%)
Clove oil			
1	*trans*-Caryophyllene	18.80	8.00
2	α-Humulene	20.52	0.87
3	Benzenemethanol	27.40	12.14
4	Caryophyllene oxide	31.71	0.40
5	Eugenol	39.63	76.35
6	Eugenol acetate	43.48	1.21
	Total content		98.97
Cinnamon oil			
1	Benzaldehyde	16.63	1.02
2	Cinnamaldehyde	34.14	96.24
3	Ethyl cinnamate	37.86	0.38
4	Cinnamic acid	63.87	1.49
	Total content		99.13

3. Growth of mold on rubberwood

The more complex growth environment on wood than in laboratory media might interact with essential oils and reduce their efficiency or provide microbial cells with greater protection from antimicrobial agents. Therefore, the concentrations of clove and cinnamon oils were made at 2 and 4-MIC. Growth of *A. niger* PSU1 on essential oil treated rubberwood at 100% RH, 25 °C is displayed in Figure 3.1. Growth of *A. niger* PSU1 on ethanol treated wood samples reached 100% after 12 weeks. Clove oil were not effective against *A. niger* PSU1 on wood even its concentrations were made at 2 and 4-MIC (10 and 20 µl ml^{-1}) as well as cinnamon oil at MIC=10 µl ml^{-1}. However, cinnamon oil at the 2 and 4 fold-MIC (20 and 40 µl ml^{-1}) completely inhibited growth of *A. niger* PSU1 on rubberwood (rated zero) for at least up to 12 weeks.

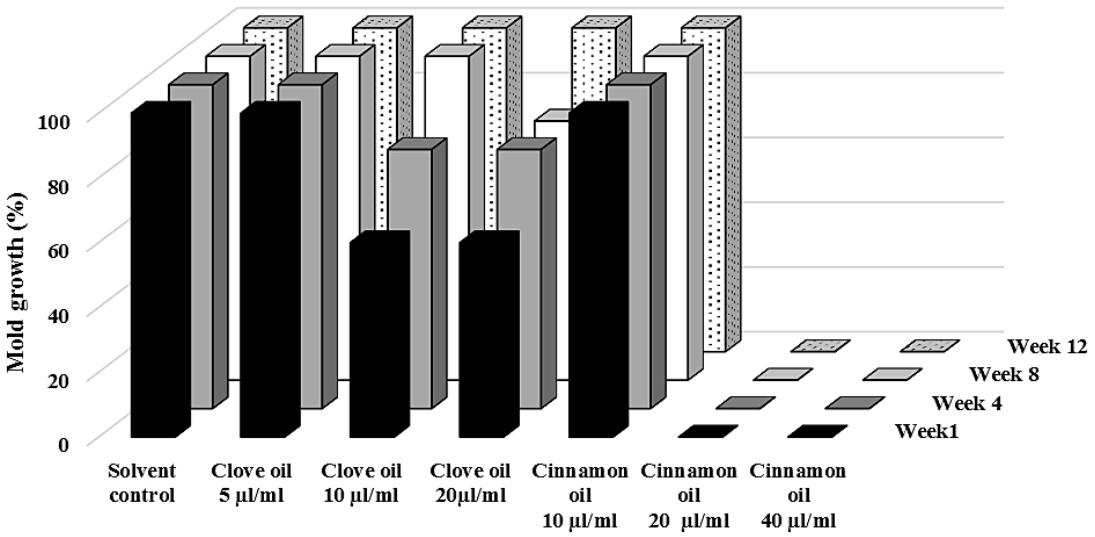


Figure 3.1 Growth of *A. niger* PSU1 on essential oil solution dip treated rubberwood (n=3) after 12 weeks of incubation at 100% RH, 25 °C.

Discussion

The present study showed that the essential oils had antifungal activities. Clove and cinnamon oil showed notable antifungal activities against three molds. The MIC of 5 µl ml^{-1} for clove oil indicates much stronger activity than the MIC=100 µl ml^{-1} in a prior study (Matan *et al.*, 2011) against *Penicillium* sp. and *A. niger*. The MIC values of cinnamon oil are also much lower than those reported by Matan *et al.* (2011), who found MIC = 50 µl ml^{-1} for clove oil against *Penicillium* sp. and *A. niger*. Obviously, each essential oil showed the different pattern of activity against each fungal species tested. The inhibitory processes of essential oils may be affected by the constituents of essential oils and target fungal species (Kumar *et al.*, 2014). Based on the current MIC results, cinnamon and clove oils were selected for the study on dip treatment of rubberwood.

Major components of clove and cinnamon oils were eugenol and cinnamaldehyde, respectively. These results were in accordance with previous reports (Jin and Cho, 2011) with some differences in the percentage of chemical compositions which may be attributed to seasonal variation, adaptive metabolism, parts of plant used, distillation process and other factors (Naveed *et al.*, 2013). The results of major constituents of essential oils is related to their inhibitory effects against fungi

(considering their detected MIC). The highest antimicrobial effects of individual constituents of the essential oils generally range as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons (Ballester-Costa *et al*., 2013). This findings supported the consistency of the low MIC values found for clove oils as eugenol (phenolic) was found to be its major component, on the other hand cinnamondehyde (aldehydes) was found to be the major constituent of cinnamon oil. Omidbeygi *et al*. (2007) suggested that eugenol played a key role in antifungal activity of clove oil against *Aspergillus flavus*.

The result of growth of *A. niger* PSU1 on wood samples are clearly not concordant with the inhibition MIC values against this fungus in PDB. The MIC of clove oil was lower than that of cinnamon oil but it was not effective against *A. niger* PSU1 on wood although its concentrations were up to 2 and 4-MIC. Clove oil might be susceptible to degradation or volatility, or possibly interacted with rubberwood and had no efficacy. It may be related to the more complex structure of wood than culture medium. However, higher concentrations of clove oil may be more effective but safety studies may be important.

Cinnamon oil exhibited a strong wood protecting activity. In a related study, Matan and Matan (2007) reported that a combination of cinnamon and clove oil (5:1; 50 µl ml^{-1}) inhibited the growth of *A. niger, Penicillium chrysogenum* and *Penicillium* sp. on rubberwood for at least 12 weeks. Cinnamaldehyde is a major component of cinnamon oil (about 96%; Table 3.2) and may play a key role in wood protection. Research conducted by Chittenden and Singh (2011) showed that cinnamaldehyde could reduce the percentage of weight loss of radiata pine wood compared to untreated wood. Clearly, this result shows that cinnamon oil is excellent against mold and may be further explored as a potential wood preservation agent.

Conclusion

Essential oils are known to be effective against a wide spectrum of molds. This study indicated that essential oils possess antifungal activity against molds isolated from rubberwood. The application by dip treatment of cinnamon oil could protect wood from the mold for a period of time under laboratory condition. This

findings may have considerable significance on its potential use as wood protectant. For outdoor uses, the leaching properties should to be examined. Furthermore, retention of essential oils within wood tissues is hindered due to their susceptible to light, temperature and oxygen availability and they have to be co-impregnated with agents that can shield them from degradation.

CHAPTER IV

Resistance of rubberwood treated with chitosan or silane against molds

Abstract

The antifungal activities of chitosan samples, C1 (Mw 37 kDa), C2 (Mw 5.4 kDa) and C3 (Mw 3.5 kDa), were tested against *Aspergillus niger* BAM 4 and *Penicillium decumbens* CBS 121928 at concentrations ranging from 0.063 to 0.5% w/v. Chitosan C1 and C3 at 0.5% exhibited strong antifungal activity against both molds. The solutions of chitosan C1 and C3 were made at 1 and 2%. Rubberwood were either dipped in each chitosan solution for 15 s or vacuum treated at 60 mbar, 2 h. After the leaching test, the content of chitosan in wood was well retained in both dipped and vacuum treated wood. The vacuum treated wood with chitosan C1 and C3 at 1% w/v had strong resistance against *A. niger* BAM 4. However, dipped rubberwood with chitosan solutions showed lower resistance against *A. niger* BAM 4. On the other hand, both dipped and vacuum treated rubberwood with chitosan had no resistance against *P. decumbens* CBS 121928. Additionally, treatment of rubberwood with silane was studied at 0.125-0.25 M silane solution. Both dipped and vacuum treated wood with silane showed no resistance to fungal growth.

Introduction

Utilization of natural products like essential oils as rubberwood preservative has been recently developed (Matan and Matan, 2008; Matan *et al.*, 2009; Ma-in *et al.*, 2014). However, essentials oils are sensitive to light, temperature and oxygen availability (Turek and Stintzing, 2013). Therefore, finding a rubberwood protecting method that is not prone to degradation, low toxic and cost-effective is urgently needed. For instance, chitosan and silane are less harmful to humans and have not been studied as rubberwood protecting agents. Chitosan and silane have been reported in inhibiting of decay fungi by many researchers and they were applied into wood by pressurized process which requires special equipment and expensive (Donath *et al.*, 2004; Eikenes *et al.*, 2005a). Hence, in this work, chitosan and silane is reported as an alternative for rubberwood protection against molds. Besides, simple dip and vacuum method were used for treatment of rubberwood.

Chitosan is biodegradable, biorenewable and non-toxic biopolymers. Several authors have reported the antifungal activity of chitosan, both *in vitro* and *in vivo*. Ziani *et al.* (2009) reported that chitosan could inhibit the growth of *Aspergillus niger*, *Alternaria alternata* and *Rhizopus oryzae* in culture media. An application of chitosan as a wood preservative in prevention of wood decaying fungi has been studied. Decaying rate in pine wood treated with chitosan at 2.5 and 5.0% w/v decreased in wood samples tested with *Poria placenta*, but the leaching test has shown a reduced antifungal effect of chitosan (Alfredsen *et al.*, 2004). High Mw chitosan (215 kDa) has been shown to be more efficient against wood decaying fungi than chitosan with lower Mw (35 kDa) (Eikenes *et al.*, 2005a).

Many types of silicon compounds have been used for wood modification and reduction of fungal attack. Silanes are the silicon analogs (Si_nH_{2n+2}) of alkane hydrocarbons and long known as modification agents. They are used for hydrophobication of ceramics, scratch resistant surfaces and anti-graffiti coatings or as adhesion promoters between organic and inorganic materials (Donath *et al.*, 2004; Hill *et al.*, 2004). Silanes are unstable in water and are hydrolyzed to silanols which subsequently condense to three dimensional structures as shown in the following equations:

Hydrolysis

$$\equiv SiOR + H_2O \rightarrow \equiv Si\text{-}OH + ROH \quad (1)$$

Water condensation

$$\equiv Si\text{-}OH + \equiv Si\text{-}OH \rightarrow \equiv Si\text{-}O\text{-}Si \equiv + H_2 \quad (2)$$

The silanol groups of silanes can react with hydroxyl groups of cell wall polymers forming a covalent bond between the silicon compound and cell wall polymers (Donath *et al.*, 2004). Various silanes have been used to modify wood and the modified wood shows improved durability against decay fungi (Hill *et al.*, 2004; Panov and Terziev, 2009).

Wood can be treated with a preservative through either pressurized or non-pressurized processes depending on the characters of the wood and types of application. A combination of vacuum and pressure treatment is the most appropriate for industrial applications (Richardson, 1993). However, non-pressure preservative treatments (dip or vacuum treatment) are the simplest method and more practical for less industrialized areas (Baillères *et al.*, 2004).

The objective of this study was to assess the potential application of different chitosan preparations against mold growth on rubberwood. Additionally, this work is aimed at investigating the efficacy of methoxysilane to increase the resistance of wood against molds. Rubberwood samples were either treated with chitosan or silane by vacuum or dip treatment and the results were compared. Concerns about the effects of leaching that would occur in service, laboratory leaching test was also studied.

Materials and Methods

1. Chitosan solutions

Three different chitosan samples were prepared and tested for their antifungal activity on rubberwood; two chitosan samples (C1 and C3), which were soluble in weak acid solutions and one different water soluble chitosan oligomer sample (C2).

The chitosan sample (C1) was KitoFlokk chitosan (Teta Vannrensing, previous Norwegian Chitosan, Kløfta Norway) with a fraction of acetylation (F_A) 0.15, analyzed by nuclear magnetic resonance spectrometry (^{1}H-NMR) (Figure 4.1) with an

Ascend^{TM} 400 instrument from Bruker (Sørbotten *et al.*, 2005). This chitosan is prepared from chitin of snow crabs. The intrinsic viscosity of this chitosan sample was analyzed on a cone-plate Rheometer. The Mark-Houwink-Sakaruda equation was used to calculate the Mw from the intrinsic viscosity (Kasaai *et al.*, 2000). The degree of polymerization (DP_n) of C1 was 220, which corresponded to an average Mw of 37 kDa. The chitosan was dissolved in 1% v/v acetic acid solution at a concentration of 2% w/v. The pH of the chitosan solution was adjusted to 5.5 before it was used in further experiments.

C1 was the starting material for preparing the C2 chitosan oligomer sample. C1 (20 g l^{-1}) was dissolved in 0.5% v/v HCl and incubated with rotation (500 rpm) at 37 °C until all the chitosan was dissolved. After adjusting the pH to 5.5 with 1 M NaOH, Chimax-35 chitosanase (Amicogen, South- Korea) was added to the chitosan solutions to a final concentration of 0.1 mg g^{-1} chitosan and the reaction was continued with rotation (250 rpm). The reaction was stopped by decreasing the pH to 2.5 using 18.5% v/v HCl followed by increasing the temperature to 60 °C to permanently inactivate the enzymes. The chitosan starting material contains small amounts (about 1%) of insoluble particles (silicates from the catching of the snow crabs), which were removed from the hydrolyzed products using a Whatman filter. The F_A and DP_n of the chitosan samples was found using ^{1}H-NMR. The F_A of this sample was 0.15. The DP_n of C2 was 32 and this corresponded to an average Mw of 5.4 kDa.

The C3 chitosan sample was prepared from a chitosan provided by Dr. Ausa Chandumpai, Prince of Songkla University. This chitosan sample was prepared from chitin of squid. The chitosan (2% w/v) was dissolved in 1% v/v acetic acid. When the chitosan was completely dissolved, an aqueous solution of 4% w/v potassium nitrite was added dropwise, while continuously mixing at 50 ºC until the solution became lighter in viscosity. The C3 had a F_A 0.32 and DP_n 20, which corresponded to Mw of 3.5 kDa. The pH of the solution was adjusted to 5.5. Chitosan is active in an acidic condition and preliminary tests showed that acetic acid solution at pH 5.5 was ineffective against the growth of the two molds.

2. Silane

Commercial methoxysilane, [3-(2-Aminoethylamino)propyl] tri-

methoxy silane (AEAPTMEOS) was obtained from Sigma–Aldrich Chemicals (Munich, Germany).

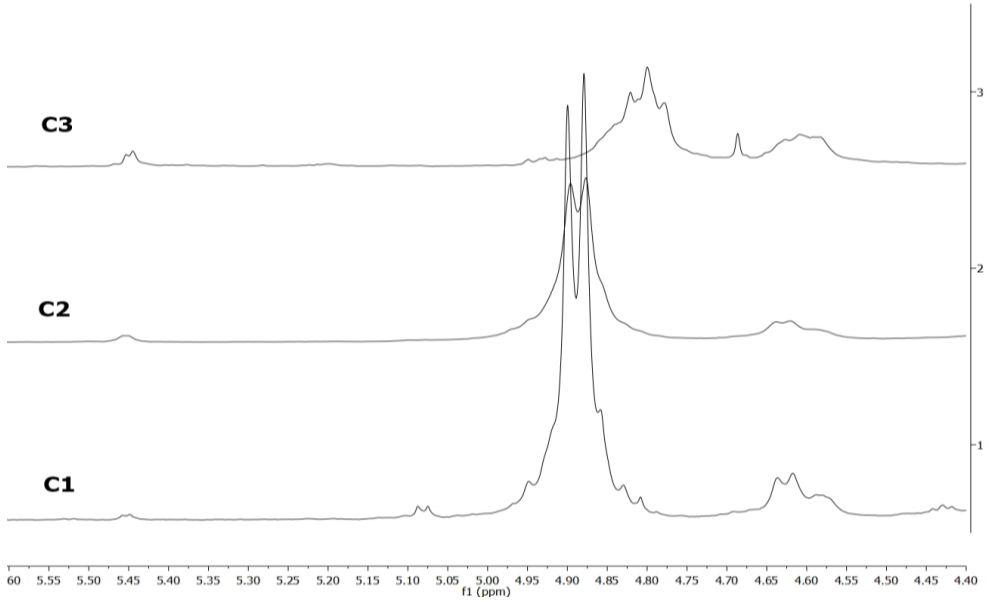


Figure 4.1 ^{1}H NMR spectra of chitosan C1 (DPn = 220, F_A = 0.15), C2 (DPn = 32, F_A = 0.15) and C3 (DPn = 20, F_A = 0.32).

3. Fungal cultures

Two strains of fungi, *Aspergillus niger* BAM 4 and *Penicillium decumbens* CBS 121928 were selected as the model fungi for the study of mold growth on rubberwood. Fungi were grown on malt extract agar (MEA; Bacto, BD Biosciences) at 25 ºC for 7 days and fungal spores were collected by flooding the surface of the plates with 5 ml sterile tween 80 solution (0.1% v/v) and counted using a haemocytometer. The suspension was standardized to 10^6 spores ml^{-1} before use.

4. Antifungal activity of chitosan on agar plate

The antifungal activity of chitosan against two fungi was determined by the reduction in colony size. The stock solution of chitosan was added to double strength sterile molten MEA to obtain the final concentrations at 0.5, 0.25, 0.125 and 0.063% w/v. A standard 9-cm Petri dish containing 20 ml chitosan-supplemented medium was inoculated with a 3-mm fungal plug at the center of the plate. The inoculated plates were incubated at 25 °C. The reduction in colony size was determined

by the radial growth of the colony on the 7th days of cultivation and calculated as follows (Alfredsen *et al*., 2004):

$$\text{Reduction in colony size (\%)} = (GC\text{-}GT)/GC \times 100$$

where GT is a diameter of the growth zone in the plates supplemented with chitosan and GC is growth zone in the control plate. Each experiment was repeated five times.

5. Treatment of rubberwood

5.1 Treatment with chitosan

Chitosan samples that showed strong antifungal activity for both fungi were selected for treatment of rubberwood and their concentrations were 1 and 2% w/v. Rubberwood samples were either dip or vacuum treated with the chitosan solutions.

5.1.1 Dip treatment

Rubberwood samples (*n=6*) were dipped for 15 s in chitosan solutions, following the method of the American Society for Testing and Material (ASTM, 1998).

