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EFFECT OF *Bacillus subtilis* ON QUALITY OF ESSENTIAL OIL DISTILLED FROM *Aquilaria crassna*

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This research was made possible by the support of

Mae fah Luang University

2012

ACKNOWLEDEGMENT

The author would like to sincerely thank Assoc. Prof. Dr. Sugunya Wongpornchai from the Department of Chemistry, Faculty of Science, Chiang Mai University for value comments and the identification of compounds in this research. Great appreciation is given to the Scientific and Technological Instrument Center (STIC), Mae Fah Luang University for GC-MS instrument. We express our great appreciation to the division of Research Services, Mae Fah Luang University for funding support.

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Abstract

This research is the study on the effect of *Bacillus subtilis* on quality of essential oil distilled from *Aquilaria crassna. A. crassna* wood was collected from Rayong province. Firstly, *A. crassna* was fermented with various microbial for 7 days before subjected to hydrodistillation for 48 hrs. The essential oils obtained from microbial fermentations were then analyzed their chemical constituents, biological and antioxidant activities, respectively. As the results, essential oils of *A. crassna* obtained from microbial and without treatment extracted using a modified Likens-Nickerson apparatus appeared as yellow viscous liquids with percentage yields of 0.03-0.21 w/w. Highest yield was detected in essential oil obtained from TN-51 followed by Best-195, S1-13, *S. cerevisiae* and *L. bulgaricus*, respectively. In addition, it was found that number of volatile components was different significantly in *A. crassna* essential oils treated with various microbes. As can be observed, the different antimicrobial activities were found among these essential oils which may be due to the different major compounds in these essential oils. However, *A. crassna* essential oils obtained from different microbial treatment exhibited lower antioxidant activity than α-tocopherol and butyl hydroxyl toluene.

บทคัดย่อ

งานวิจัยครั้งนี้เป็นการศึกษาผลของ *Bacillus subtilis* ต่อคุณภาพน้ำมันหอมระเหยที่กลั่นจากต้นกฤษณา *Aquilaria crassna* ซึ่งไม้กฤษณาพันธุ์ *Aquilaria crassna* ถูกเก็บมาจากจังหวัดระยอง ในขั้นแรก ไม้กฤษณาพันธุ์ *Aquilaria crassna* ถูกหมักกับเชื้อจุลินทรีย์ชนิดต่างๆ เป็นเวลา 7 วัน ก่อนจะนำไปกลั่นไอน้ำเป็นเวลา 48 ชั่วโมง หลังจากนั้นนำน้ำมันหอมระเหยกฤษณาที่ได้จากการหมักด้วยเชื้อจุลินทรีย์ชนิดต่างๆ ไปวิเคราะห์องค์ประกอบทางเคมี, ฤทธิ์ทางชีวภาพ และฤทธิ์การต้านอนุมูลอิสระตามลำดับ จากผลการทดลองพบว่าน้ำมันหอมระเหยกฤษณาที่ได้จากการหมักและไม่ได้หมักด้วยเชื้อจุลินทรีย์ต่างๆ ซึ่งสกัดโดยใช้ชุดกลั่น Likens-Nickerson มีลักษณะเป็นของเหลวหนืดสีเหลือง โดยมีเปอร์เซ็นต์โดยน้ำหนักอยู่ในช่วง 0.03-0.21 น้ำมันหอมระเหยกฤษณาที่ได้จากการหมักกับเชื้อจุลินทรีย์ชนิด TN-51 ให้ปริมาณน้ำมันหอมระเหยกฤษณามากที่สุด รองลงมาคือ Best-195, S1-13, *S. cerevisiae* และ *L. bulgaricus* ตามลำดับ นอกจากนี้ยังพบว่าจำนวนองค์ประกอบทางเคมีในน้ำมันหอมระเหยกฤษณาที่ได้จากการหมักด้วยเชื้อจุลินทรีย์ชนิดต่างๆ มีจำนวนองค์ประกอบแตกต่างกันอย่างมีนัยสำคัญ จาการสังเกตยังพบความแตกต่างของฤทธิ์การต้านแบคทีเรียในน้ำมันหอมระเหยกฤษณาต่างๆ ซึ่งอาจจะเป็นผลมาจากความแตกต่างขององค์ประกอบหลักในน้ำมันหอมระเหยต่างๆ อย่างไรก็ตามยังพบว่าน้ำมันหอมระเหยกฤษณาที่ได้จากการหมักด้วยเชื้อจุลินทรีย์ชนิดต่างๆ มีฤทธิ์การต้านอนุมูลอิสระต่ำกว่า α-tocopherol และ butyl hydroxyl toluene

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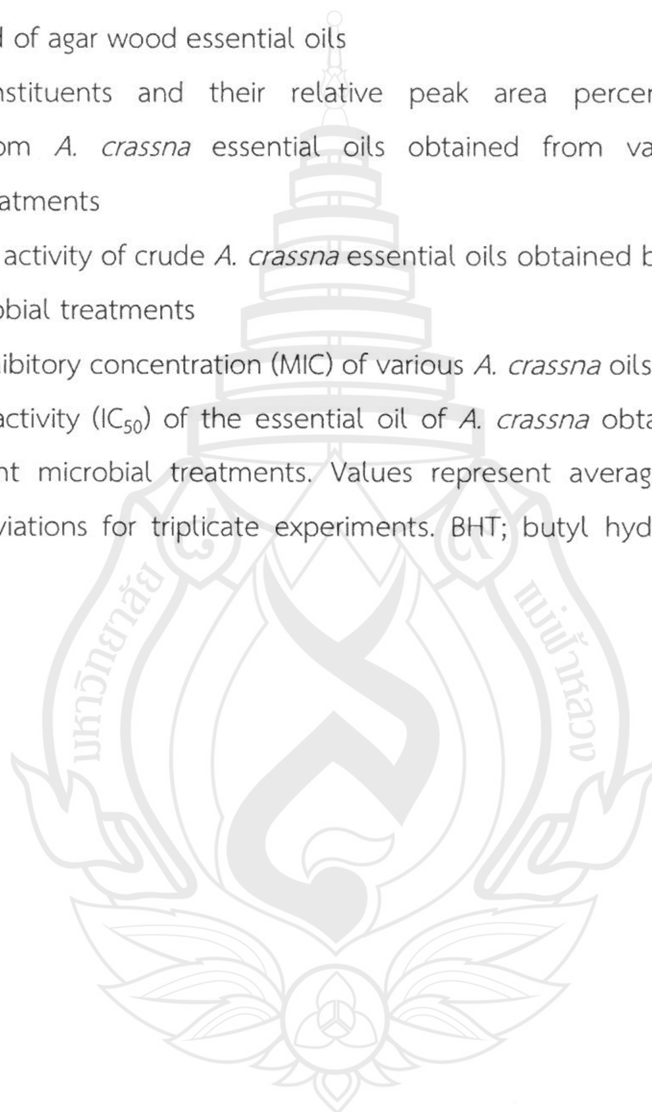

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CHAPTER 1
INTRODUCTION

1.1 Introduction

Agarwood is the resinous heartwood from *Aquilaria* trees, large evergreens native to Southeast Asia. The trees occasionally become infected with mold and begin to produce an aromatic resin in response to the fungus attack. The resin is commonly known as "jinko" in Japanese and as "aloeswood", "agalloch" or "eaglewood" in English [1]. Agarwood and its essential oils are economically important natural products used for the production of incense, perfumes, and traditional medicines throughout Asia [2, 3]. Additionally, its essential oils had great cultural and religious significance in ancient civilizations around the world. The aroma of agarwood is a complex mixture of many volatile constituents which give it unique and elegant oriental aroma characters [4-6]. In recent decades, agarwood has usually been harvested from *A. malaccensis*; *A. agallocha* and *A. secundaria* are synonyms for *A. malaccensis* [7, 8]. Other agarwoods can also be collected from *A. crassna* and *A. sinensis* plants.

There are increasing demands for agarwood-based products for use in medicine, perfume, and incense. Agarwood is traded in the form of product derivatives such as wood chips, powder, and oil. Agarwood is the heartwood produced by a number of *Aquilaria* species in Southeast Asian countries such as Indonesia, Malaysia, Vietnam, Cambodia, Thailand and Laos. *Aquilaria* species could find in rainforests and dry evergreen east parts such as Rayong, Trad, Nakhonnayok, and Prachinburi provinces. Agarwood is used in religious and spiritual ceremonies, perfumery, incense, cosmetic and pharmaceutical industries.

Table 1 Scientific classification of *Aquilaria crassna* [1-3]

Scientific name	*A. crassna* Pierre
Kingdom	*Plantae*
Phylum	*Tracheophyta*
Class	*Magnoliosida*
Order	*Myrtales*
Family	*Thymelaeaceae*
Cambodian name	Chan crassna

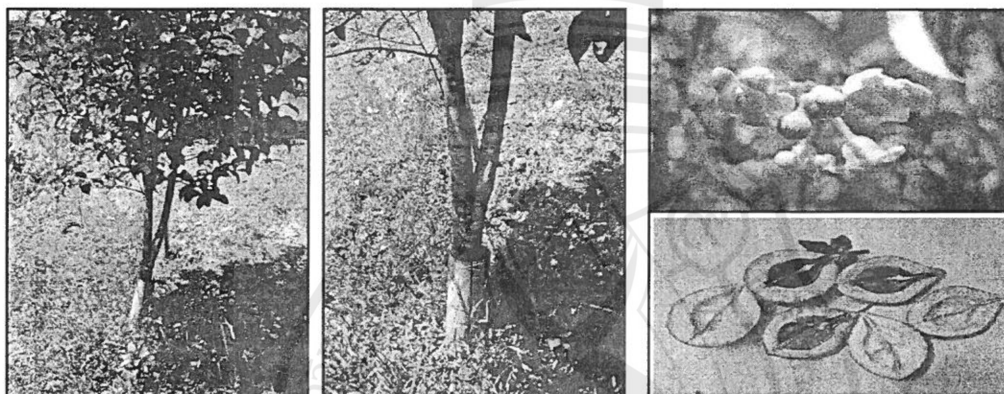


Figure 1 Parts of *A. crassna* Pierre [1-3]

The volatile odor components of agarwood and its essential oils have been investigated by many researchers. Meier et al. [8] analyzed the volatile constituents from *A. malaccensis* using gas chromatography-mass spectrometry (GC-MS). Agarospirol and jinkoh-eremol were identified as the major constituents with anisyl acetone as a minor component. Ishihara et al. [6] identified oxygenated guaiane, eudesmane derivatives and oxo-agarospirol as the major sesquiterpene components in Vietnamese agarwoods. Moreover, other components have also been reported as the constituents of agarwoods such as sesquiterpenes of eremophilane [9-11], prezizaane-type [9], 2(2-phenylethyl)chromone derivatives [12-14], diepoxy tetrahydrochromones, oxidoagarochromones etc [15]. In these previous reports most researchers focused on

the study of the chemical composition of *A. malaccensis* species for which sesquiterpenes and chromone derivatives were found to be the major constituents. However, the volatile compounds and aromas of other agarwood species have not yet been investigated.

Microbes are important in several biotransformation processes which have been proved from the reviews published addressing the production of flavor and fragrance chemicals by microorganisms ([16, 17]. The majority of microorganisms perform useful function in the environment and also in some branches of food industry, such as production of wine, beer, bakery productions, dairy production etc. On the other hand unwanted spoilage of foods is generally caused by micro-organisms and contamination of food with pathogens cause food safety problems

The microorganism occurring on and or in food is from a practical point of view divide in three groups: molds, yeast and bacteria. Molds are generally concerned in the spoilage of food; their use in food industry is limited. Yeast is the most widely used microorganisms in the food industry due to their ability to ferment sugar to ethanol and carbon-dioxide. Some type of yeast, such as baker's yeasts is grown industrially and some may be uses as protein sources, mainly in animal feed. Important of microorganism in food microbiology may be divided into groups according to the product of fermentation, e.g. lactic acid bacteria, acetic acid bacteria, probioticacid bacteria. Bearing in mind the food constituent attacked, proteolytic, sacharolytic and lipolytic bacteria may be distinguished. Their systemic classification is based primarily on morphological and physiological properties. Lactic acid bacteria are widely used in the dairy industry and acetic acid bacteria in vinegar production. Many bacteria are known as microorganisms that cause spoilage and some are pathogens e.g. salmonellae, staphylococci, etc.