5.1.2 Vacuum treatment

Rubberwood samples (*n=6*) were vacuum treated with chitosan solutions. The wood samples were completely soaked in each solution and vacuumed at 60 mbar for 2 h. A study carried by Matsumura *et al*. (1999) used vacuum treatment at 200 mbar for 30 min and that vacuum schedule showed equilibrium uptake of preservative. Therefore, in this study, vacuum treatment at 60 mbar for 2 h definitely provided equilibrium for chitosan reacting with wood.

5.2 Treatment with silane

Silane was dissolved in water at concentrations of 0.125 and 0.25 M. The treatment of rubberwood with silane solutions was carried out following the methods described in treatment of rubberwood with chitosan. Water was served as a control solution since it was used as a solvent for chitosan and silane; thus, it is needed

to confirm that water did not affect the efficacy of chitosan and silane on wood.

After treatment, wood samples were wiped off with paper and weighted. Samples were dried at room temperature overnight and subsequently cured in the oven at 40 ºC for 48 h. The samples were then dried at 103 ºC for 18 h and the dry mass of each sample was recorded.

6. Leaching study of chitosan and silane treated wood samples

Chitosan or silane treated wood samples (*n=6*) were leached according to European standard EN84 (1997). The samples were placed in a beaker with a glass sphere on top to prevent them from floating. The beakers were filled with fresh water to a ratio of approximately five volumes of water to one volume of wood before vacuum at 40 mbar for 20 minutes. After the vacuum stage, the samples were left for 2 hours in the beaker and the water was changed every day for 10 days. Then the wood samples were dried at room temperature for 24 h and oven dried at 40 ºC for 48 h. These wood samples were analyzed for chitosan or silicon content.

7. Quantification of chitosan content

The quantification of chitosan content in wood samples was analyzed by an acid hydrolysis method described by Eikenes *et al.* (2005b). The surfaces of three wood samples (1 mm thick) were cut with further grinding to reduce the particle size and ground to very fine wood powder with ball milling process. Each grounded wood sample (100 mg) was hydrolyzed with 1.5 ml of 6 M hydrochloric acid (HCl) in a 2-ml centrifuge tube at room temperature overnight and then at 100 ºC for 48 h. The samples were stored at 4 ºC until further use. An appropriate internal standard (2 mmol l^{-1} homocysteic acid) was used for all samples. The hydrolyzed samples containing glucosamine were then analyzed by an Agilent 1100 series liquid chromatograph (Agilent technologies, California, USA) equipped with a Shimadzu RF-551 fluorescence detector (Shimadzu, Duisburg, Germany). The analytical column was Zorbax Eclipse XDB-C8 (4.6×75 mm, 3.5mm particle size, Agilent technologies). The results of the chemical analysis expressed in micromoles per liter were converted to mass of chitosan by the use of an average molar mass of 174.5 g mol^{-1} following this

equation:

$$C = \frac{GCA \times DF \times v_o \times Mw\ Chitin}{m_o \times 10^6}$$

Where C—the chitosan amount (mg g^{-1} dry wood powder); GCA—glucosamine con- (µmol l^{-1}); DF—dilution factor; v_o—calculated volume; Mw chitin—174.5 g mol^{-1}; m_o—sample weight (dry wood powder; g).

8. Quantification of silicon content

The quantification of silicon content in wood samples was analyzed by Inductively Coupled Plasma (ICP) technique. The surfaces of three wood samples (1 mm thick) were cut into small pieces. Each sample (100 mg) was transferred to test tube and 2 ml of HCl (37% v/v) was added. Samples were microwave-assisted digested in closed vessels microwave digestion system (Milestone, Sorisole, Italy) loaded with 120 ml of H_2O and 5 ml of H_2O_2 (30% v/v). The heating program was performed in three steps (1.—temperature 1 (T1) = 120 ºC/temperature 2 (T2) = 60 ºC/9 min/110 bar, 2.—T1 = 220 ºC/T2 = 120 ºC/7 min/120 bar and 3.—T1 = 220 ºC/T2 = 120 ºC/8 min/120 bar; 1500 W power was applied). After digestion, each sample was transferred to a volumetric flask and the volumes was adjusted to 12 ml using distilled-deionized water. The standard reference material, tomato leaves (NIST 1573a, National Institute of Standards and Technology, USA) was employed to evaluate accuracy. The content of silicon in the samples was analyzed by ICP using a Thermo Scientific iCAP 6000 Series ICP-OES Spectrometer (Waltham, MA, USA) equipped with an autosampler.

9. Growth of mold on rubberwood

All chitosan and silane treated rubberwood samples (*n=6*) were tested against mold growth according to a modified version of the European Standard EN 152 (1988). Untreated rubberwood was used as a control sample. Wood samples were packed in an air-tight plastic bag and sterilized by Gamma irradiation of 25 KGy. Samples were inoculated with 50 µl of each fungal spore solution and incubated at 25 ºC, 98% RH with periodic observations of fungal growth for 12 weeks. The fungal growth on each sample was rated on a scale of 0–3 as follows:

0 = no fungal growth can be detected visually on the surface.
1 = only individual small fungal spots can be detected none larger than 1.5 mm in width and 4 mm in length and not more than 5 in number.
2 = the surface is covered by the fungi up to a maximum of one third or half of the total area.
3 = the surface is more than half covered by the fungi.

6. Statistical analysis

Results were statistically analyzed using ANOVA and LSD tests were used to evaluate the significant difference between results within the confidence interval of 95 %.

Results

1. Antifungal activity of chitosan

Figure 4.2A shows the antifungal activity of three chitosan samples against *A. niger* BAM 4 on MAE plates. Chitosan C1 at 0.5% w/v completely inhibited the fungal growth with 100% of reduction in colony size. Chitosan C3 at 0.5% w/v showed moderate antifungal activity with 54% reduction in colony size but there was no growth inhibition at 0.063-0.25% w/v. However, chitosan C2 at all concentrations had no effect on the growth of *A. niger* BAM 4. The results of the antifungal activity of chitosan samples against *P. decumbens* CBS 121928 are shown in Figure 4.2B. Chitosan C1 and C2 exhibited 100% reduction in colony size against *P. decumbens* CBS 121928 at the concentrations of 0.125-0.5% w/v while chitosan C3 showed 100% inhibition at 0.5% w/v.

2. The concentrations of chitosan and silicon in the wood samples

A The rubberwood samples were treated with chitosan by vacuum or dip treatment. The highest concentrations of the chitosan solution in this study was limited to 2% w/v because of the viscosity of the chitosan. The results clearly showed that chitosan concentrations in vacuum treated wood samples were higher than in dip treated samples (Figure 4.3A and B). The concentrations of chitosan in vacuum and dip treated

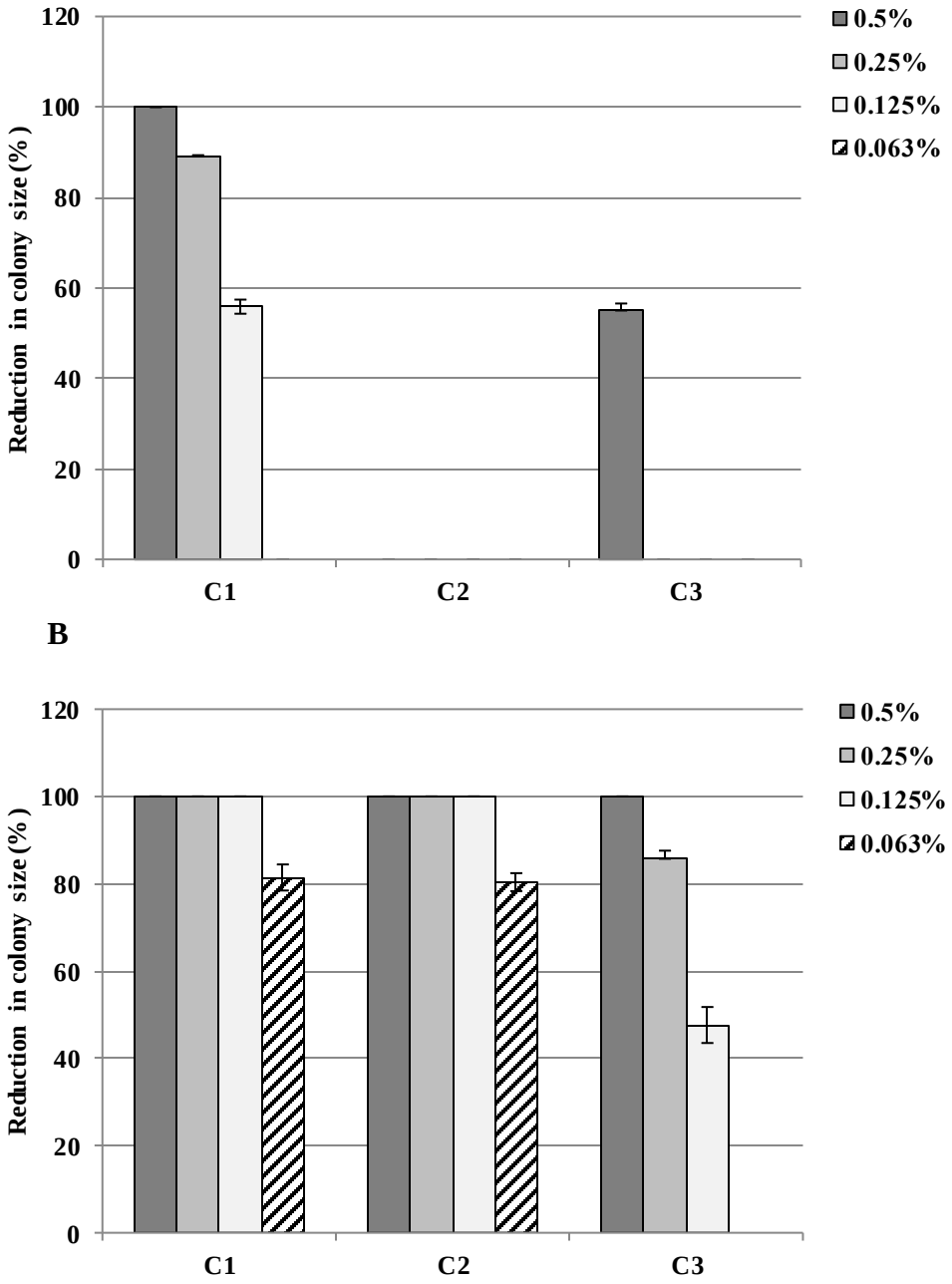


Figure 4.2 Reduction in colony size of *Aspergillus niger* BAM 4 (A) and *Penicillium decumbens* CBS 121928 (B) on media amended with different kinds of chitosan. Growth inhibition of the fungi is expressed as a percentage of fungal colony size in chitosan amended plates compared with control. Plates were incubated at 25 ºC and radial growth of the colony was measure on the 7th days of cultivation.

wood samples after leaching were not much different from unleached samples. The relative content of chitosan in wood after leaching (content of chitosan in wood after leaching divided by content before leaching) indicated the interaction between chitosan and the wood cell wall—the higher relative content, the better interaction. The relative content of chitosan increased with increasing Mw. For instance, calculated from vacuum treated sample with 1% chitosan solution, C1 had 100% relative content while the relative content of C3 was approximately 78%.

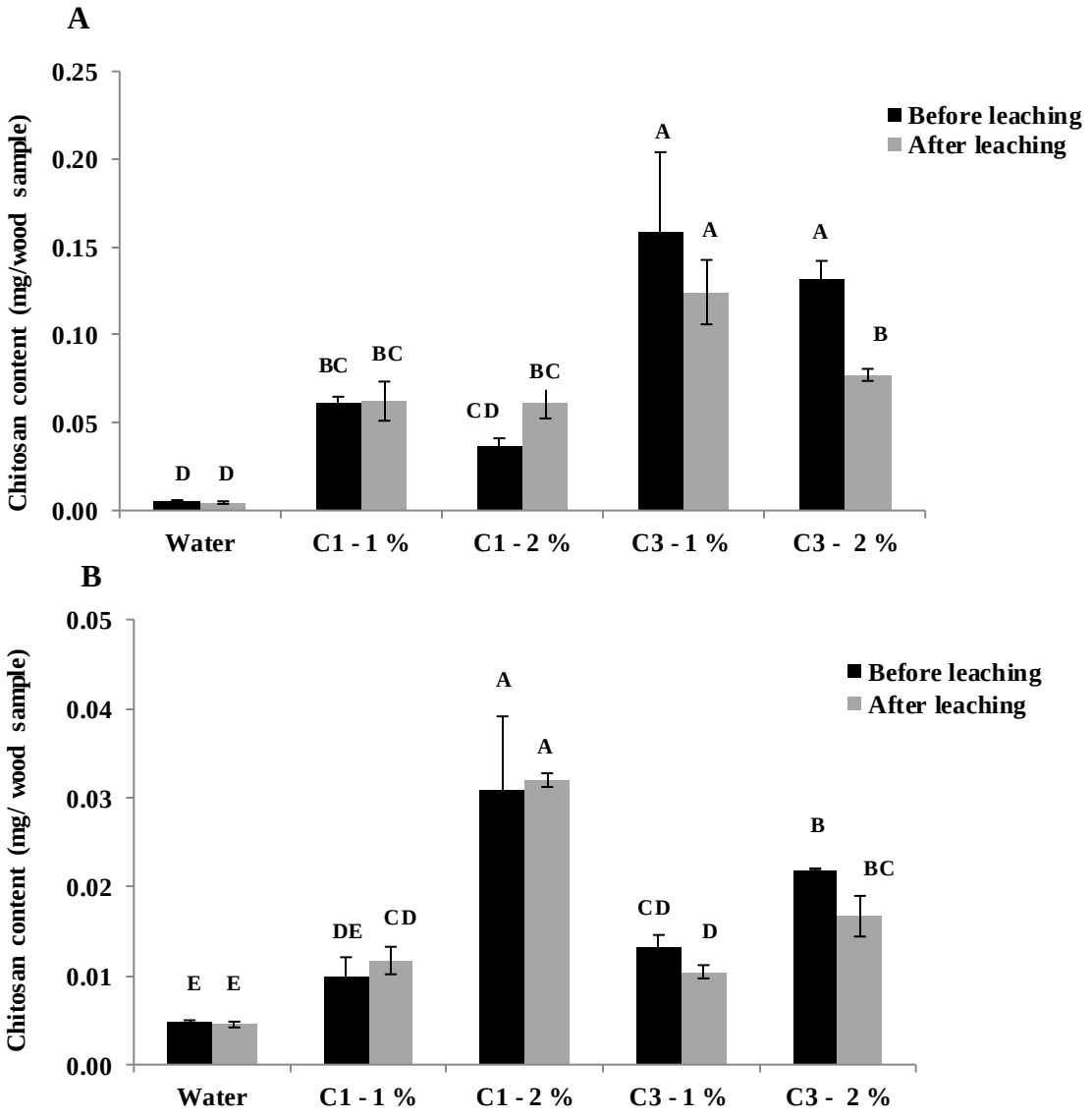


Figure 4.3 Chitosan concentrations in wood samples treated with different kinds of chitosan by vacuum (A) and dip treatment (B) before and after leaching. The results are presented as milligrams of chitosan per wood sample (±1 SD).

The silicon content in vacuum treated wood samples was higher than in dip treated wood samples (Figure 4.4A and B). These results clearly show that vacuum treatment is regarded as a better method than dip treatment because of better penetration and retention. With increasing concentration of silane solution, the contents of silicon in wood increased but the relative content decreased.

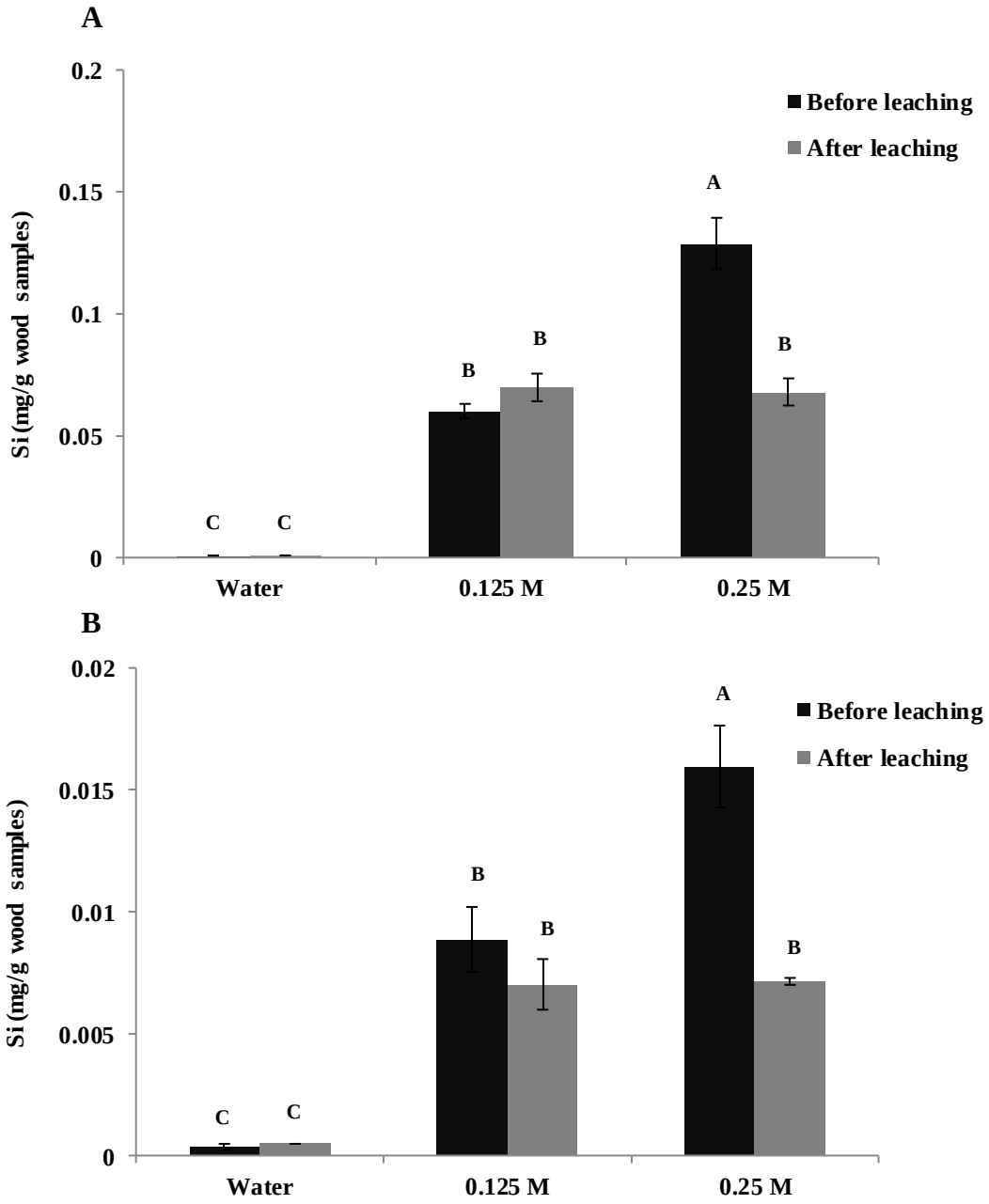


Figure 4.4 Silicon concentrations in wood samples treated with methoxysilane by vacuum (A) and dip treatment (B) before and after leaching. The results are presented as milligrams of silicon per wood sample (±1 SD).