Latobacillus bulgaricus is gram positive, non-spore forming, catalase negative, microaerophilic rods with homo fermentative metabolism, whereas some are hetero fermentative [18]. Lactobacilli form the normal flora of human body in the gut and vagina.

Saccharomyces cerevisiae belongs to kingdom fungi because it has a cell wall made of chitin, it has no peptidoglycan in its cell walls, and its lipids are ester linked. It also uses DNA template for protein synthesis and it has larger ribosomes. It is then consider yeast because it is a unicellular organism which cannot form a fruiting body; like other fungi [19]. It is used extensively in batch fermentations to convert sugars to ethanol for the production of beverages and biofuels. Identification of these constraints represents an important step toward the development of improved organisms and process conditions for more rapid ethanol production [20].

Bacillus subtilis, a gram-positive, endospore forming bacteria, is usually found as the predominant microorganism in fermented soybean foods. The beneficial effect of *B. subtilis* fermentation, particularly on *natto*, has been well- established including the presence of genistein and some proteolytic enzymes that candegrade staphylococcal enterotoxin [21].

GC-MS has been a powerful tool for the identification and quantification of volatile constituents in essential oils. However, this technique cannot specifically identify those compounds which are odor-active; that is, which have a sensory perceptual impact.

In this study, *A. crassna* was fermented with some microbes. After 7 days, the mixture was subjected to hydrodistillation. The essential oil compositions obtained were investigated by using gas chromatography-mass spectrometry (GC-MS) prior analysis their antibacterial activities by using disc diffusion method.

1.2 Scopes of study

1. Microbial fermentation of *A. crassna* sample
2. Extraction of *A. crassna* essential oil after microbial treatment
3. Analysis of chemicals composition of *A. crassna* essential oils by gas chromatography-mass spectrometry (GC-MS)
4. Antibacterial assay of *A. crassna* essential oils
5. Antioxidant activity of *A. crassna* essential oils

1.3 Objectives

1. To study chemical composition of *A. crassna* oils
2. To study antimicrobial activity of *A. crassna* oils
3. To study antioxidant activity of *A. crassna* oils

1.4 Executive summary

Agarwood oil has unique and elegant oriental aroma which is a complex mixture of terpenic constituents. The aromatic components are produced from the heartwood when it is injured by insects, physical cuts, bacterial infections and chemical stimulation. Agarwood is considered to be an economically important plant in the production of perfumes. Hydrodistillation is the classical method used for extraction the agarwood essential oil. Agarwood is fermented with water for 7-10 days before it is subjected to the hydrodistillation technique. Undesired odor may be produced during fermentation process and affected on quality of agarwood oil. *Thua nao* is a conventional fermented soybean, *Bacillus subtilis*, obtained from the northern part of Thailand. A dramatic increase of several volatile components was found in soybean fermentation. It was noted that enzymatic action from *B. subtilis* can increase the amounts of volatiles in different soybeans products. The aim of this research is to study an alternative method for agarwood fermentation by using *B. subtilis* and various microbial strains. The volatile constituents of agarwood oil from various microbial treatments and their antimicrobial activities were also investigated. As the results, content of *A. crassna* essential oil was enhanced by using various microbes resulting from the activity of the intracellular bacteria which was involved with the essential oil cells in the glands of agarwood chip. These microbes could be biotransformed the essential oil precursors. Essential oil of *A. crassna* exhibited the border antibacterial activities. It can be concluded that the *B. subtilis* TN-51 and *S. cerevisiae* could be considered for use in fermenting and developing the treatment method for essential oil of many aromatic plants.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material

Stem wood chips of *A. crassna* chips aged 9 years were collected in Rayong province, Thailand. The wood chips of all plants were dried for 10 days under the shade and then pulverized into a fine powder using a blender (AIM 5CF Double ribbon blender, CapPlus technologies, USA) before being subjected to simultaneous distillation and extraction (SDE).

Figure 2 The powder of *A. crassna*

2.2 Microorganism

a. *B. subtilis* strain S1-13 (shrimp paste) , TN-51 (Thao nao) and Best 195 (Natto)
b. *S. cerevisiae* isolated from baking yeast
c. *L. bulgaricus* isolated from yogurt

2.3 Microbial starter culture preparation

In general, a single colony of each microbe was cultured in appropriate media: Nutrient agar for *B. subtilis*, bacilli MRS for *L. bulgaricus*, and Yeast extract peptone dextrose agar for *S. cerevisiae*: incubate at 37 ° C for *B. subtilis*, *L. bulcaricus* and 30 ° C for *S. cerevisiae*. They were then grown in media broth for starter culture and incubate overnight. For media broth we used 1000 ml of each flask and inoculate cell suspension of microbe, incubate overnight. The cell concentration we measure at 600 nm until to the O.D. equal 0.8 before to fermentation.

2.4 Fermentation of *A. crassna*

The microbial cells were cultured in 1000 ml of media broth until the O.D. 600 of 0.80 was obtained. 180 g of *A. crassna* sample was then added in the cell suspension. The fermented *A. crassna* was then incubated at room temperature for 7 days before extraction the essential oil.

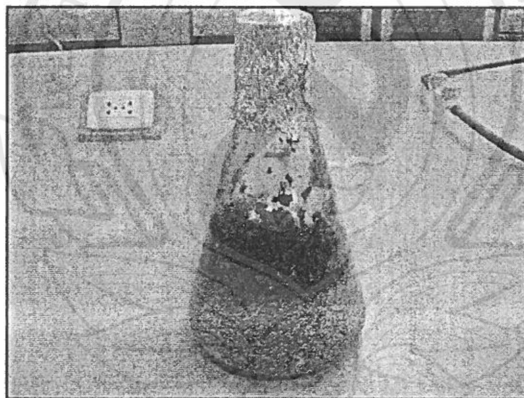


Figure 3 Fermented *A. crassna* with *B. subtilis* strain

2.5 Extraction of essential oils

Essential oils of *A. crassna* were extracted by hydrodistillation method for 48 hrs. After that, the distillate from hydrodistillation method is partitioned with dichloromethane by using separatory funnel before elimination of water by anhydrous Na_2SO_4. The essential oils were concentrated by using rotary evaporator prior stored at 4 °C for further analysis.

2.6 Analysis of essential oils chemical compositions

GC-MS analysis was performed with an HP model 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP model 5975C mass-selective detector. The capillary column was HP-5 ms (30 m × 0.25mm i.d., 0.25 μm film thickness, Agilent Technologies, Palo Alto, CA, USA). The oven temperature was initially held at 60 °C and then increased at a rate of 3 °C/min to a final temperature of 250 °C. The injector temperature was 250 °C. Purified helium was used as the carrier gas at a flow rate of 1 ml/min. EI mass spectra were collected at 70 eV ionization voltages over the range of *m/z* 30-550. The electron multiplier voltage was 1059 V. The ion source and analyzer temperatures were set at 230 °C and 200 °C, respectively. Identification of volatile components was performed by comparison of their retention indices, relative to C_6-C_{20} *n*-alkane mixture (ASTM D2887, Supelco, Bellefonte, PA, USA) and comparison of the mass spectra of individual components with the reference mass spectra in the W8N05ST databases. Results are presented in terms of percent relative peak areas as no external or internal standards were used in this work.

2.7 Antimicrobial activity assay

- Bacterial strains

The bacterial strains tested were *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa* , *Enterobacter aerogenes, Serratia marcescens, Salmonella typhimurium* and *Micrococcus luteus.*

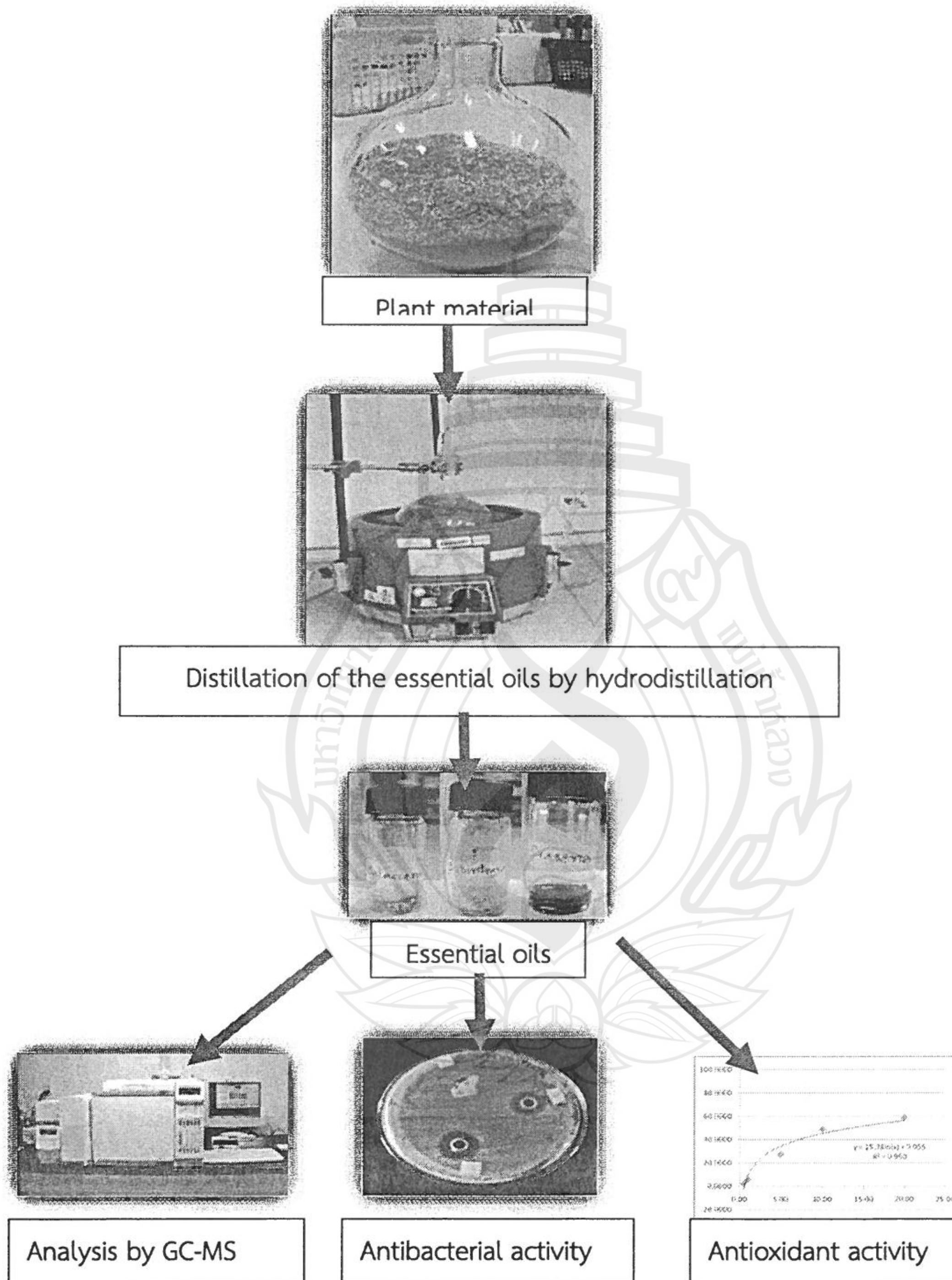
- Disc diffusion assay

The antibacterial assays were carried out by the disc-diffusion. Briefly, filter paper discs (6 mm) and bacterial stain was placed into the nutrient agar (NA) plate by using sterile cotton swab. Prepared discs by diluted with dichloromethane ratio 1:1 of tested agents were placed on the surface of inoculated nutrient agar (NA). Plates were incubated at 37 °C for 24 hrs and zone of inhibition was measured (mm).Each experiment was performed in triplicate. In addition, growth inhibition and minimal inhibitory concentration (MIC) were also determined.