3. Growth of molds on rubberwood

3.1 Growth of molds on chitosan treated rubberwood

Mold-inhibiting activities of chitosan C1 and C3 on wood against *A. niger* BAM 4 are shown in Figure 4.5. Fungal growth on untreated wood samples reached rate 3 in two weeks. Both chitosan C1 and C3 at the lowest concentration tested (1% w/v) could completely protect rubberwood from *A. niger* BAM 4 on vacuum treated wood samples (Figure 4.5A) after 12 weeks. In contrast, on leached samples, chitosan could not totally prevent the growth of *A. niger* BAM 4 (Figure 4.5B). It is obvious that the leaching weakened the performance of chitosan on wood against mold. Interestingly, the dip treatment also reduced the growth of *A. niger* BAM 4 on wood but it was not effective as vacuum treatment method (Figure 4.5C and D). The mold test on wood clearly shows that the capabilities of chitosan C1 and C3 in prevention of mold growth on wood were insignificantly different.

The resistances of chitosan treated wood samples against *P. decumbens* CBS 121928 are presented in Figure 4.6. The infection value of untreated and water treated wood samples reached a score 3 on the second week (Figure 4.6A and B). When Treated with C1 or C3, by vacuum or dip treatment, mold infection also reached a score 3 in two weeks. Wood samples treated with chitosan C1 and C3 showed no resistance against the growth of *P. decumbens* CBS 121928.

3.2 Growth of molds on silane treated rubberwood

Growth of *A. niger* BAM 4 and *P. decumbens* CBS 121928 on silane (AEAPTMEOS) treated wood samples obtained from vacuum and dip treatment did not differ from untreated wood after 12 weeks (Figure 4.7). It was revealed that treatment of rubberwood with silane could not inhibit molds growth.

Discussion

Inhibition of fungal growth with increasing chitosan concentrations in agar plates has been reported in several studies (Guerra-Sánchez et al., 2009; Li *et al.*, 2009; Al-Hetar *et al.*, 2011; Cota-Arriola et al., 2011; Rahman *et al.*, 2014). Although

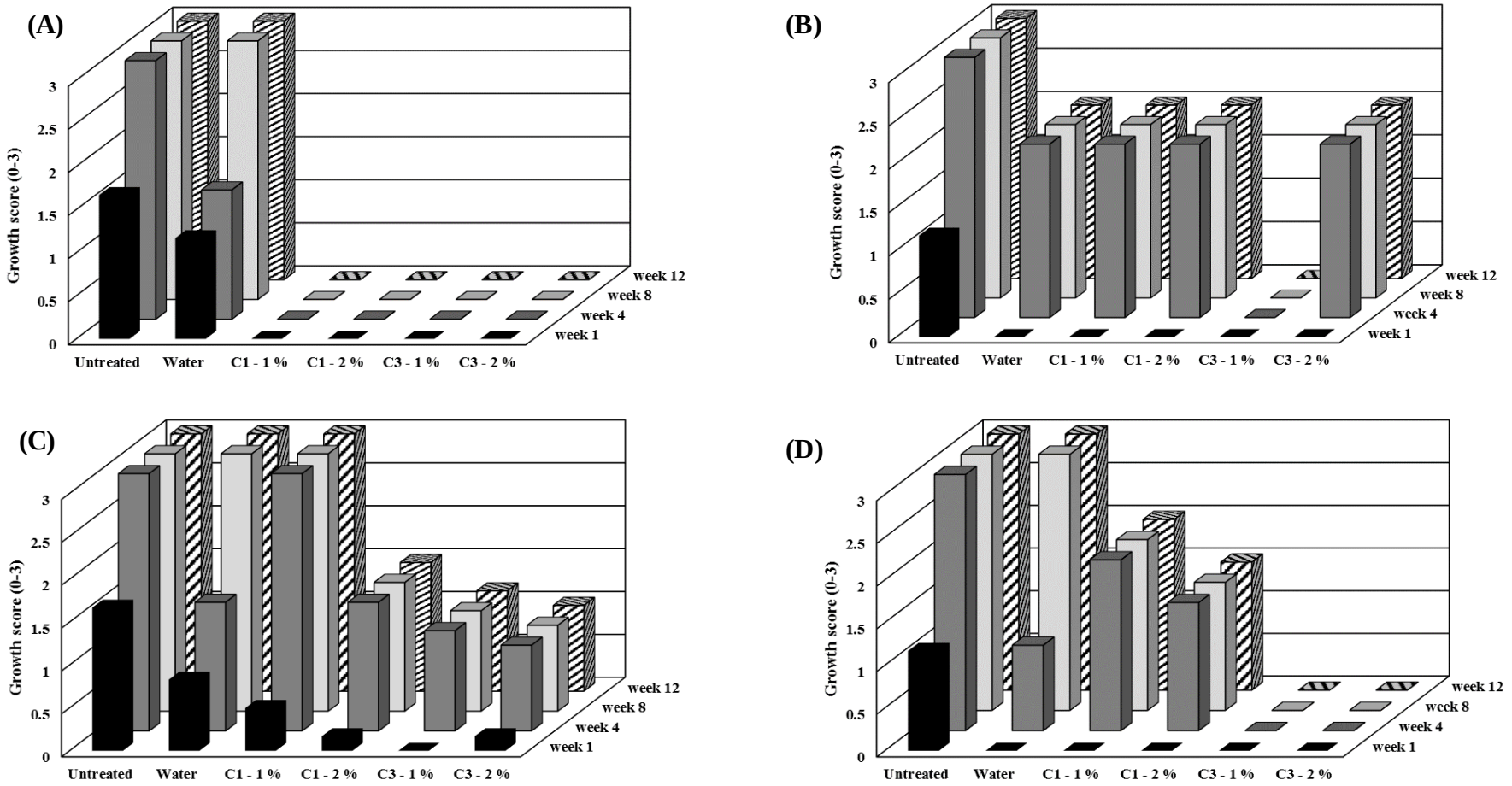


Figure 4.5 Growth of *A. niger* BAM 4 on chitosan treated wood samples incubated at 98% RH, 25 ºC: vacuum treated wood samples (A), vacuum treated wood samples after leaching (B), dip treated wood samples (C) and dip treated wood samples after leaching (D).

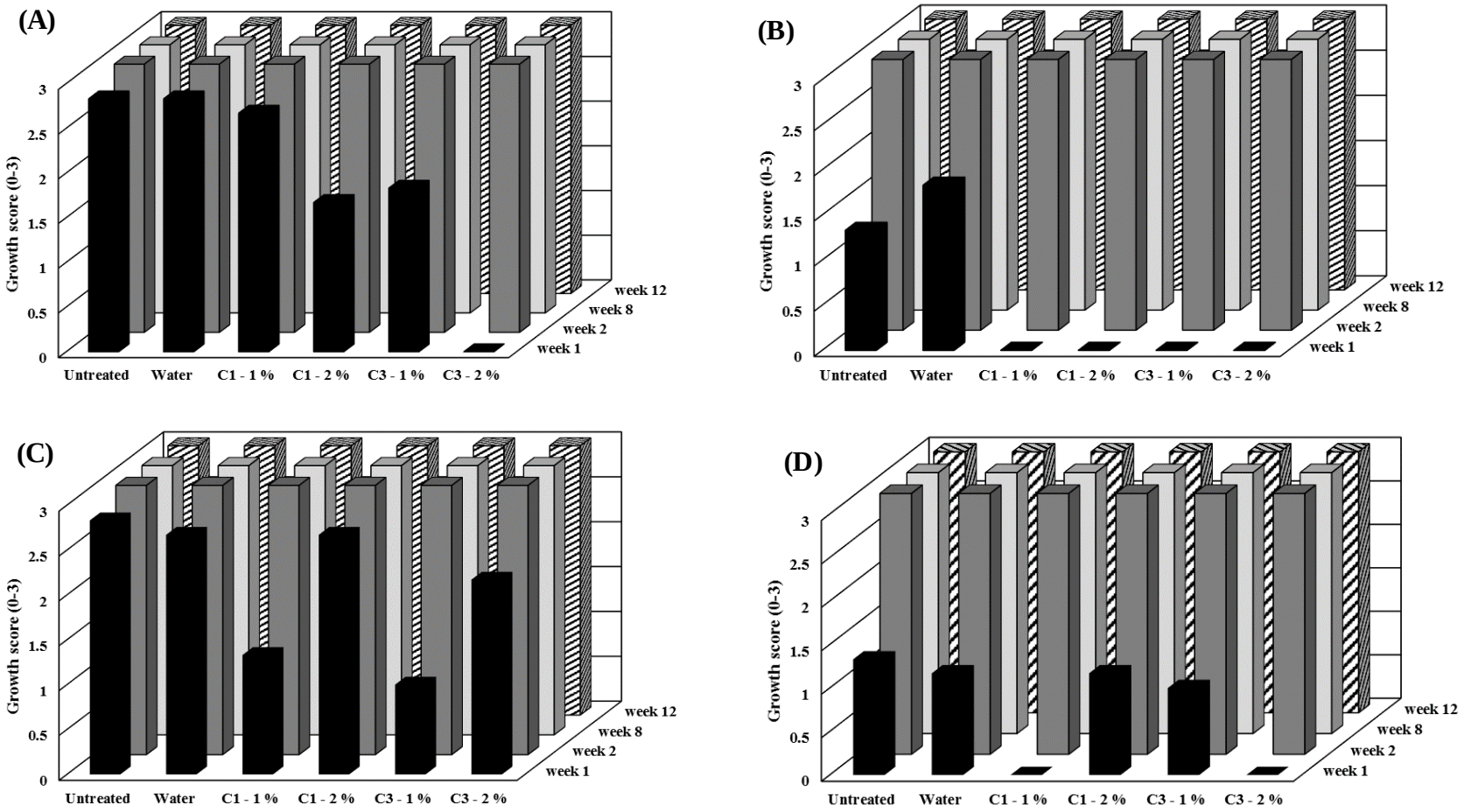


Figure 4.6 Growth of *P. decumbens* CBS 121928 on chitosan treated wood samples incubated at 98% RH, 25 ºC: vacuum treated wood samples (A), vacuum treated wood samples after leaching (B), dip treated wood samples (C) and dip treated wood samples after leaching (D).

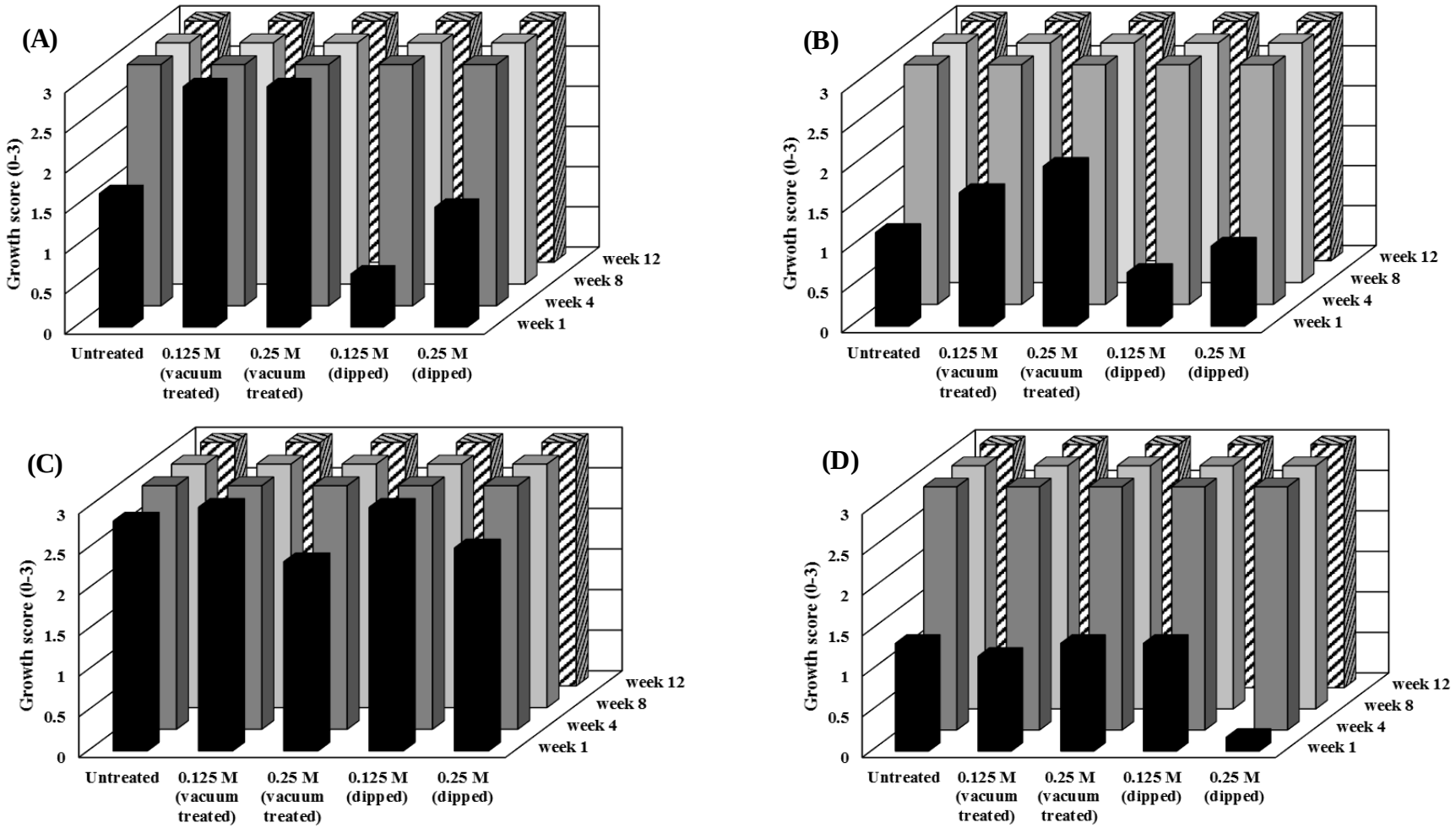


Figure 4.7 Growth of molds on methoxysilane treated wood samples incubated at 98% RH, 25 ºC: *Aspergillus niger* BAM 4 on unleached (A) and leached wood samples (B) and *Penicillium decumbens* CBS 121928 on unleached (C) and leached wood samples (D)

increasing the concentrations of chitosan may increase the antimicrobial efficacy of chitosan on many fungi and bacteria, this trend depends on several factors such as Mw, FA, targeted pathogen and pH (Younes *et al.*, 2014). The study by Guo *et al.* (2008) showed that the antifungal activity of chitosan decreased with decreasing Mw. Besides, Cota-Arriola *et al.* (2011) found that higher Mw showed stronger inhibitory effect. Our result clearly indicated that the effectiveness of chitosan against fungi was dependent on species of target fungi. For example, chitosan C2 could inhibit the growth of *P. decumbens* CBS 121928, but not the growth of *A. niger* BAM 4. Additionally, *P. decumbens* CBS 121928 was more sensitive with 100% reduction in colony size for all the chitosan samples tested at a 0.5% w/v concentration. This finding is in agreement with other studies, where the antifungal activity of chitosan has been reported to be specific with fungal species (Laflamme *et al.*, 2000; Singh *et al.*, 2008). Further, the sensitivity of a fungus to chitosan is influenced by its plasma membrane composition. Palma-Guerrero *et al.* (2010) found that the plasma membranes of chitosan-sensitive fungi contained more polyunsaturated fatty acids than chitosan-resistant fungi. The antifungal properties of chitosan were reported against various fungi including *P. digitatum*, *P. italicum*, *A. niger*, *A. parasiticus* at concentrations ranged from 0.05 to 0.4% w/v (Sebti *et al.*, 2005; Chien and Chou, 2006; Cota-Arriola *et al.*, 2011). C1 and C3 showed strong antifungal activity at 0.5 % w/v for both molds; so, these chitosan samples were chosen for the treatment of rubberwood.

The leaching results show that chitosan still well retained in the wood after leaching and the fixation of chitosan in the wood was good. Fixation of wood preservative is considerable for long-term persistence. The electrostatic interactions between chitosan and the hydroxyl groups of cellulose is important for the formation of chitosan with wood. Hydroxyl groups has a strong tendency to form hydrogen bonds with chitosan. Therefore, binding of chitosan with wood is facilitated by the affinity between charged cellulose and chitosan (Eikenes *et al.*, 2005a; Patel *et al.*, 2013). Chitosan with higher Mw interacted with wood better than that lower Mw. Larnøy *et al.* (2010) reported a similar result shown that the higher Mw of chitosan had better fixation in wood. This can be explained by the DP_n of chitosan which decides Mw of chitosan. DPn has effects upon the biological and functional properties of the chitosan. The binding reaction between chitosan and wood is also influenced by the density of

charged amino groups of chitosan. Chitosans with lower DP_n have lower number of amino groups in the polymer chains, therefore they have the lower binding capacity (Hejazi and Amiji 2003). The DP_n of chitosan C1 (DP_n = 220) is higher than chitosan C3 (DP_n = 20), thus it has better interaction with wood.

The concentrations of silicon in wood were well correlated with the concentrations of silane in the impregnation solutions. Silanes are hydrolyzed to silanol groups and these groups will form the covalent bonds with the hydroxyl groups of wood cell walls (Donath *et al.*, 2004). This interaction is important for the binding of silane with wood. The ability of silane to interact with the wood cell wall was reduced by increasing the concentration. Higher concentration of silane in the solution may indicate a certain saturation level of silane adsorbed to the cell wall of wood.