2.8 *Antioxidant activity (DPPH radical scavenging assay)*

The radical scavenging abilities of *F. fragrans* flowers oil and various crude extracts were analyzed compared to a standard butyl hydroxyl toluene (BHT) and α-tocopherol based on the reaction with 2,2-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$). This method was evaluated using a spectrophotometric method. One millilitre of various concentrations of each sample in methanol was added to 1 ml of a 0.003% methanol solution of DPPH and the reaction mixture was shaken vigorously. The tubes were allowed to stand at room temperature for 30 min. Each reaction mixture was then placed in the cuvette holder of a Perkin Elmer-Lamda 25 UV/Vis spectrophotometer and monitored at 517 nm against blank which used methanol as the baseline correction. The scavenging ability was calculated as follows: Scavenging ability (%) = 100 × [Absorbance of control – Absorbance of sample/Absorbance of control]. The antioxidant activity of all samples was expressed as IC_{50} which was defined as the concentration (in μg/ml) of oil required to inhibit the formation of DPPH radicals by 50%. The experiment was carried out in triplicate.

Flow chart of method



CHAPTER 3
RESULTS AND DISCUSSION

3.1 Percent yield of *A. crassna* essential oils

Essential oils of *A. crassna* obtained from Rayong province after microbial treatment extracted using a modified Likens-Nickerson apparatus appeared as yellow viscous liquids with percentage yields of 0.03-0.21 (w/w) as shown in Table 2. Highest yield was detected in essential oil obtained from TN-51 followed by Best-195, S1-13, *S. cerevisiae* and *L. bulgaricus*, respectively when compared with essential oil of *A. crassna* without microbial treatment (control). All essential oils extracted by hydrodistillation method comprise different color and odor. Essential oil of *A. crassna* obtained from microbial fermentation including *B. subtilis* strain TN-51 has light brown color with strong odor while S1-13, *L. bulgaricus* and Best-195 contain brown color as shown in Figure 4.

Table 2 Percent yield of *A. crassna* essential oils

Microbial treatment	% yield
TN-51	0.21
S1-13	0.11
Best-195	0.20
S. cerevisiae	0.09
L. bulgaricus	0.08
Control (7 days)	0.03

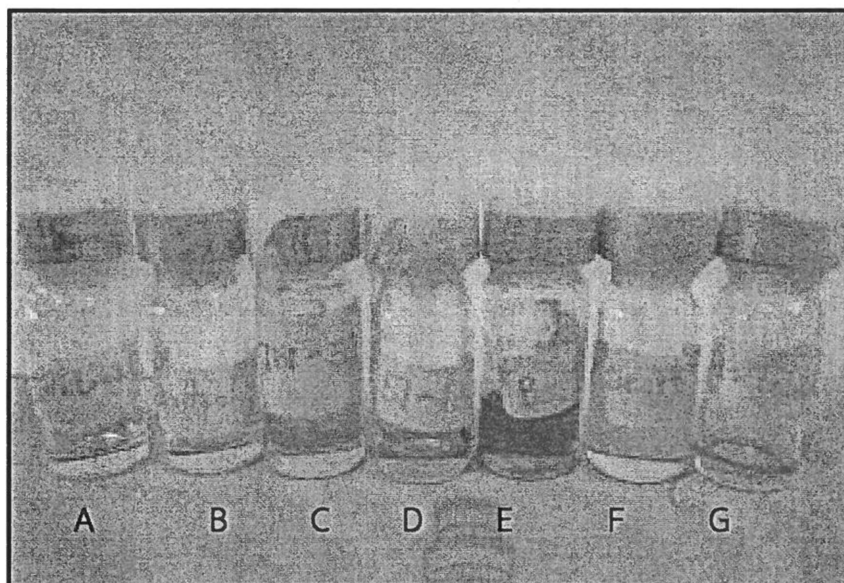


Figure 4 Essential oils of oils *A. crassna* fermented with microbes; Control (A), control (fermented with water for 7 days) (B), *B. subtilis* strain TN-51 (C), *B. subtilis* strain S1-13 (D), *B. subtilis* strain Best-195 (E), *S. cerevisiae* (F) and *L. bulgaricus* (G)

3.2 Chemical composition of *A. crassna* oils

Results of the fingerprint in terms of volatile components of *A. crassna* essential oil from Rayong province and their fermentation with bacterium supernatants of various strains analyzed by GC-MS and quantities determined with the compound to internal standard are summarized in Figure 5 and Table 3. Similar characteristics of all fermentations were illustrated. Thirty volatiles of *A. crassna* essential oil ground with water for 7 days without microbial treatment representing 91.01% of the total peak area were identified. The major components were hexadecanoate (13.38%), β-agarofuran (10.34%), kusunol (8.20%), dehydrojinkoh-eremol (7.34%) and 9,11-eremophiladien-8-one (6.29%), respectively. Eighty-one volatiles were identified among the *A. crassna* essential oil with *S. cerevisiae* (yeast) treatment. Agarospirol, β-agarofuran, karanone, benzeneacetaldehyde, α-(Z)-atlantone and jinkoh-eremol were found to be the major components in *A. crassna* essential oil with *S. cerevisiae* treatment. Small amounts of kusunol, selina-3,11-dien-14-al, β-epi-eudesmol and oxo-agarospirol were also detected. Essential oil obtained by *L. bulgaricus* treatment from yogurt contained agarospirol, β-agarofuran, p-anisaldehyde, karanone, jinkoh-eremol and nor-ketoagarofuran as the key odor

constituents, while monoterpene components such as α-(Z)-atlantone, α-terpinene, selina-3,11-dien-14-ol and selina-3,11-dien-14-al were minor components. The dominant components of *B. subtilis* TN-51-treatment were neo-dihydro carveol acetate, cubenol, jinkoh-eremol, agarospirol, 1-epi-cubenol and β-agarofuran. They were accompanied by the small amounts of acorone, benzyl acetone, β-epi-eudesmol and β-acoradienol. *A. crassna* fermented with *B. subtilis* S1-13 culture supernatants produced the dominant components of benzyl acetone, kusunol, cyperotundone, agarospirol, jinkoh-eremol, β-epi-eudesmol and dehydrojinkoh-eremol. Other components such as m-cymenene, allo-cedrol, 1,3,8-p-menthatriene, α-(Z)-atlantone and epoxybulnesene were detected in lower amounts. Essential oil obtained by *B. subtilis* Best-195 treatment contained acorenone B, agarospirol, jinkoh-eremol, allo-cedrol, selina-3,11-dien-14-al, α-(Z)-atlantone, epoxybulnesene, mayurone and selina-3,11-dien-9-ol as the key odor constituents, while cyclocolorenone, geranyl benzoate, karanone, caryophyllene oxide, α-agarofuran, 9,11-eremophiladien-8-one and oxo-agarospirol were minor components.