Chitosan has been reported to work well for postharvest protection of various products (Cruz-Romero *et al.*, 2013; Aloui *et al.*, 2014; Simonaitiene *et al.*, 2015) and wood samples (Alfredsen *et al.*, 2004; Eikenes *et al.*, 2005a). In this study, the results implies that chitosan possessed inhibitory activity against molds on wood and there were differences in responses to the two fungi between different chitosan sources and different treatment methods. Chitosan samples have the ability to inhibit or reduce the development of *A. niger* BAM 4 on rubberwood for at least 12 weeks. The results of vacuum treatment showed that 1% w/v C1 and C3 was adequate to protect wood against *A. niger* BAM 4. Li and Yu (2001) also reported that chitosan decreased the incidence of brown rot fungi of postharvest peaches fruit at a concentration of 1% w/v. However, the concentration of chitosan at 5% w/v was reported for total protection of wood against decay fungi (Alfredsen *et al.*, 2004; Eikenes *et al.*, 2005a). Wood is sometimes exposed to water and wood preservative may be released. Obviously, after leaching, the inhibition effect of chitosan was weakened. The growth score of *A. niger* BAM 4 on leached sampled was higher than that of unleached sample. This result is concordant with the studies of Eikenes *et al.* (2005a) and Alfredsen *et al.* (2004) who found that the leached wood samples had an increasing decay rate compared to unleached samples. Further investigations are needed to improve the better fixation in wood to obtain a more complete protection.

Chitosan C1 and C3 showed insignificantly strong inhibition against *A. niger* BAM 4. This demonstrated that Mw had no effect on their antifungal activities

on wood. Several studies indicated the relation of Mw of chitosan and its antifungal activities. The study by Chittenden *et al*. (2003) suggested that the antifungal effects of low Mw chitosan was more effective than that of high Mw chitosan. On the contrary, the study of Alfredsen *et al*. (2004) and Eikenes *et al*. (2005a) presented that high Mw chitosan perfectly reduced the decaying rate of wood decay fungi while low Mw chitosan was less efficient. However, the study of Younes *et al*. (2014) showed that decreasing or increasing Mw was not related to the antifungal activity of chitosan but it depended on the particular type of fungi. It is plausible that when chitosan is applied in wood, not only the function of Mw bus also number of factors such as pH and type of wood impact its mold-inhibiting capability.

Chitosan was not effective against *P. decumbens* CBS 121928 on wood samples either vacuum or dip treated although it could completely inhibit this fungus in agar plate. The 2% w/v chitosan solutions was insufficient to inhibit *P. decumbens* CBS 121928 on wood. The type of microorganism is one of the factors which influences the antimicrobial activity of chitosan. In addition, the conditions on wood might be different from in the agar plate for example, pH, nutrients and the structure of wood (Fadillah *et al*., 2014). These might influence the effectiveness of chitosan against *P. decumbens* CBS 121928 on wood. However, it should be noted that 98% RH is an extremely high humidity and with lower humidity or in the natural environment the performance of chitosan might have been better.

Previous studies report the efficacy of silane on the improved properties of wood such as resistance to fungal attack and enhanced strong hydrophobation (Donath *et al*., 2004; Mai and Militz, 2004). Recently, it was reported that [γ-(methacryloxy)propyl] trimethoxy silane and vinyl trimethoxy silane were effective against fungal decay in *Pinus nigra* (Hill *et al*., 2004). These improvements are due to a stable incorporation of silane in the wood as a result of internal crosslinking in the treated material. Moreover, wood species, wood moisture content and other properties affect the effectiveness of wood modification. One possible explanation is that methoxysilane might not entirely form covalent bonds with the hydroxyl group of rubberwood cell walls and it was not fixed in the cell wall, thus its ability to preserve wood was not achieved. It could be affected by the treatment procedures which also influence the effectiveness of wood preservatives. It is feasible that dip and vacuum

treatment applied in this study might not provide an increasing penetration depth of silane into wood cells. Treatment processes that involve pressure where the preservatives have penetrated deeply may improve the performance of silane on wood protection.

Regarding the Biocide Directive, the leaching of chitosan or silane can happen in field tests, but this will not cause an environmental problem or human effect. Chitosan is used in pharmaceutical industry as a diet pill for human and for water Purification. Silanes are relatively friendly to the environment and have been used as surface treatment to control of microbial growth for food and beverage industry (Kregiel, 2014). The concentration of leached chitosan or silane would be unable to act as a biocide (Alfredsen *et al.*, 2004). There is no harm to the environment or humans by chitosan and silane.

In the present study, rubberwood treated with chitosan by vacuum and dip treatment method had improved properties against mold growth. Furthermore, leaching results showed a good fixation of chitosan in vacuum and dip treated wood. Both wood treatment methods are simple and practical to be used in less industrialized area (Baillères *et al.*, 2004). Undoubtedly, dip treatment is cheaper and does not require a vacuum chamber; so dip treatment method may be more cost-effective and better for application of chitosan in prevention of rubberwood.

Conclusion

The present study showed that chitosan C1 and C3 were effective on rubberwood against mold. Chitosan is not sensitive to be degraded and it could be used to protect mold growth on wood instead of chemical preservatives. Thus, the application of chitosan as wood preservative should be considered for its use on an industrial scale. However, an improvement of the fixation need to be investigated to develop the durability of chitosan treated wood exposed to water. Vacuum and dip treatment were effective method of applying chitosan to rubberwood. Interestingly, dip treatment is a simpler method and may be more useful for superficial protection against molds. Silane was not efficient in inhibiting mold growth on rubberwood. Further studies looking closer into treatment processes that involve pressure may improve the efficiency of silane used in rubberwood.

CHAPTER V

Treatment of rubberwood with maleic anhydride to prevent molds

Abstract

Rubberwood samples were treated with 0.5-10% MA solutions. Treatment of rubberwood with 2.5% MA was adequate to prevent growth of molds on wood for up to 12 weeks. The viable count of *A. niger* PSU1 on the MA treated wood indicated that fungal spores were not killed. The moisture contents of MA treated and untreated wood samples were not significantly different. The concentration of MA released from treated wood in the leachate was 0.02 mg mm^{-3} after 60 min leaching in water. However, after leaching MA treated wood still had high resistance to mold growth. Agar well diffusion showed that the leachate had no antifungal activity. The diffusion test showed that MA treated wood slice showed no fungal growth inhibition. The leachates from both MA treated and untreated wood samples had similar cytotoxic effect. The SEM study showed that the surface of MA treated wood had almost smooth surface while the untreated wood showed rough surface. The weathering test was conducted and weathered samples were seriously infected. Treatment of rubberwood with MA by dip and spray treatment together with decreasing the reaction temperature from 90 to 70 and 50 °C were studied. Additionally, with and without soaking stage after the heat treatment were compared. The results showed that dip treated samples reacted with MA at 70 and 50 °C and without soaking stage had strong resistance against molds compared to the sprayed samples.

Introduction

An alternative method of improving the fungal growth resistance of wood without the use of chemical biocides is chemical wood modification. The chemical modification of wood using di- and tricarboxylic acid anhydrides has been reported (Papadopoulos and Hill, 2002; Iwamoto and Itoh, 2005). Treatments of wood with various organic anhydrides have the potential to increase fungal resistance and to replace the use of biocides for wood preservation (Schiopu and Tiruta-Barna, 2012). Wood chemical modification using anhydrides is accomplished by reacting the hydroxyl groups of the cell wall polymers with selected anhydride, to form a covalent bond without leaving toxic residues within the wood (Papadopoulos *et al.*, 2008). If free hydroxyl groups are occupied and the access to water is prevented, the susceptible to fungal attack is reduced (Hill *et al.*, 2004).

Maleic anhydride (MA) is one of the chemicals that is usually used for chemical wood modification (Tjeerdsma *et al.*, 2005; Hill, 2006; Clemons *et al.*, 2007). This chemical bears non-polar endings capable of bonding with polar structures such as wood (Hill, 2006). The reaction of wood with MA is displayed in Figure 5.1. Maleic anhydrides do not yield a by-product when reacting with the hydroxyl groups of wood (Hill and Mallon, 1998). Thus, it can be suggested that a chemical modification such as treatment of wood with MA, which can eliminate hydrophilic groups from the wood, would improve wood's performance against molds.

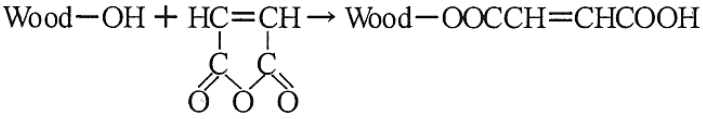


Figure 5.1. Reaction of wood with MA
Source: Iwamoto and Itoh (2005)

Previous studies showed that treatments of wood with MA enhanced the resistance of treated wood that has been exposed to the attack of brown and white-rot fungi. Maleic anhydrides treated samples of different softwoods have been tested against different standard fungal species, e.g., *Fomitopsis palustris* and *Trametes versicolor* and it has been observed that a better durability is achieved through this pro-

cess (Fujimoto, 1992; 1995; Iwamoto and Itoh, 2005).

The information about the protective effect of MA in rubberwood, especially against molds, is rare. Therefore, the present study investigated the possibility of using MA as wood treatment in order to improve resistance of rubberwood against molds. This present work also aimed to apply MA in wooden toys. Children may obtain MA by saliva during chewing and leachable MA may be toxic against human cells, so, cytotoxicity of leachates from MA treated wood was investigated. In this study, the reaction between MA and wood was carried at high 90 °C and the treatment method was time consuming for the industrial scale. Reduced reaction temperature and time of treatment process are important for industrial area as they may be more cost effective. Therefore, effects of reaction temperatures and treatment methods on mold growth resistance of MA treated wood were also determined. Additionally, dip and spray treatment were applied and the results were compared.

Materials and Methods

1. Fungal cultures

A. niger PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3 were isolated from the contaminated rubberwood samples. Fungal spores were collected from the mold grown on PDA plate at 25 °C for 7 days and counted using a haemocytometer. The spore suspension was standardized to 10^6 spores ml^{-1} before use as an inoculum.

2. Preparation of MA solutions and wood treatment

Maleic anhydrides was dissolved in water as concentrations of 0.5, 1.0, 1.5, 2, 2.5, 5 and 10% (w/v). The rubberwood samples (n=5 for each concentration) were dipped in the MA solutions for 5 min (five volumes of MA solution to one volume of wood), immediately wrapped in foil and placed in an oven at 90 °C for 2 h. Samples were soaked in water for 10 min to remove non-reacted MA and then dried at 90 °C for 2 h.

3. Growth of fungi on rubberwood and viable count

Maleic anhydride treated and untreated wood samples were packed in an air-tight plastic bag and sterilized by Gamma irradiation of 25 KGy at Thailand Institute of Nuclear Technology, Thailand. Samples were inoculated with 50 µl of spore suspension of *A. niger* PSU1, *A. flavus* PSU2 or *P. citrinum* PSU3 on surface and incubated at 25 ºC and 100% RH. The fungal growth on the surface of the wood samples was observed weekly for 52 weeks. The fungal growth on each sample was rated on a scale of 0–5, following the method of ASTM (ASTM, 1998). The lowest concentration showing total protection against molds were selected for further studies.

For a viable count, *A. niger* PSU1 was selected as a model fungus. Maleic anhydrides treated and untreated rubberwood samples were inoculated with 50 µl of *A. niger* PSU1 spore suspension and kept in the humidity chamber at 25 °C and 100% RH. At predetermined time points (3 h and 1 to 7 days), rubberwood samples (n = 3) were removed from the chambers and placed in a sterile flask containing 5 ml of 0.1% (w/v) tween-80 solution and shaken for 30 min at room temperature. Sample (1 ml) was removed from each flask and serially diluted with sterile 0.1% (w/v) tween-80 solution and a 100 µl aliquot was spread on PDA containing ampicillin (50 mg l^{-1} of PDA) plate and incubated at 25 °C for 72 h. The viable count of *A. niger* PSU1 was compared with the viable count on day 0 as follow:

$$\text{Viable count (\%)} = (D_n \times 100)/D_0$$

where D_0 = the number of fungus counted on day 0 and D_n = the number of fungus counted on day 1 to day 7.

4. Determination of wood moisture content

Both MA treated and untreated wood samples were dried at 103 °C for 18 h and their oven-dried weights were measured. Wood samples were incubated in the humidity chamber maintained at 25 °C and 100% RH. The weight of the wood samples were determined periodically until they reached constant mass and the equilibrium moisture content of the materials was calculated as follows:

$$EMC = (M_{final}-M_{initial})/M_{initial} \times 100$$

where EMC is the equilibrium moisture content (%); $M_{initial}$ is the final weight of wood

material at 103 °C (g) and M_{final} is the final weight of moist wood kept at 100% RH (g).

5. Leaching study

Both maleic anhydrides treated and untreated wood samples were leached in mammalian cell culture medium (Dulbecco's Modified Eagle Medium; DMEM). Samples of MA treated and untreated wood were sterilized by autoclaving. The wood samples (n = 3) were separately placed in 100-ml sterile laboratory glass bottle containing 20 ml of sterile DMEM (five volumes of DMEM to one volume of wood). The samples were then shaken at 200 rpm and the leachates (2 ml) were collected at 5, 15, 30 and 60 min and 2 ml of DMEM was added to make up the volume each time. The leachates were used further in antifungal and cytotoxicity tests. The contents of released MA in the leachates were analyzed by a HPLC.

6. HPLC analysis

The leachates of MA treated and untreated wood samples were analyzed in comparison with the standard solution of the MA (1.25, 2.5, 5, 10 mg ml^{-1}). The analyses were performed using an Agilent 1200 series (Agilent Technologies, Germany) liquid chromatography coupled with a diode array detector (DAD). The analytical column C18 (Agilent 5 µm, 250 × 4.6 mm) was thermostatically controlled at 25 °C. The mobile phase was dicyclohexylamine:formic acid:methanol:water (0.5:0.5:25:74). The UV detector wavelength was set at 254 nm. The flow-rate was 1.5 ml min^{-1} and the injection volume was 20 µl and run time was 10 min.

7. Antifungal activity of leachates and diffusion test

The antifungal activity of leachates was tested against *A. niger* PSU1 by an agar diffusion method. A 0.4 ml of fungal spore suspension (10^8 spores ml^{-1}) was added to 3.6 ml of sterile molten PDA then overlaid onto a PDA plate (16 ml) and allowed to solidify. Wells of 5 mm diameter were aseptically bored into the agar and 50 µl of sterile filtered leachates were added to the wells.

The diffusion test was performed to measure whether there was any inhibition of the fungal growth when MA treated wood samples were placed together

with the tested fungus. The MA treated and untreated round wood slice (20 mm diameter×5 mm thick) samples (n = 3) were placed in the center of inoculated agar plates. The plates were incubated at 25 °C for 72 h and the diameter of the inhibition zone was measured.

8. Cytotoxicity assay

The cytotoxic activity of leachates of both MA treated and untreated wood samples was studied against human keratinocyte cells using MTT assay (Mosmann, 1983). In this method, the optical density of the solution containing the formazan produced by metabolically active cells is measured spectrophotometrically. Briefly, the cells ($1x10^4$ cells/well, cultured in 96-well microplates) were incubated at 37 ºC and 5% CO_2 for 3 days. The leachates (n=3) collected at 60 minutes of leaching test were sterilized by filtration method (0.2 µm syringe filter) and added to seeded wells in triplicate and incubated at 37 ºC for 1 h. Control wells was DMEM. At the end of the incubation time, cultured plates were washed with a sterile phosphate buffer saline (PBS, pH 7) solution. The MTT solution (5 mg ml^{-1} in PBS) was subsequently added to each well and plates were incubated at 37 ºC for another 3 h. Supernatants were then discarded and 100 µl of DMSO was added to the cultures and mixed thoroughly. Formazan quantification was performed using an automatic plate reader (Multiskan™ GO, Thermo Scientific, USA) at 562 nm. The percentage of viability was determined using the following formula:

viability (%)= (treatment absorbance×100)/(control absorbance)

9. Microscopic examination

Scanning electron microscope (SEM) was used to examine the surface of the rubberwood samples. The MA treated and untreated rubberwood samples were fixed in 3% glutaraldehyde, dehydrated in graded series of alcohol; air-dried and then coated using a gold sputter coater. The coated specimens were examined with a SEM (Quanta400, FEI, Czech Republic) at the Scientific Equipment Center, Prince of Songkla University.

10. Weathering test

Weathering test was conducted following the method modified from Japanese Industrial Standard (JIS) K 1571 (JIS, 2004). Wood samples were immersed in deionized (DI) water in a ratio of 10 volumes of water to 1 volume of wood and shaken at 200 rpm, 25 °C for 8 h and followed by drying at 60 °C for 16 h. This cycle was repeated 5 times. Weathered samples were tested against fungal attack following the method of ASTM as described in section 4.

11. Resistance of rubberwood treated with MA by dip and spray treatment against molds: reduced reaction temperature and soaking or non-soaking treatment process

Maleic anhydride was dissolved in water with a concentration of 2.5% (w/v). The rubber wood samples (n=3) were either dip treated or sprayed with the MA solution (Scheme 1).

- **Dip treatment**

Rubber wood samples were dipped for 5 min in MA solution (five volumes of MA solution to one volume of wood), immediately wrapped in foil and placed in an oven at 50, 70 and 90 °C for 2 h.

- **Spray treatment**

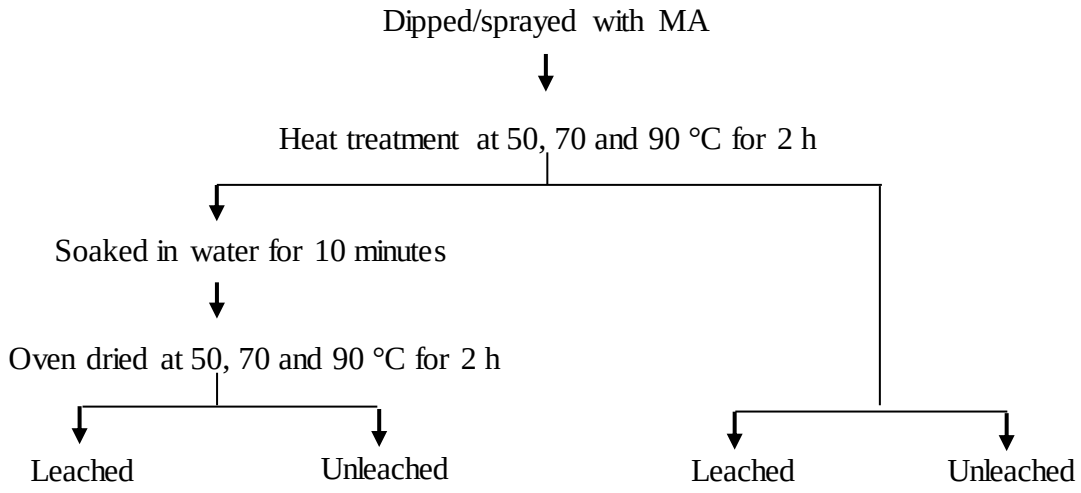
Rubber wood samples were sprayed with MA solution (approximately 1.85 ml of MA solution to one wood sample) and left at room temperature for 5 min. The sprayed wood samples were wrapped in foil and placed in an oven at 50, 70 and 90 °C for 2 h.

After oven drying, samples of dip treated and sprayed wood was separated into two sets. Two methods were applied; the samples from the first set were soaked in water for 10 min then dried at 50, 70 and 90 °C for 2 h before further use, while the samples from the second set were used for further experiments without soaking and drying stages.

For preparation of leached samples, half of the samples from each above mentioned treatment and untreated wood were leached in water (five volumes of water

to one volume of wood) for 60 minutes and dried at room temperature for 48 h.

Growth of molds was evaluated on unleached and leached samples. All wood samples were sterilized by Gamma irradiation of 25 KGy. The samples were inoculated with fungal spore and growth was rated following the method of ASTM as described in section 4.



Scheme 5.1 The treatment methods for MA and rubberwood

12. Statistical analysis

Results were statistically analyzed using ANOVA and LSD tests were used to evaluate the significant difference between results within the confidence interval of 95 %.

Results

1. Growth of molds on rubberwood

Growth of *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU2 on MA treated rubberwood at 25 °C and 100% RH after 52 weeks of incubation is displayed in Figure 5.2 and 5.3. Growth of molds on 0.5-1.5% MA treated wood was 100% By contrast, when 2% MA was used, *A. flavus* PSU2 and *P. citrinum* PSU3 were inhibited but growth of *A. niger* PSU1 was 100%. However, rubberwood treated with 2.5-10% MA showed no fungal growth at least up to 52 weeks, at which time observation was

discontinued. Based on this result, 2.5% MA was used to treat wood samples for further study. The samples treated with 2.5% MA was leached in water and after 12 weeks, they also had no fungal growth.

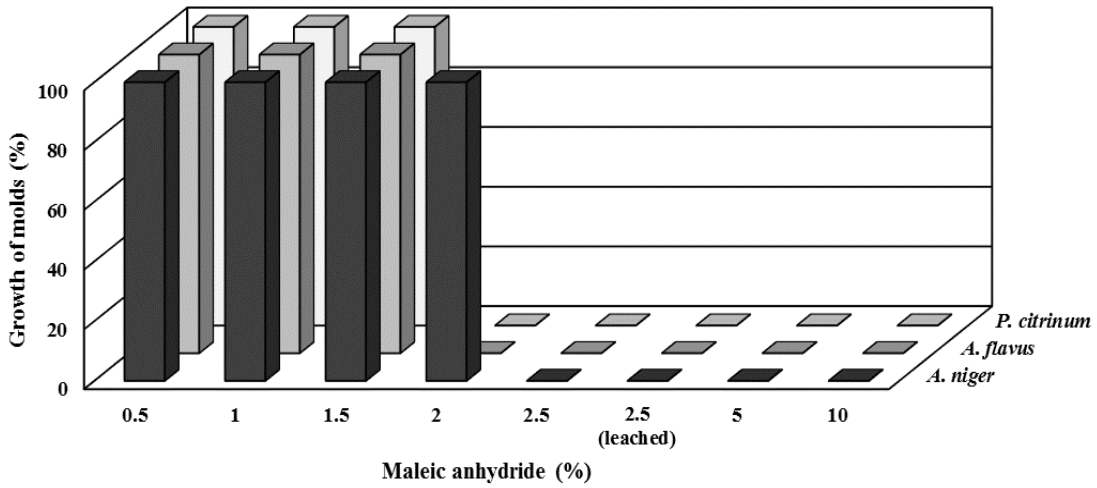


Figure 5.2 Growth of inoculated molds: *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3 on maleic anhydride treated and untreated rubberwood (n=5) incubated at 25 °C and 100% RH for 52 weeks.

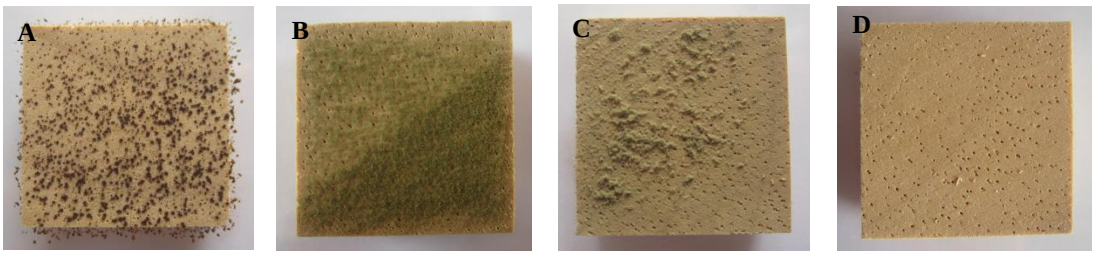


Figure 5.3 Colonization of *A. niger* PSU 1 (A), *A. flavus* PSU 2 (B), *P. citrinum* PSU3 (C) on untreated rubberwood and no visible mold colonization on MA treated rubberwood. All samples were incubated at 25 °C and 100% RH for 52 weeks.

2. Viable count

The percentage of relative viabilities of *A. niger* PSU1 on MA treated wood monitored for 7 days are presented in Figure 5.4. After 24 h, the percentage of viability of *A. niger* PSU1 was approximately 90% and subsequently maintained at 85

to 60% in 5 days. On day 7, 53% of viability was observed

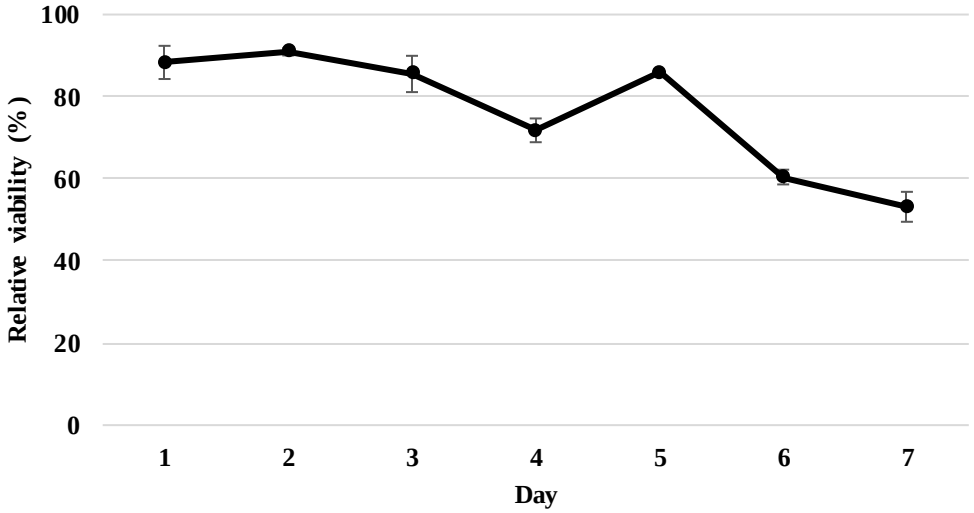


Figure 5.4 Plots of the relative viability (%) of *A. niger* PSU1 on 2.5% maleic anhydride treated rubberwood at 100% RH, 25 °C.

3. Moisture content of rubberwood

The moisture contents of MA treated and untreated rubberwood samples kept at 25 ºC and 100% RH after 8 days were 16 and 17.5%, respectively (Figure 5.5). The moisture content of MA treated wood was not significantly different in comparison with that of untreated wood ($p < 0.05$).

4. Leaching of MA treated wood

Rubberwood samples treated with MA at 2.5% were leached in Dulbecco's Modified Eagle Medium. The concentrations of the leached MA are shown in Figure 5.6. Maleic anhydride was leached out from the treated wood. When the leaching duration increased, the concentrations of released MA increased gradually. Total concentration of MA leached from rubberwood for 60 min was approximately 0.02 mg mm^{-3}.

5. Antifungal activity of leachates

The result of agar well diffusion for antifungal activity of the leachates

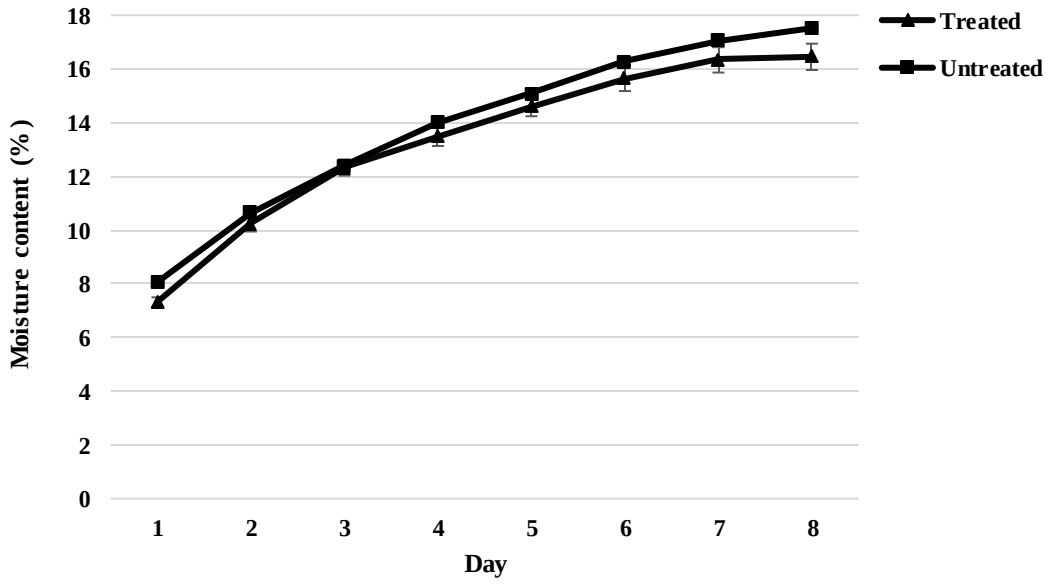


Figure 5.5 Moisture contents of maleic anhydride treated and untreated rubberwood samples incubated at 100% RH, 25 °C.

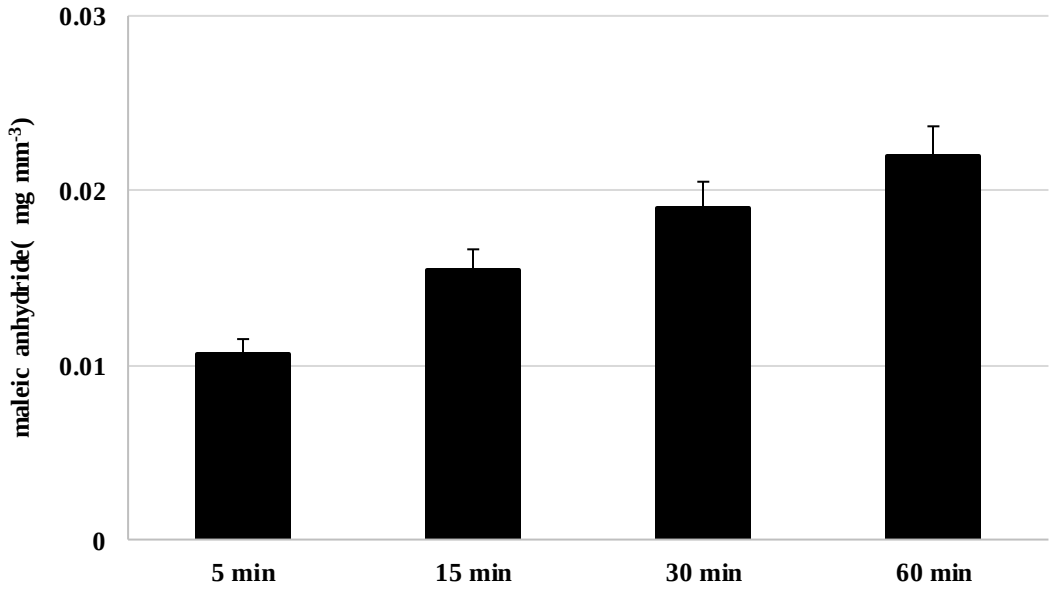


Figure 5.6 Effect of leaching time on the maleic anhydride leached out from the treated wood.

from MA treated and untreated wood collected after 60 min of leaching showed that there was no fungal inhibition zone against *A. niger* PSU1. Both kinds of leachates

affected mold growth at the same rate. Although the leachate from MA treated wood contained MA, it did not show antifungal activity.

6. Wood diffusion test

Maleic anhydride treated and untreated round wood slice were placed on the center of the inoculated plate and the inhibition zone was measure. After 72 h of incubation, no inhibition zone was observed on agar plates placed with both types of wood samples. The fungal grew on the agar plate normally. It is obvious that there was no inhibition of the fungal growth when inoculated together with MA treated wood (Figure 5.7).

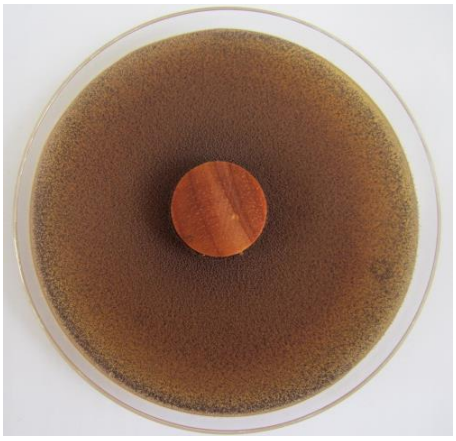


Figure 5.7 *A. niger* PSU1 inoculated together with MA treated wood slice after 72 h of incubation.

7. Cytotoxicity assay

No significant differences ($p > 0.05$) were seen between the survivals of keratinocyte cells in the absence and presence of MA in leachates (Figure 5.8). The cell viability incubated with both leachates were about 50% after 60 minutes incubation. Both kinds of leachates were highly toxic against human cell lines.

8. Microscopic examination

The rubberwood treated with MA results in significant changes of wood surface morphology. The SEM result showed that the treated wood had smooth surface

while the untreated wood had rough surface (Figure 5.9A and B), suggesting that the rough surface on wood are physically changed.

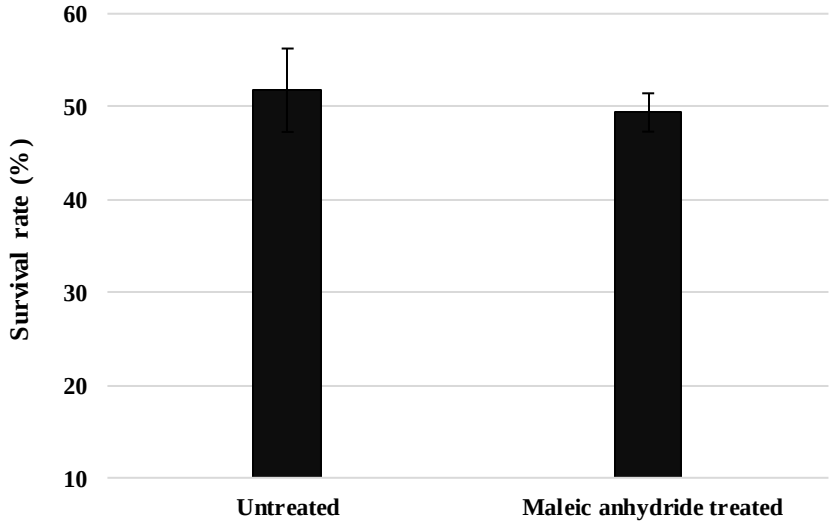


Figure 5.8 Cytotoxic activity on keratinocyte cells of leachates from untreated wood samples and maleic anhydride treated wood samples collected at 60 min of leaching.

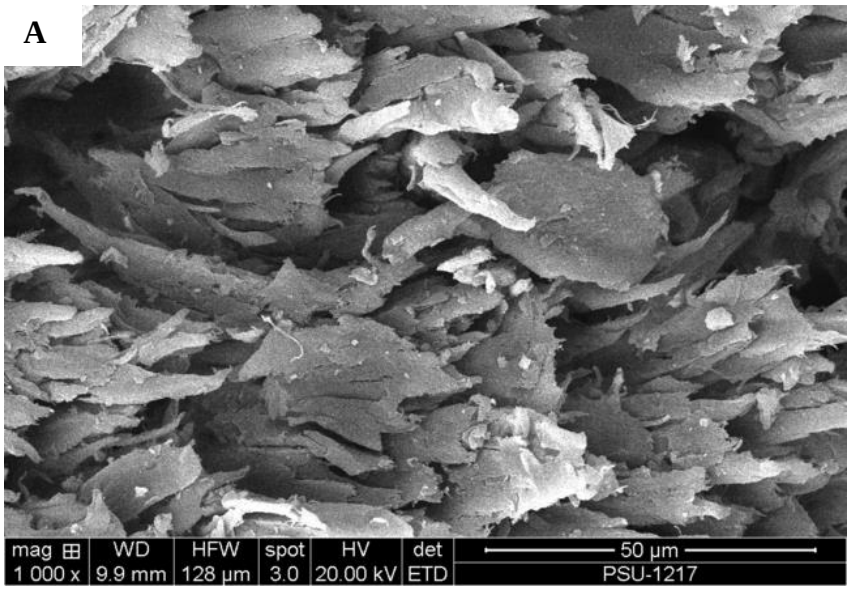


Figure 5.9 Scanning electron micrographs (SEM) of untreated rubberwood (A) and maleic anhydride treated rubberwood (B).