Our results are similar from most published studies. Agarospirol was found to be the predominant constituent in the *A. crassna* essential oil as was reported by Meier et al. [8]. It was found that greater number of volatile components was detected significantly in *A. crassna* treated with various microbes while compared to the control. Microbial cultures fermentation of could induce increasing amounts of various volatile components due to enzyme production of each strain. Total volatiles significantly increased from its original non-fermented sample. These occurrences happened could be explained that the existing form, amount and kind of aroma precursors were different in different tea explained by Su et al. [22]. Besides, the enzyme could show different substrate specificity to different aroma precursors. It seems that agarospirol, jinkoh-eremol and β-agarofuran play important role in the special odor of *A. crassna* essential oil. Increased number of components might be also affected from enzymatic production of microbes which enzymatic activities produced by microbes, such as protease, amylase and galactosidase [23, 24] improved odor volatile components during fermentation. Furthermore, various

isolated *B. subtilis* could produce several extracellular enzymes with the same function, such as nattokinase, protease, amylase, phytase, lipases and glutamyl hydrolase [25-29]. Enzymatic degradation products might be generated further complex odorous compounds through other reactions. Culture supernatants of *S. cerevisiae* (yeast) and *B. subtilis* TN51 strains improved the high yield of essential oil with their effectiveness on increasing of volatile components.

3.3 Antibacterial activity of *A. crassna* oils

The results of antibacterial activities of all crude essential oils of *A. crassna* using the agar diffusion method are shown in Table 4. The essential oil of *A. crassna* from microbial treatments demonstrated the moderate antibacterial activity against most test bacteria. *B. subtilis* strain TN-51 and *S. cerevisiae* showed the greatest antibacterial activities against all bacteria consisting of *M. luteus*, *S. marcescens*, *P. aeruginosa*, *E. aerogenes*, *S. aureus*, *B. cereus*, *S. typhimurium* and *E. coli.* The essential oil of Bacillus strain S1-13 provided moderate antibacterial activity against 7 bacteria consisting of *M. luteus, S. marcescens, P. aeruginosa, E. aerogenes, S. aureus, B. cereus, E. coli* and no activity on *S. typhimurium* was detected. Essential oils with Best-195 treatment also present the moderate antibacterial activity against 7 bacteria consisting of *M. luteus, S. marcescens, P. aeruginosa, E. aerogenes, S. aureus, B. cereus* and *E. coli.* For *L. bulgaricus* treatment, lower antibacterial activity was found against 6 bacterial strains; *M. luteus, S. marcescens, P. aeruginosa, E. aerogenes, S. aureus* and *B. cereus* while essential oil without microbial treatment against only 4 bacteria; *M. luteus, P. aeruginosa, S. aureus* and *B. cereus.* Evaluation of minimum inhibitory concentration (MIC) of all essential oils is shown in Table 5. Inhibition zones from some *A. crassna* essential oils with various concentrations are shown in Figure 5. MIC of essential oils of *A. crassna* with *B. subtilis* strain TN-51, S1-13, Best-195, *S. cerevisiae, L. bulgaricus* treatment and without microbial treatment were 62.25-1000, 62.25-1000, 500-1000, 125-1000, 500-1000 and 250-500 mg/ml, respectively. As noticed, essential oils of *A. crassna* with *B. subtilis* strain TN-51 and S1-13 inhibited *P. aeruginosa* significantly at concentration of 62.25 mg/ml compared to that obtained from various microbial treatment as well as control. As the result,

the activity of each essential oil depends on each microbe used for fermentation because enzyme produced from each microbe provides different ability to breakdown cell of *A. crassna* oil resulting various percent yield and chemical composition. The different inhibition among these essential oils may be due to the different major compound in these essential oils which greater inhibition was found in the *B. subtilis* strain TN-51 followed by yeast (*S .cerevisiae*), S1-13, *L. bulgaricus* from yogurt and Best-195, respectively.

3.4 Antioxidant activities of essential oil of *A. crassna*

According to using various microbes, the antioxidants properties are considered to be different. Antioxidant activities of the essential oil of *A. crassna* were tested by the DPPH radical scavenging. The violet color of the radical disappeared when mixed with the substances in the sample solution that donate a hydrogen atom. Antioxidant activities of all samples, standard BHT and α-tocopherol are presented in Table 6 in which lower IC_{50} values indicate higher antioxidant activity. The essential oil of *A. crassna* obtained from control (without treatment), *S. cerevisiae* (yeast), TN-51, S1-13, Best-195 and *L. bulgaricus* (yogurt) treatment were able to reduce the stable free radical DPPH with an IC_{50} of 184.71, 107.36, 98.69, 144.99 and 154.47 µg/ml whereas IC_{50} of standard BHT and α-tocopherol were lower at 30.37 and 50.27 µg/ml, respectively. In this study, the essential oils of *A. crassna* obtained from different microbial treatment exhibited lower antioxidant activity than the synthetic antioxidant α-tocopherol and BHT. Although the essential oil contained different monoterpenes and sesquiterpenes, poor antioxidant activity was detected among all essential oils. It was shown that these terpene hydrocarbons, whose antioxidant activity was not closed to that of phenolic compounds. Antioxidant activity of various essential oils of *A. crassna* obtained from microbial treatment should be not related to major compounds such as agarospirol, β-agarofuran and p-anisaldehyde, neo-dihydro carveol acetate, cubenol and jinkoh-eremol.