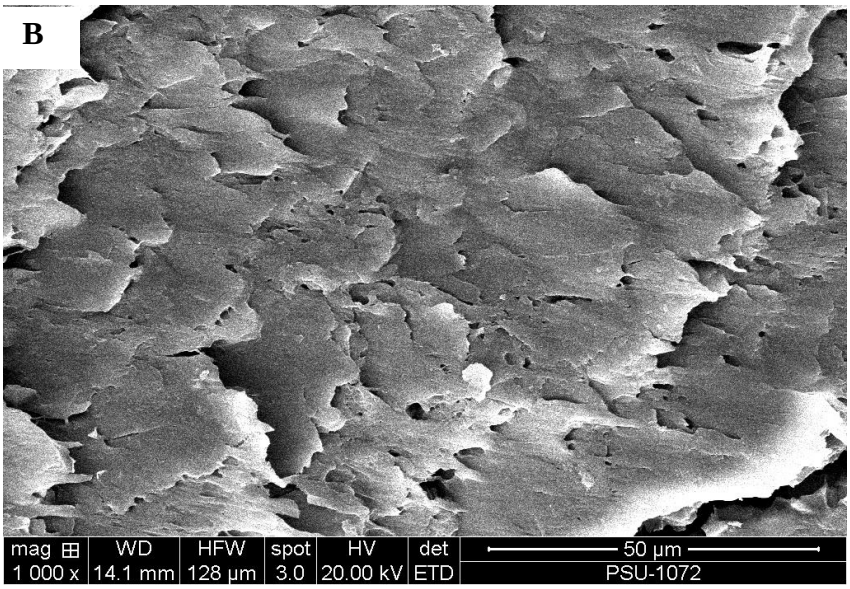


Figure 5.9 (continue)

9. Resistance of weathered samples to molds growth

The weathering test was conducted following the Japanese Industrial Standard (JIS) K 1571. After one week incubation, growth of *A. niger* PSU1 was 100% on weathered samples. *A. flavus* PSU2 and *P. citrinum* PSU3 showed 100% growth in three and two weeks, respectively.

10. Resistance of rubberwood treated with MA by dip and spray treatment against molds: Reduced reaction temperature and treatment process with or without soaking stage

The wood samples were dipped or sprayed with MA. The reaction temperature was set at 50-90 ºC for 2 h. The non-reacted MA was removed by soaking in water for 10 minutes and oven-drying for 2 h. The complete protection of molds was only observed on dip treated samples prepared at 90 ºC. The dip treated samples prepared at 70 and 50 ºC had a resistance against growth of *A. flavus* PSU2 and *P. citrinum* PSU3, but not that of *A. niger* PSU1 (Figure 5.10). However, no complete protection was obtained when wood samples were sprayed with MA. Although the reaction temperature was 90 ºC, sprayed samples had no resistance against *A. niger* PSU1 (Figure 5.11).

When the samples were not soaked in water for 10 minutes after treated with MA, a reaction temperature of 70 and 50 ºC also provided a total protection on dip treated wood against molds as well as a temperature of 90 ºC (Figure 5.12). On the other hand, sprayed samples with MA at 70 and 50 ºC were seriously infected by *A. niger* PSU1 after 12 weeks, but only sprayed samples prepared at 90 ºC showed strong resistance against tested molds (Figure 5.13).

The leaching test reduced the prevention of mold growth by MA. For example, in Figure 5.10, *A. niger* PSU 1 could grow on leached samples prepared at 70 and 50 ºC but, this fungus was inhibited on unleached samples prepared at 70 and 50 ºC.

Discussion

Chemical modification with MA afforded biological protection of rubberwood against surface molds. Rubberwood samples treated with 2.5, 5 and 10% MA had no fungal growth for 1 year even it was inoculated with fungal spores and kept at 100% RH. A concentration of MA at 2.5% was adequate for total protection of wood against tested fungi. The treatment of wood with MA could be used to treat rubberwood for long-term prevention of surface mold growth. Many research studies have been carried out on chemical modification of wood using anhydrides and focused on decay resistance of modified wood. The effectiveness of cyclic anhydrides used in this study has already been demonstrated (Iwamoto and Itoh, 2005) where it was found that MA treated Sugi sapwood (*Cryptomeria japonica* D. Don) had strong resistance against attack by *Fomitopsis palustris* and *Trametes versicolor*. The MA studied here is known to form covalent bonds with OH groups of wood (Iwamoto and Itoh, 2005). By reducing free OH groups in wood, the sorption of water in the cell walls is prevented, the wood moisture content is reduced and resistance of wood to fungal attack is increased. The present study showed that the moisture content of MA treated wood was slightly reduced compared to the control indicating that MA might not prevent water absorption by wood. This result is not correlated with the results previously reported on wood modified with MA (Chauhan *et al.*, 2001). At least three mechanisms have been presented to explain the protection provided by wood modification: (1) changes of the cell wall polymers that become unrecognizable for fungal enzymes; (2) a reduction of

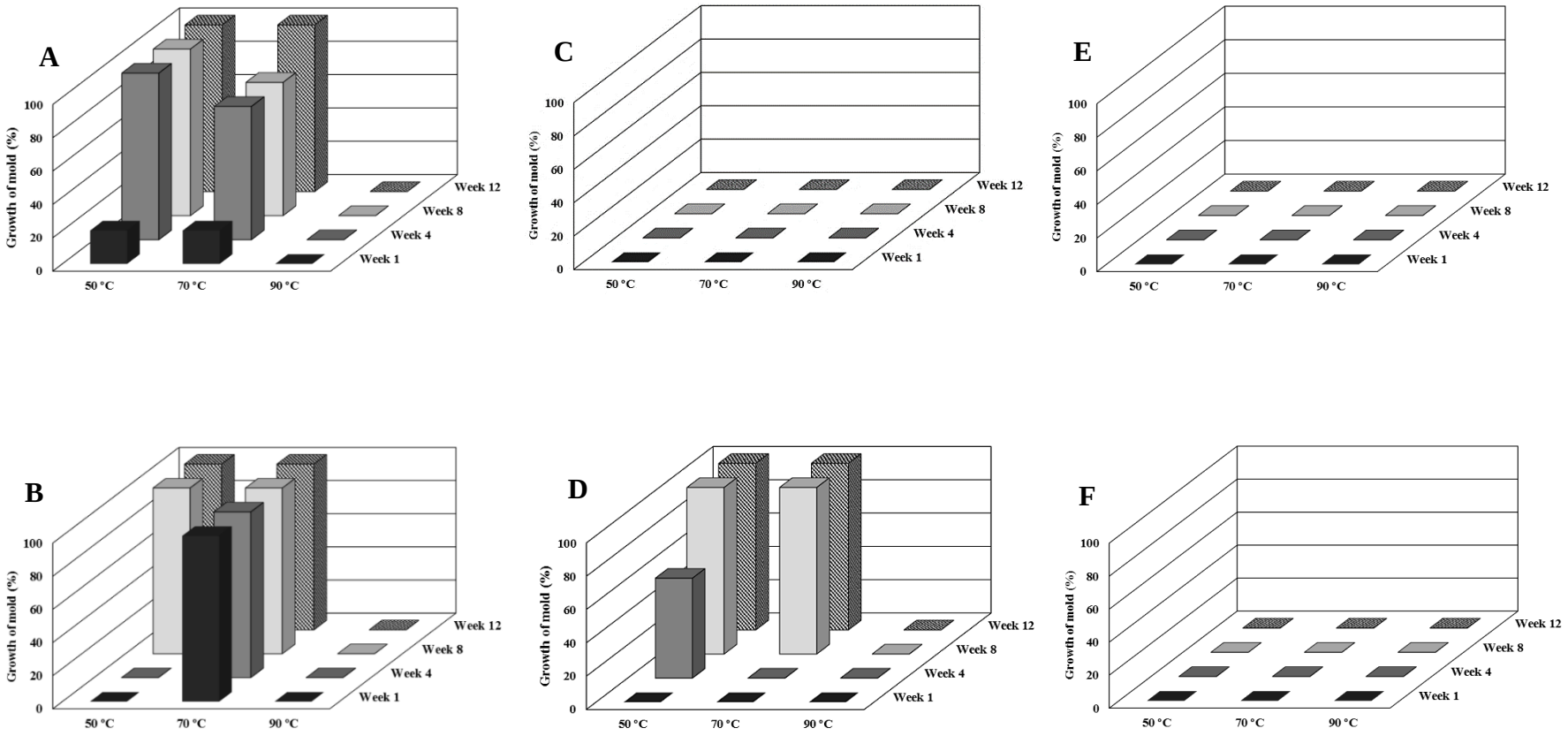


Figure 5.10 Growth of molds on rubberwood treated with MA by dip treatment carried at 50 °C, 70 °C and 90 °C with soaking stage: *A. niger* PSU1 on unleached (A) and leached samples (B), *A. flavus* PSU2 on unleached (C) and leached samples (D) and *P. citrinum* PSU3 on unleached (E) and leached samples (F) incubated at 25 °C and 100% RH for 12 weeks.

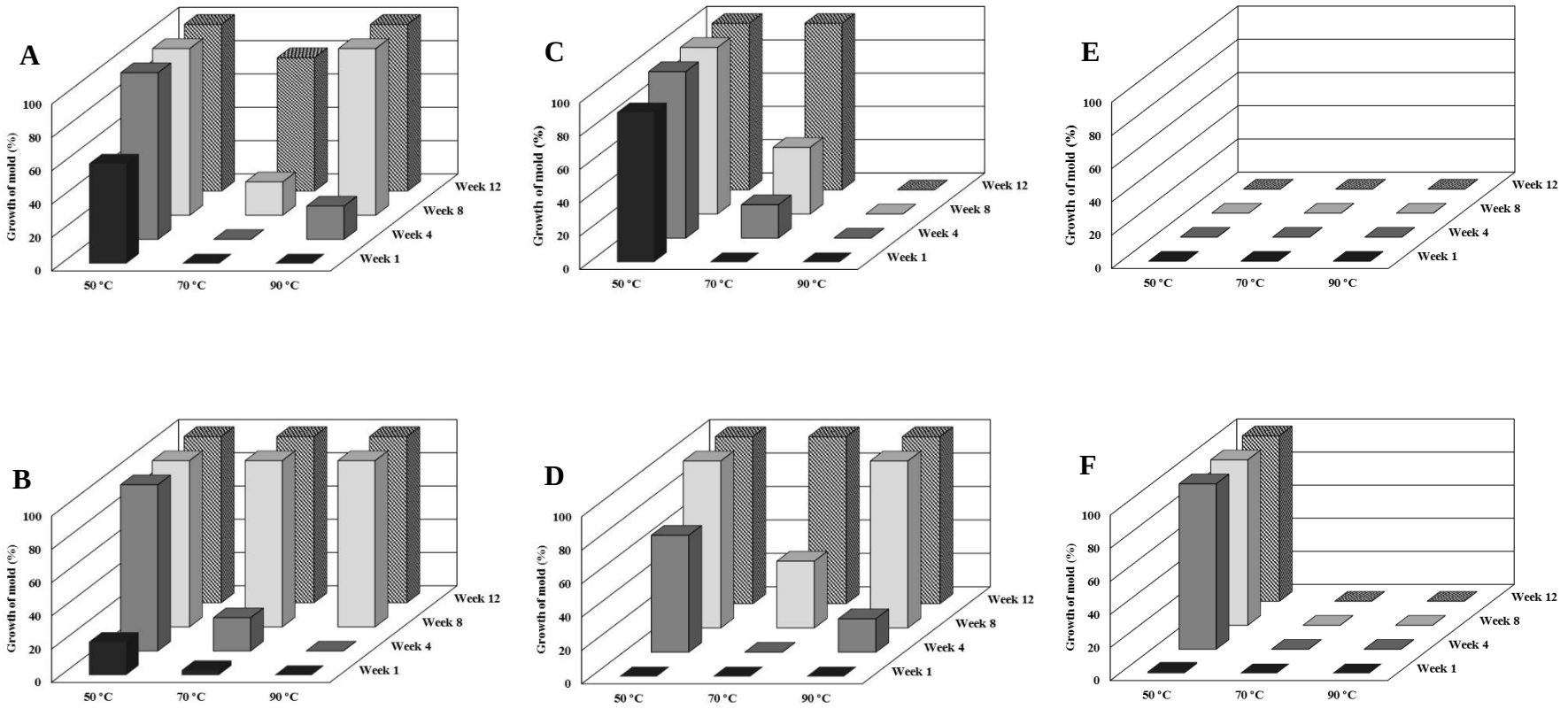


Figure 5.11 Growth of molds on rubberwood treated with MA by spray treatment carried at °C, 70 °C and 90 °C with soaking stage: *A. niger* PSU1 on unleached (A) and leached samples (B), *A. flavus* PSU2 on unleached (C) and leached samples (D) and *P. citrinum* PSU3 on unleached (E) and leached samples (F) incubated at 25 °C and 100% RH for 12 weeks.

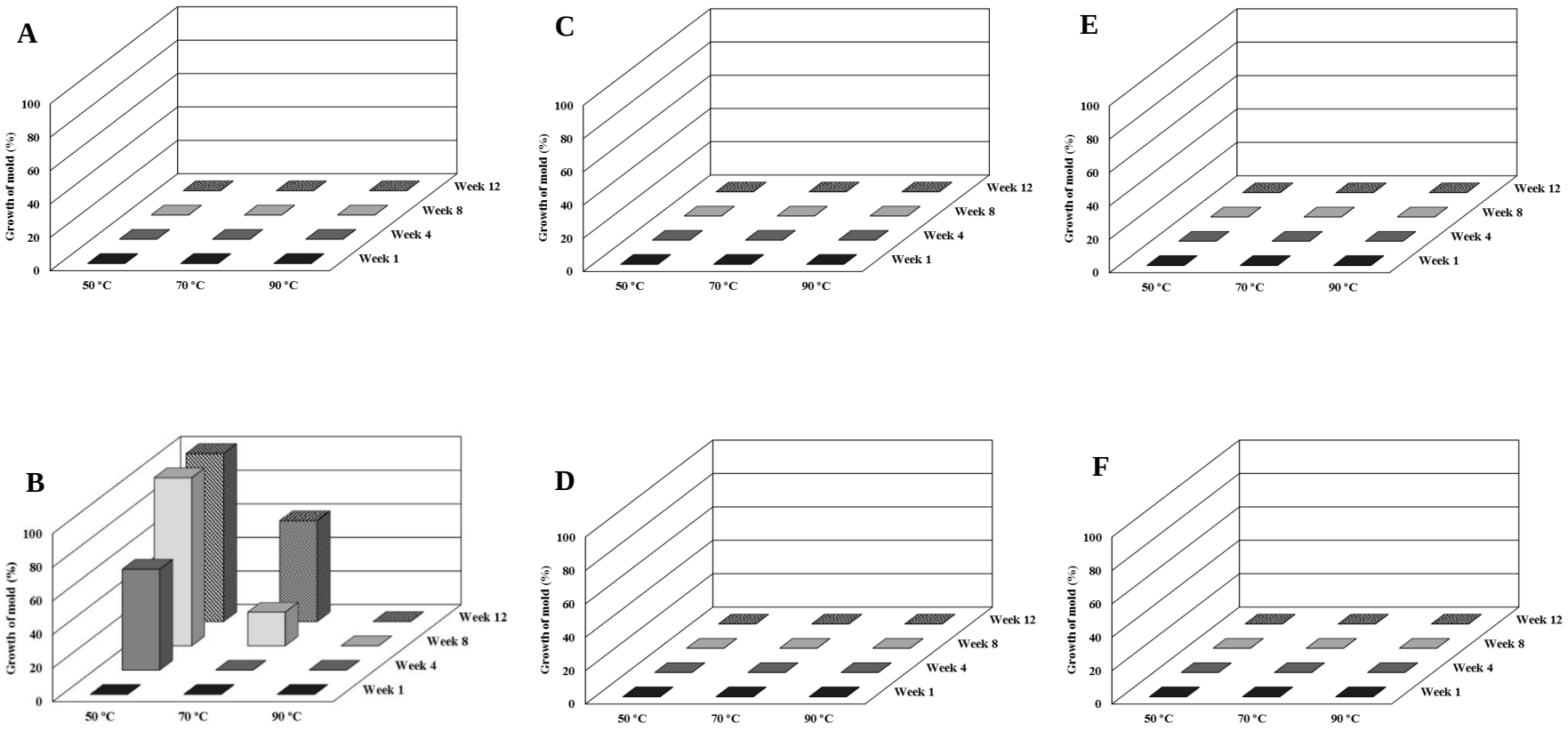


Figure 5.12 Growth of molds on rubberwood treated with MA by dip treatment carried at °C, 70 °C and 90 °C without soaking stage: *A. niger* PSU1 on unleached (A) and leached samples (B), *A. flavus* PSU2 on unleached (C) and leached samples (D) and *P. citrinum* PSU3 on unleached (E) and leached samples (F) incubated at 25 °C and 100% RH for 12 weeks.