Table 3 Volatile constituents and their relative peak area percentage obtained from *A. crassna* essential oils obtained from various microbial treatments

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
1	Benzaldehyde	6.40	976	0.58	0.15	1.13		0.71	
2	5-Methyl furfural	6.67	987	4.27					
3	α-Terpinene	7.85	1017		1.44	0.89		3.71	
4	Limonene	8.15	1029		0.12				
5	Benzeneacetaldehyde	9.55	1073	7.06		0.75	1.33		
6	Salicylaldehyde	9.95	1084	0.03		0.89			
7	Acetophenone	10.08	1088	0.23					
8	p-Cresol	10.24	1092	0.10					
9	2-Methoxy ethyl benzene	10.38	1096	0.04	1.26				
10	m-Cymenene	10.64	1103	0.05		2.84		0.89	
11	trans-Linalool oxide (furanoid)	10.71	1105	0.04					
12	o-Guaiacol	10.82	1108	0.14					
13	6-Camphenone	11.23	1118	0.03					
14	Linalool	11.30	1119	0.05					

Table 3 (continued)

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
15	Phenyl ethyl alcohol	11.71	1129	0.06	0.05				
16	1,3,8-p-Menthatriene	11.92	1135	0.03		2.55			
17	Chrysanthenone	12.18	1141	0.07					
18	Dihydro-linalool	12.63	1152	0.03		0.59			
19	p-Vinyl anisole	13.11	1164	0.09	0.14	0.73	1.49	0.63	
20	Neo-allo-ocimene	13.53	1174	0.13	0.05	0.68	1.37	0.59	
21	δ-Terpineol	14.05	1176		0.14				
22	Benzyl acetone	17.37	1265	1.64	3.42	8.96	0.42	2.04	
23	p-Anisaldehyde	17.57	1270	0.08	0.24	0.78	1.38	5.84	
24	p-Anisyl alcohol	18.82	1299	0.06	0.08	0.65	0.32		
25	iso-Isopulegyl acetate	18.99	1303	0.04	0.05	0.60		0.65	
26	neo-Dihydro carveol acetate	19.99	1327	0.07	11.21	0.67	0.45		
27	Methyl thujate	20.37	1336	0.04	0.09	0.64		0.64	
28	3-Hydroxy-4-phenyl-2-butanone	21.71	1368	0.07	0.13	0.70	0.38	0.64	
29	Silphiperfol-6-ene	22.95	1397	0.17	0.31	0.59	1.46	0.64	

Table 3 (continued)

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
30	β-Elemene	23.22	1403	0.06	0.06	0.62	0.65	0.54	
31	α-Funebrene	23.65	1414	0.19	0.09	0.74	1.41	0.65	
32	α-cis-Bergamotene	23.93	1421	0.09	0.07	0.65	0.60	0.56	
33	E-α-Ionone	24.87	1444	0.05	0.07	0.75	1.35	0.57	
34	trans-α-Bergamotene	25.05	1448	0.08	0.06	0.64	0.38	0.54	
35	α-Guaiene	25.28	1454	0.23	0.57	1.12	1.96	1.22	
36	α-Humulene	25.47	1454		0.71				
37	β-Agarofuran	26.79	1491	10.52	4.45	0.68	1.57	6.61	10.34
38	Germacrene D	26.85	1492	0.24	0.23	0.95		0.62	
39	β-Selinene	26.94	1494	0.09	0.05	0.64	1.38	0.62	
40	Viridiflorene	27.08	1498	0.07	0.05	0.64	1.35		
41	*p*-methoxybenzylacetone		1504	0.09	0.11	0.14	0.21	0.08	0.47
42	α-Selinene	27.31	1504	0.17	0.66	1.80	1.87	0.67	
43	4-epi-cis-Dihydroagarofuran	27.49	1508	1.02	0.88	1.15	0.89	1.18	
44	Anisyl propanoate	27.66	1512	0.51	0.22				

Table 3 (continued)

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
45	α-Bulnesene	28.08	1523	0.39	0.45	1.15		1.14	
46	α-Agarofuran	29.23	1553	0.89	0.66	1.34	2.07	1.37	0.21
47	cis-Muurol-5-en-4-α-ol	29.94	1571	0.86	0.88	1.24	1.93	1.59	
48	Caryophyllene oxide	30.68	1590	0.11	1.37	1.62	2.21		
49	allo-Cedrol	30.99	1597	0.96	2.34	2.78	4.03		
50	nor-Ketoagarofuran	31.29	1605	0.12	0.36	0.77	1.44	5.08	1.54
51	Epoxybulnesene	31.72	1617	0.41	0.17	1.06	2.73		3.04
52	β-epi-Eudesmol	32.26	1631	2.54	3.15	3.60		4.35	
53	1-epi-Cubenol	32.77	1638		4.81				
54	Agarospirol	33.18	1656	13.71	5.15	5.02	5.19	8.83	5.77
55	Cubenol	33.47	1646		7.25				
56	7-epi-α-Eudesmol	33.90	1663		1.79				
57	4-(-Hydroxy-3-methoxyphenyl)-2-butanone		1665	0.14	0.05	0.14	0.09	0.11	0.45
58	epi-Zizanone	34.02	1670		1.52				

Table 3 (continued)

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
59	valerianol		1679	0.09	0.12	0.11	0.08	0.05	0.33
60	γ-eudesmol		1679	0.89	0.32	0.24	0.16	0.29	4.22
61	dehydrojinkoh-eremol		1680	0.78	2.14	3.54	1.18	0.98	7.34
62	Jinkoh-eremol	34.18	1682	5.22	5.44	4.34	4.76	5.09	4.47
63	selina-3,11-dien-9-one		1683	0.08	0.14	0.24	0.11	0.07	1.12
64	Kusunol	34.65	1695	3.55	4.63	5.25	1.75		8.20
65	Acorenone B	34.91	1702	1.54	0.66	1.37	7.10		1.09
66	Cyperotundone	35.14	1708	2.11	0.34	5.16	1.66	1.31	
67	Melaleucol	35.22	1709		1.84				
68	α-(Z)-atlantone	35.49	1718	6.39	0.31	2.39	2.93	4.55	
69	rotundone		1719	0.47	0.35	0.21	0.64	0.89	1.22
70	Mayurone	35.62	1722	1.38	0.84	0.83	2.41		
71	Selina-3,11-dien-9-ol	36.08	1735	0.87	1.37	1.20	2.64	0.98	2.85
72	(E)-Nerolidol acetate	36.60	1745	0.25	0.66	0.14	0.22	0.31	0.11
73	Selina-3,11-dien-14-al	36.90	1747	3.10		1.15	3.24	3.04	0.08

Table 3 (continued)