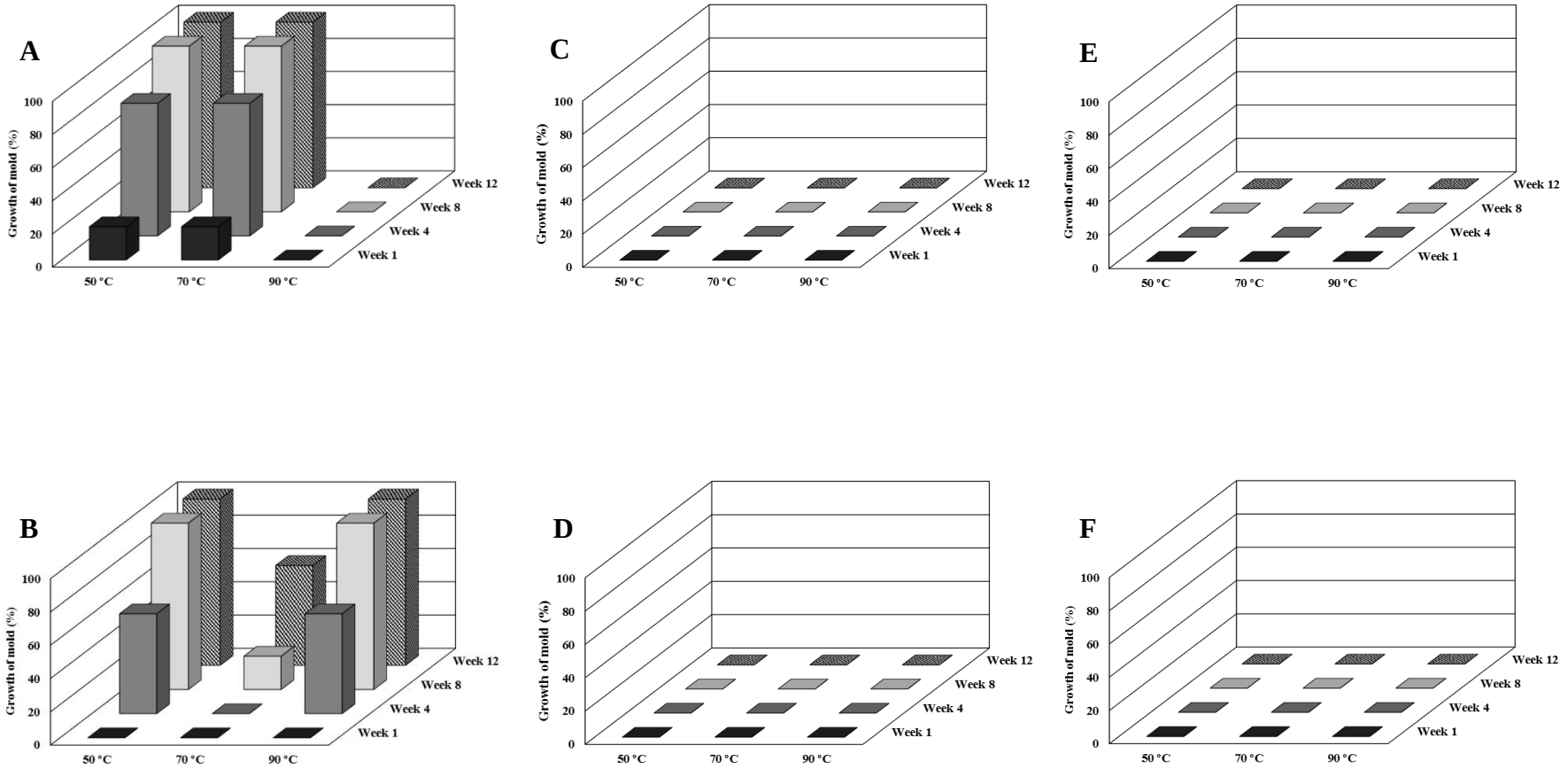


Figure 5.13 Growth of molds on rubberwood treated with MA by spray treatment carried at °C, 70 °C and 90 °C without soaking stage: *A. niger* PSU1 on unleached (A) and leached samples (B), *A. flavus* PSU2 on unleached (C) and leached samples (D) and *P. citrinum* PSU3 on unleached (E) and leached samples (F) incubated at 25 °C and 100% RH for 12 weeks.

the moisture content; and (3) a lower micro-pore size in the wood cell wall (Li *et al.*, 2011). It is obvious that in this study, fungal growth resistance of MA treated wood was not provided by reduction of moisture content but it is feasible that the cell wall of treated rubberwood became unrecognizable for fungal enzyme and a pore size of wood cell wall was lower. In addition, one possible explanation is the resistance to mold growth of the MA treated wood might due to the smoothness of the surface that was clearly seen from SEM image. A study by Bardage and Bjurman (1998) showed that surface roughness contributed to the adhesion of fungal spores. An increase in substratum surface roughness increases the retention of microorganisms when dealing with food contact surfaces, since spores are more easily attached to damaged or uneven surfaces than to smooth ones (Verran *et al.*, 2000). Lugauskas *et al.* (2003) suggested that surfaces or surfaces with cracks might concentrate nutrients and moisture more easily and provide favorable conditions for fungal attachment and growth. Furthermore, physical treat of rubberwood with MA might regulate the permeability of O_2 and water vapor, thereby preventing fungal growth.

The leachable MA quantity present in wood was determined by a leaching test and the result showed that 0.02 mg mm^{-3} MA was leached from treated wood. The high concentrations of released MA at the initial period of leaching may be because of high content of MA on the surface or in the pore of treated wood that is subject to very early loss. Once wood preservatives fix well during the reactions with wood, they should resist to leaching. This means that the protective agent will not leach. Although the results from the chemical analysis of leachates showed that MA was leached from the treated wood, the wood samples still had high fungal resistance. The concentration of non-reacted leachable MA from MA treated wood was 0.02 mg mm^{-3} but did not contribute to fungal growth resistance. The leaching test indicated that MA was leached but the fixed MA in wood was enough for prevention of mold growth.

The result of viable count of *A. niger* PSU1 on MA treated wood showed that there was about 90% viable count of *A. niger* PSU1 after inoculation for 24 h. This result indicated that fungal spores were not largely reduced on MA treated wood. Fungicidal activity was defined as a reduction in fungal growth of ≥ 3 $\log_{10}$ in CFU ml^{-1}, resulting in about 99.9% reduction in CFU ml^{-1} relative to the initial inoculum (Ernst *et al.*, 2002). Clearly, MA did not exhibit fungicidal activity against mold on wood.

Additionally, the result of wood diffusion test showed similar antifungal activity for untreated and MA treated wood. In addition, the agar well diffusion showed that the amount of released MA in leachates did not contribute to any antifungal effects. These results suggested that MA used in this study did not act as a fungicide. This was clearly seen from both the antifungal study and the measurement of growth inhibition test on mold. Although there was 0.02 mg mm^{-3} released MA in the leachate from MA treated wood, it had cytotoxic effect the same as the leachate from untreated wood. The survivals of cell incubated with leachates from MA treated and untreated wood showed that both types of leachates had high toxicity to cell lines. One reason for high leachates toxicity could be that the wood contained high natural extractive content such as phenolic compounds and esters (Simatupang *et al.*, 1992; Vetter *et al.*, 2008). These results indicate that the MA treated rubberwood had a similar cytotoxic effect as the natural rubberwood. This study showed that treatment of rubberwood with MA is an environmentally friendly alternative to prevent molds instead of using highly toxic preservatives.

A 5 cycles of weathering process might cause excessive leaching and release of the components, so the weathered samples had no resistance against molds. One possible explanation is that MA has high solubility in water and when MA treated wood were soaked in water for 8 h following the weathering process, the retention of MA on wood was reduced. Previous work conducted by Iwamoto and Itoh (2005) showed that the decay resistance of MA treated wood after weathering was remarkably enhanced compared to untreated wood. The treatment method used in that referred study was a vapor phase system in vacuum vessel with a pressure of 7 hPa, which is more efficient than dip treatment and heat treatment applied in this study so, the retention of MA in wood was better.

The important step of treatment of wood with MA is the heat treatment where the reaction between MA and wood is performed. An increase of reaction temperature from 50 to 90 °C led to an increase in fungal growth resistance. This suggests that MA was effectively fixed and the reaction was more complete at higher temperature—the higher the reaction temperature is, the higher the fungal growth inhibition. A higher temperature would enhance the mobility of the molecules and the diffusion MA, therefore, increasing reaction rates (Liu *et al.*, 2007). Iwamoto *et al.*

(2005) indicated that higher reaction temperature (180 °C) between MA and wood resulted in low weight loss of treated samples against attack by decay fungi.

Two methods were compared—with and without soaking stage after heat treatment. Obviously, without soaking stage, wood had strong resistance against molds compared to with soaking stage. The soaking stage after the heat treatment might reduce the retention of MA reacted with wood. However, when wood samples were treated at high temperature, the reducing effect of soaking stage was not as much as when treated at low temperature. When the samples were reacted with MA at high temperature, the reaction rate was high and the endurance of MA with wood was slightly decreased after the soaking stage; therefore, wood still had high resistance against mold. By contrast, at low temperature, the rate of bond formation between MA and wood was low and subsequently reduced after soaking. So, treated wood had low resistance against mold attack. In conclusion, when the soaking stage was not carried, treatment of wood with MA could be conducted at low temperature to prevent mold growth. These results suggest that the reaction temperature of 50 °C is sufficient for formation of MA with wood cell wall. The related study reported by Pardo and Alfaro (2014) has shown that teak and melina samples treated with acetic anhydride at 70 °C showed good protection against fungal attack. Results obtained from this study may be more useful and practical for wooden toy manufacturers since treatment of wood with MA without soaking stage is less time consuming and more cost-effective method compared to with the soaking stage. Additionally, the low reaction temperature may be more economical compared to treatment at high temperature.

The application of wood preservative by dip or spray treatment require minimal equipment and are simple treatment methods. The effect of dip and spray treatments is superficial. Dip treatment provides little more effectiveness than spray treatment. A complete immersion probably gives greater uniformity of coverage than spray treatment and more assurance that all checks and cracks are filled (Kollmann *et al.*, 1968). The present study shows that dip treatment of MA is more effective than spray treatment. So, dip treatment may be more useful for wooden toy manufacturers.

Conclusion

In this study, MA was tested for efficacy on wood protection against molds isolated from rubberwood. 2.5% MA was effective to prevent mold growth on rubberwood for 12 weeks. The complete protection was not provided by reduction of moisture content but possibly obtained by change of recognizable site for fungal enzyme and a lower pore size of wood cell wall. After leaching, MA treated rubberwood still resisted to mold growth. MA used in the present study is not a fungicide, environmentally friendly and its cytotoxic effect against human cells is similar to untreated wood. MA has a potential to be used as a low toxic anhydride for wood modification, thereby increasing the wood's utility for various applications related to human such as wooden toys, kitchen wares and furniture. The bond formation between wood and MA was increased by the increasing a reaction temperature. However, treatment of wood with MA could be carried under a low temperature and resulted in a significant mold inhibition. Dipping of MA with wood resulted in mold growth prevention and this result is important for the wooden toys manufacturer since dipping is a cheap and simple method.

CHAPTER VI

Conclusions and suggestions

Conclusions

Three dominant molds which colonized rubberwood were *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3 (LC127087). Growth of *A. niger* PSU 1 on rubberwood was prevented when the RH was below 92%, where the equilibrium moisture content of wood was 13.69%.

Clove and cinnamon oil showed remarkable antifungal activity against *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3. When tested on wood by dip treatment, only cinnamon oil could completely prevent growth of *A. niger* PSU1.

Treatment of rubberwood with chitosan showed that both chitosan C1 and C3 were effective on rubberwood against *A. niger* BAM 4, but not *P. decumbens* CBS 12198. Vacuum and dip treatment were effective method of applying chitosan to rubberwood. However, dip treatment is a simpler method and may be more useful for superficial protection against molds compared to vacuum treatment.

Silane treated rubberwood showed no resistance against mold growth. Dip and vacuum treatment might not provide an increasing penetration depth of silane into wood cells.

Rubberwood treated with maleic anhydride (MA) showed strong resistance against molds for 52 weeks. Change of recognizable site for fungal enzyme and a lower pore size of wood cell wall might prevent MA treated wood from fungal attack. In addition, the smooth surface of MA treated wood might be one explanation of the resistance to mold growth on treated wood. Maleic anhydride did not act as a fungicide and this result indicated that MA was safe to environment. Moreover, treatment of wood with MA is safe to humans, since the cytotoxicity values of the leachates from both MA treated and untreated wood samples had similar effect.

Among the wood protectants tested, MA was the most effective. Therefore, it was used in the experiment related to an industrial scale. Rubberwood reacted with MA at 50 ºC and without soaking stage showed strong resistance against mold attack. Such treatment method is practical for the wooden toy manufacturer since it is more cost-effective and less time consuming. Additionally, to apply MA on toy production process, dip treatment was more suitable than spray treatment, since dip treated samples with MA showed stronger resistance against molds.

Suggestion

1. Toxic chemical preservatives currently still in use can be replaced by an application of essential oils as wood protecting agent. However, their leachability from wood when exposed to water should be investigated. Susceptibility of essential oils to light, temperature and oxygen availability may reduce their antifungal activity and retention on wood. Effective protection technologies that can shield them from degradation may alleviate these problems.
2. An improvement of the fixation of chitosan in wood need to be investigated to strengthen the durability of chitosan treated wood exposed to water.
3. The more effective treatment process such as pressure combined with vacuum treatment may enhance the efficacy of silane used in rubberwood.
4. Results from laboratory studies showed that MA was efficient on rubberwood protection. The application of MA as wood protectant should be considered for its use on an industrial scale.

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APPENDIX

Appendix A

1. Humidity regulated by saturated salt solution (Greenspan, 1977)

The relative humidity (RH) at 75, 80, 85, 92 and 97%, 25 °C were regulated by saturated salt solution. Saturated salt solution is prepared by dissolving sterile salt in 100 ml of sterile water:

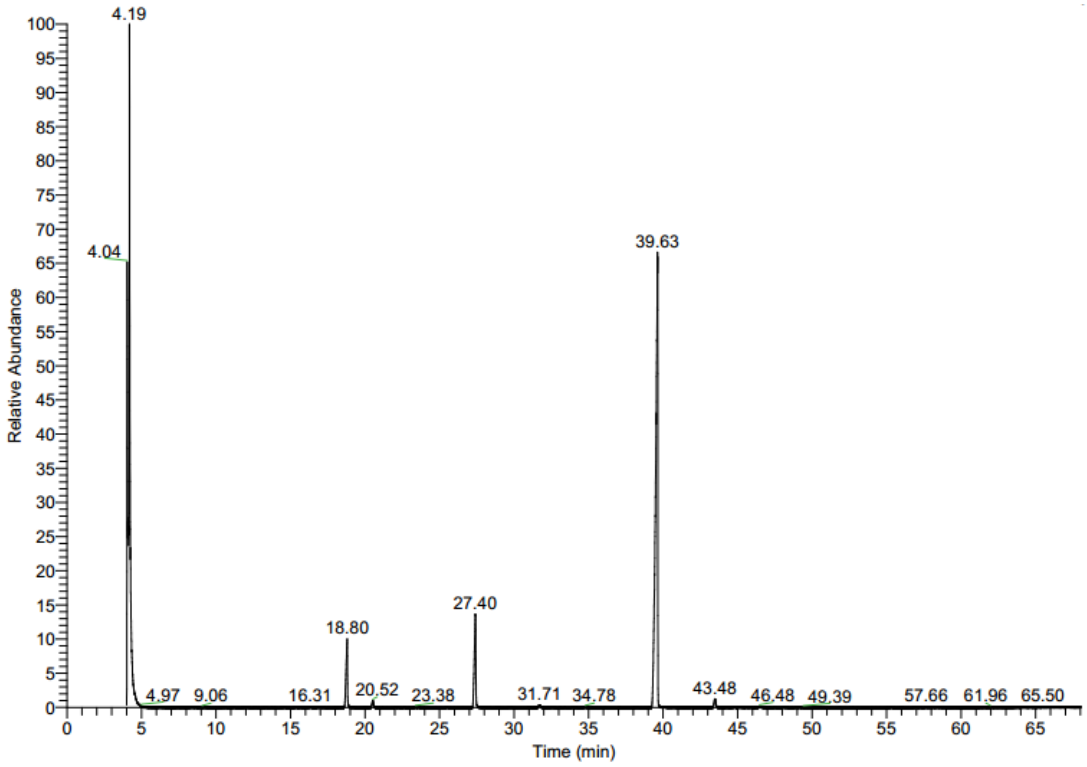
75% RH	35.8 g NaC1
80% RH	54.0 g $(NH_4)_2SO_4$
85% RH	40.0 g KCl
92% RH	40.0 g KNO_3
97% RH	13.0 g K_2SO_4

and then adding 5-10 additional grams of the salt, until sediment is formed.

Fifty milliliters of saturated salt solution was poured into sterile glass and placed in plastic container (12 cm ×16.8 cm × 6.8 cm). The lids of the containers is sealed with a Parafilm preventing salt migration. The container was placed in an incubator with constant temperature at 25 °C for 24 h for stabilization. Electronic hygrometer was placed in the container during 24 h of stabilization to measure the stability of humidity level.

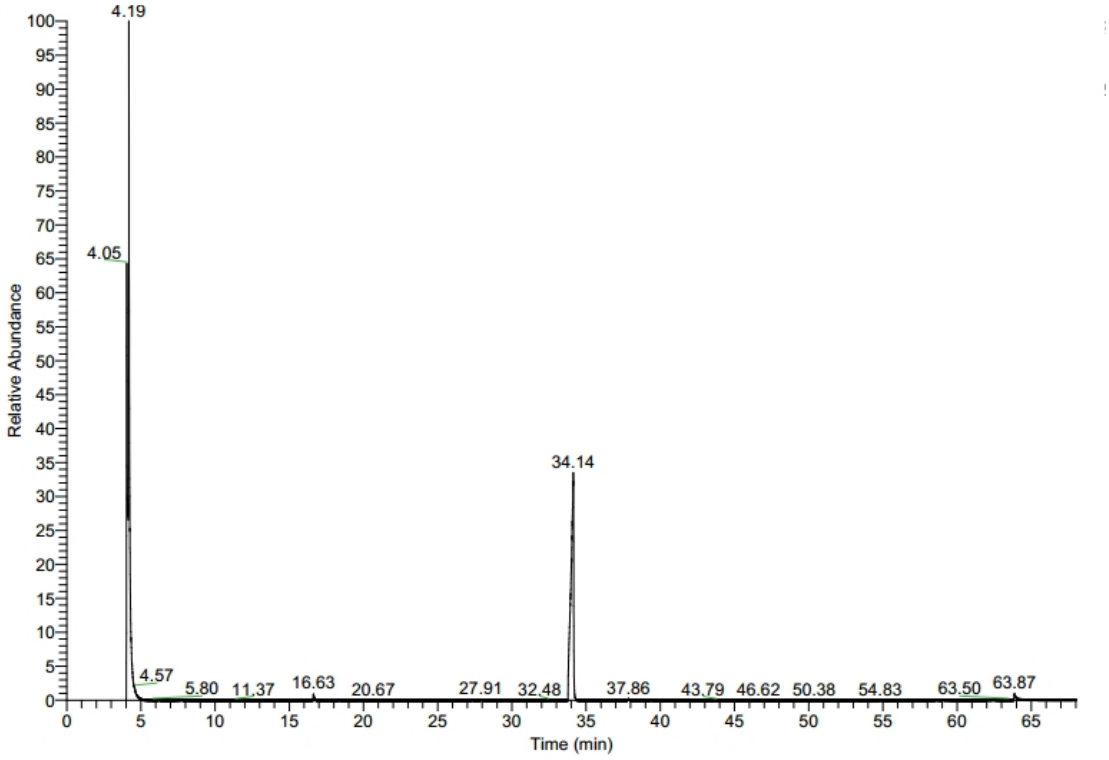
Appendix B

1. Chromatograms of GC-MS of clove oil and cinnamon oil



No.	Components	Retention time (min)	Relative content (%)
1	*trans*-Caryophyllene	18.80	8.00
2	α-Humulene	20.52	0.87
3	Benzenemethanol	27.40	12.14
4	Caryophyllene oxide	31.71	0.40
5	Eugenol	39.63	76.35
6	Eugenol acetate	43.48	1.21
	Total content		98.97

Figure 1. Chromatogram of GC-MS of clove oil.



No.	Components	Retention time (min)	Relative content (%)
1	Benzaldehyde	16.63	1.02
2	Cinnamaldehyde	34.14	96.24
3	Ethyl cinnamate	37.86	0.38
4	Cinnamic acid	63.87	1.49
	Total content		99.13

Figure 2. Chromatogram of GC-MS of cinnamon oil.

Appendix C

1. The HPLC chromatogram of a standard glucosamine solution derivatized with an o-phthalaldehyde (OPA) reagent

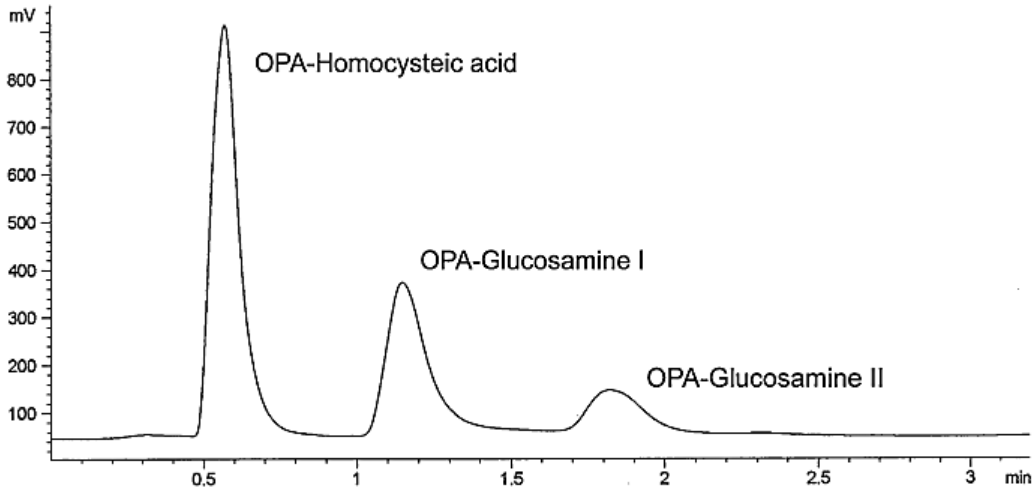


Figure 1. The HPLC chromatogram of a standard glucosamine solution derivatized with an o-phthalaldehyde (OPA) reagent.

Appendix D

1. The HPLC chromatogram of a standard maleic anhydride solution.

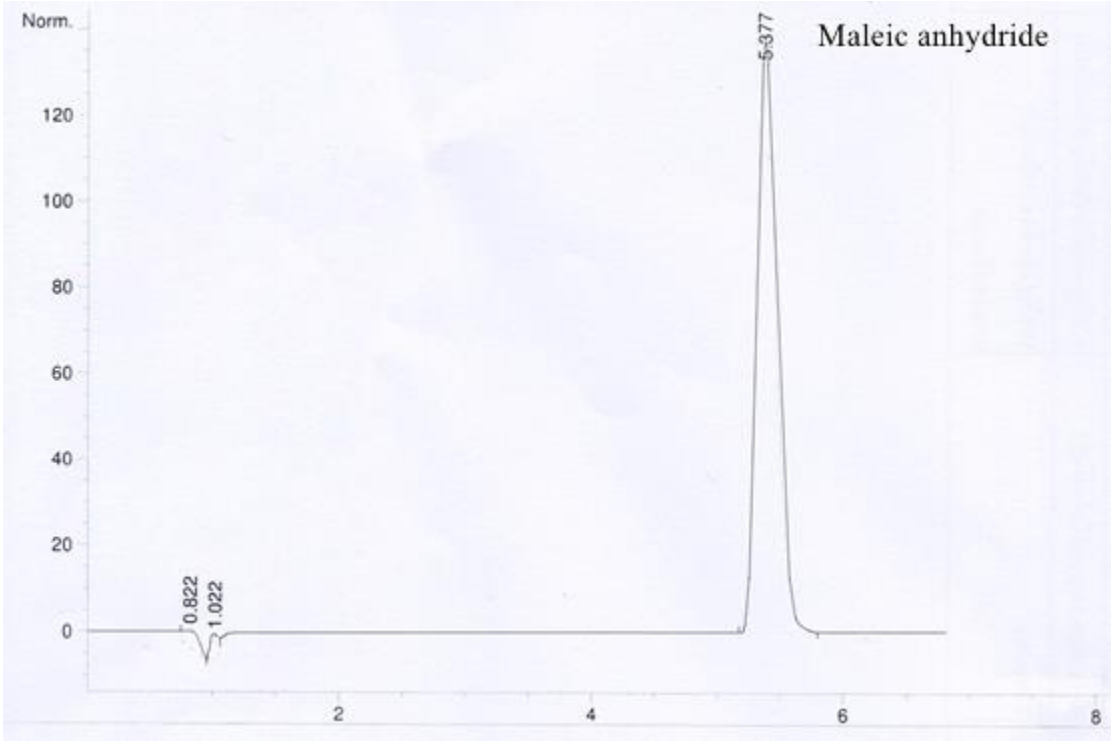


Figure 1. The HPLC chromatogram of a standard maleic anhydride solution.

Appendix E

Identification of isolated strain

Aspergillus niger strain ISSFR-019 18S ribosomal RNA gene, partial sequence

Sequence ID: gb|KT832786.1| Length: 1655 Number of Matches: 1

Range 1: 25 to 1602 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  265  TGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCC  324

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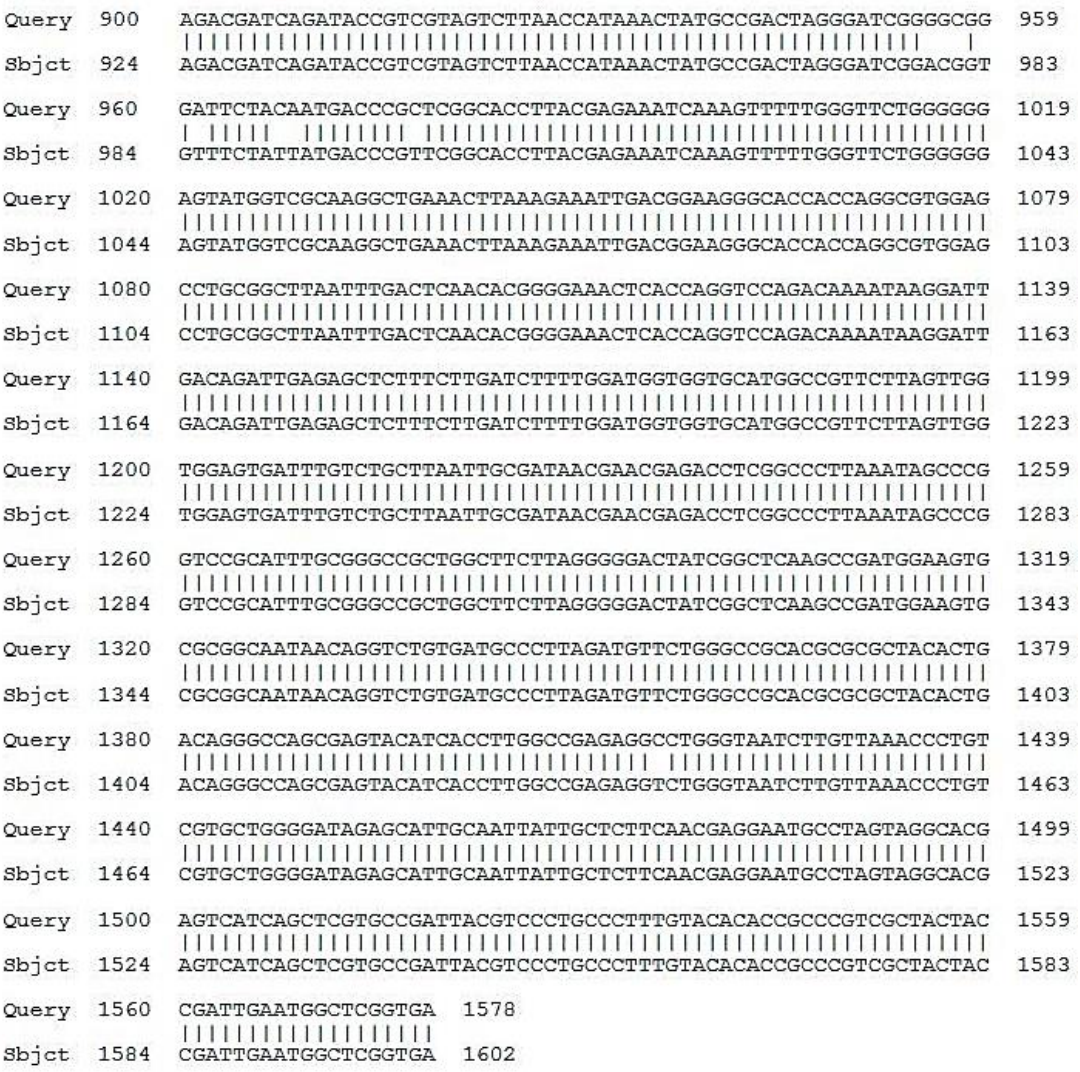

Figure 1. Alignment of DNA sequence of strain PSU1 with DNA sequence of *Aspergillus niger* strain ISSFR-019 (GenBank accession number KT832786.1).

Aspergillus flavus strain TZ1985 18S ribosomal RNA gene, partial sequence

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Range 1: 50 to 1636 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  470   GTACAATCTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGC  529

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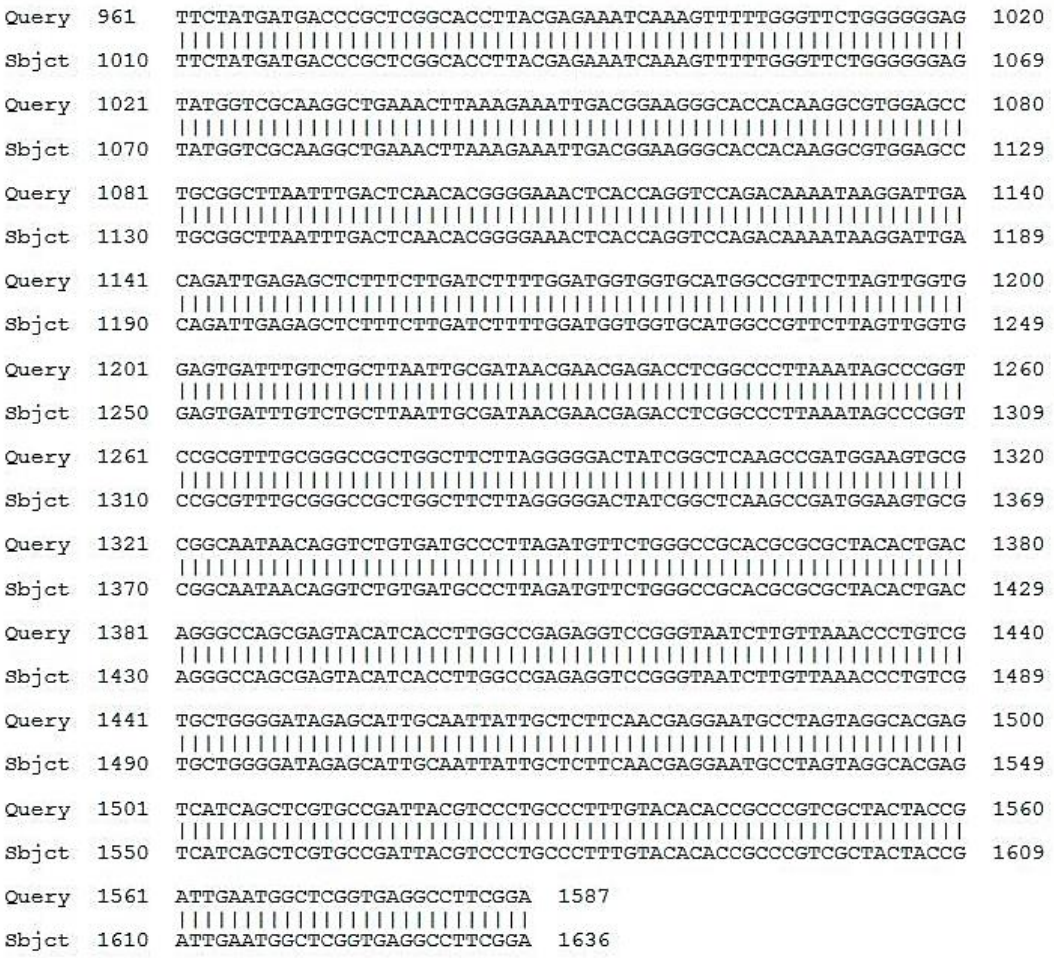

Figure 2. Alignment of DNA sequence of strain PSU2 with DNA sequence of *Aspergillus flavus* strain TZ1985 (GenBank accession number GU953210.1).

Penicillium citrinum strain TG2 18S ribosomal RNA gene, partial sequence

Sequence ID: gb|KC960012.1| Length: 1738 Number of Matches: 1

Range 1: 21 to 1613 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  141   GTATTTATTAGATAAAAAACCAACGCCCTTCGGGGCTCCTTGGTGAATCATAATAACTTA  200

Query  181   ACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTTTC  240
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Query  241   GATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTC  300
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Sbjct  321   CGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTA  380

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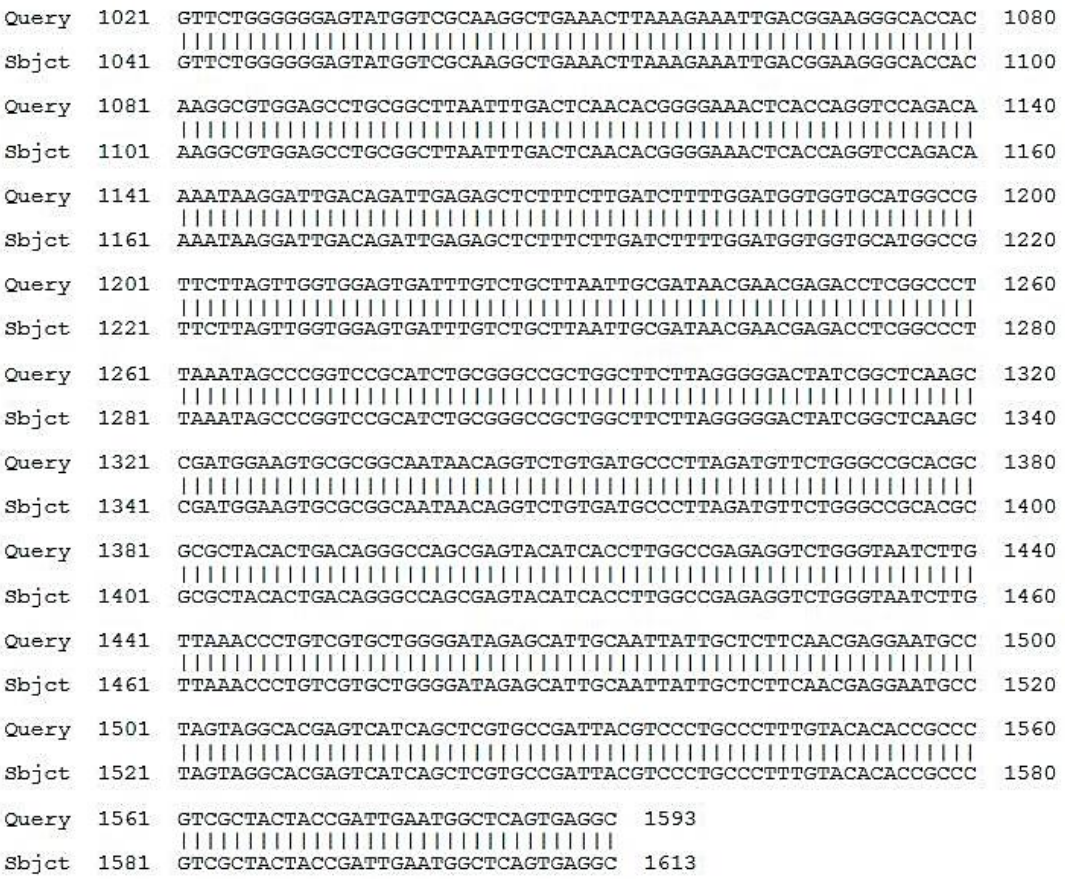

Figure 3. Alignment of DNA sequence of strain PSU2 with DNA sequence of *Penicillium citrinum* strain TG2 (GenBank accession number KC960012.1).

Paper 1

Application of plant essential oils in prevention of fungal growth on Para rubberwood

Paper 2

Treatment of rubberwood (*Hevea brasiliensis*) (Willd. ex A. Juss.) Müll. Arg. with maleic anhydride to prevent moulds

Paper 3

Resistance of rubberwood (*Hevea brasiliensis*) treated with chitosan or silane against surface molds

VITAE

Name Mrs. Kittiya Oldertrøen

Student ID 5311030025

Education Attainment

Degree	Name of institution	Year of graduation
B.Sc. Microbiology	Chiang Mai University	2007

Scholarship Awards during Enrolment

Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program

List of Publications and Presentations

Publications

Ma-in, K., H-Kittikun, A., Phongpaichit, S. 2014. Application of plant essential oils in prevention of fungal growth on Para rubberwood. Eur. J. Wood Wood Prod. 72: 413–416.

Oldertrøen, K., H-Kittikun, A., Aam, B.B. and Larnøy, E. 2016. Resistance of rubberwood (*Hevea brasiliensis*) treated with chitosan or silane against surface molds. Eur. J. Wood Wood Prod. doi: 10.1007/s00107-016-1071-9

Oldertrøen, K., H-Kittikun, A., Phongpaichit, S., Riyajan, S. and Teanpaisal, R. 2016. Treatment of rubberwood (*Hevea brasiliensis*) (Willd. ex A. Juss.) Müll. Arg. with maleic anhydride to prevent moulds. J. Forest Sci. 62: 314–321.

Presentations

Ma-in, K., H-Kittikun and A., Phongpaichit, S. 2013. Humidity requirements for growth of filamentous fungi on Para rubberwood. The 25th Annual Meeting of the Thai Society for Biotechnology and International Conference", organized jointly by the Thai Society for Biotechnology (TSB). 16-19 October 2013. Bangkok. (Poster presentation)

Ma-in, K., H-Kittikun and Larnøy, E. 2015. Antifungal activity of chitosan with different molecular weight against rubberwood surface mold. RGJ-Ph.D.

Congress XVI "ASEAN: Emerging Research Opportunities". 11-13 June 2015. Pattaya. (Poster presentation)

Ma-in, K., H-Kittikun and Larnøy, E. 2015. Resistance of rubberwood (*Hevea brasiliensis*) treated with chitosan to surface molds. 113th TRF Seminar Series in Basic Research "From Basic Research to Applications" 25 September 2015. Hat Yai. (Oral presentation)