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
74	β-Acoradienol	36.93	1763		2.80				
75	γ-Curcumen-15-al	37.07	1768		0.84				
76	selina-4,11-dien-14-al		1766	0.11	0.08	0.14	0.11	0.10	0.28
77	9,11-Eremophiladien-8-one	37.22	1767	1.80	0.74	1.06	2.03	1.39	6.29
78	methyl tridecanoate		1771	0.32	0.22	0.15	0.36	0.24	0.79
79	Selina-3,11-dien-14-ol	37.41	1772	1.40	0.62	1.10	1.54	3.32	0.22
80	β-eudesmol acetate		1776	0.11	0.23	0.38	0.65	0.41	2.24
81	epi-α-bisabolol acetate		1779	0.21	0.35	0.11	0.08	0.07	0.45
82	guaia-1(10),11-dien-15-ol		1781	0.08	0.04	0.11	0.09	0.08	1.93
83	Cyclocolorenone	37.77	1782	1.67	0.77	0.92	2.42	1.34	3.43
84	Acorone	38.77	1820		3.64				
85	Karanone	39.41	1838	7.14	0.66	1.59	2.22	5.61	1.54
86	hexadecanoate		1842	0.35	0.89	0.78	1.52	2.36	13.38
87	(*Z*)-9-octadecanoic acid		1856	0.54	0.23	0.56	0.85	0.95	1.08
88	oxo-Agarospirol	39.89	1857	2.21	0.35	0.73	2.03	1.54	1.18

Table 3 (continued)

No.	Compound	Retention time (min)	LRI	% area					
				S. cerevisiae (yeast)	TN-51	S1-13	Best-195	*L. bulgaricus* (yogurt)	Control (7 days)
89	Isoamyl dodecanoate	40.22	1869	0.68	0.26	0.87	1.42	1.34	
90	β-(E)-Santalol acetate	40.52	1881	0.96	0.26	0.86	1.41	0.77	
91	Oplopanonyl acetate	41.05	1901	0.59	0.13	0.74		0.73	
92	Geranyl benzoate	43.30	1971	0.27	0.42	0.77	2.32	1.19	
93	Ethyl hexadecanoate	44.07	1996	1.22	1.03	0.74	1.52	1.91	

LRI; Linear retention index on HP-5 column

Table 3 presents the volatile constituents and their relative peak area percentage obtained from *A. crassna* essential oils obtained from various microbial treatments. Compounds with peak area percentage provide high content which is considered to be major compound in each essential oil. Identification of all components was performed by comparing their retention indices on HP-5 column. As the results, the different major components were detected in various essential oils obtained from microbial fermentations.

Table 4 Antibacterial activity of *A. crassna* essential oils obtained by various microbial treatments

Bacteria strain	Diameter of inhibition zone, mm (mean±SD)					
	Control	TN-51	S1-13	Best-195	*S. cerevisiae*	*L. bulgaricus*
M. luteus	1.0±0.0	2.0±1.0	1.0±1.0	1.3±0.6	1.0±0.0	1.0±0.0
S. marcescens	-	2.7±1.2	1.0±0.0	1.0±0.0	3.3±1.5	1.0±0.0
P. aeruginosa	1.1±0.0	3.0±1.7	1.7±0.6	2.0±0.0	3.0±1.0	1.3±0.6
E. aerogenes	-	3.7±2.1	2.7±0.6	2.0±1.0	2.0±0.0	0.2±0.0
S. aureus	1.0±0.2	7.0±3.5	1.0±0.0	1.0±0.0	2.3±1.5	3.0±0.0
B. cereus	1.0±0.0	6.0±1.0	3.7±0.6	1.3±1.2	2.7± 2.3	1.3±1.2
S. typhimurium	-	3.0±1.0	-	-	2.6±1.2	-
E. coli	-	2.0±0.0	1.0±1.0	0.7±0.6	2.0±1.7	-

Note: * (-) no inhibition

*Diameter of zone of inhibition in mm (mean±standard deviation)

Antibacterial activity of *A. crassna* essential oils obtained by various microbial treatments was shown in Table 4. Wider diameter of inhibition present showed better antimicrobial activity. Most essential oils from various microbial fermentations exhibited good antimicrobial activities when compared to essential oil without microbial fermentation (control).

Table 5 Minimum inhibitory concentration (MIC) of various *A. crassna* oils

Bacteria strain	Diameter of inhibition zone, mm (mean ± SD)					
	Control	TN-51	S1-13	Best-195	*S. cerevisiae*	*L. bulgaricus*
M. luteus	500 (0.2±0.1)	1000 (5.1±0.7)	500 (1.8 ±0.0)	1000 (4.2±1.4)	500 (6.0±0.7)	1000 (2.0±0.0)
S. marcescens	-	1000 (2.1±0.7)	1000 (18 ±0.0)	1000 (2.2±1.4)	125 (2.2±2.1)	1000 (2.0±0.0)
P. aeruginosa	500 (0.1±0.0)	62.25 (2.0±0.0)	62.25 (0.2±0.7)	1000 (5.0± 0.0)	250 (1.0±0.0)	500 (2.0±1.4)
E. aerogenes	-	500 (2.0±0.0)	500 (0.3±0.0)	1000 (5.1±0.7)	500 (11.2±2.1)	1000 (1.0±0.0)
S. aureus	250 (0.1±0.0)	62.25 (3.1±1.4)	500 (1.0±0.7)	500 (2.2±1.4)	250 (1.0±0.0)	500 (3.0±0.0)
B. cereus	500 (0.1±0.0)	500 (2.0±0.0)	500 (1.0±0.0)	1000 (1.1±0.0)	250 (3.0±0.0)	500 (6.0±0.7)
S. typhimurium	-	1000 (3.0±1.0)	-	-	1000 (2.6±1.2)	-
E. coli	-	1000 (2.0±0.0)	1000 (1.0±0.0)	1000 (0.7±1.4)	1000 (2.0±1.4)	-

Note: * (-) no inhibition

*Diameter of zone of inhibition in mm (mean±standard deviation)

Table 5 presents minimum inhibitory concentration (MIC) of various *A. crassna* oils which most essential oils provided good antimicrobial activities with a concentration of 1000 mg/ml. However, better antimicrobial activity was detected on essential oil fermented with TN-51 which can inhibit *S. aureus* and *P. aeruginosa* with a concentration of 62.25 mg/ml. In addition, S1-13 microbe provided greater antimicrobial activity inhibiting *P. aeruginosa* with a concentration of 62.25 mg/ml.

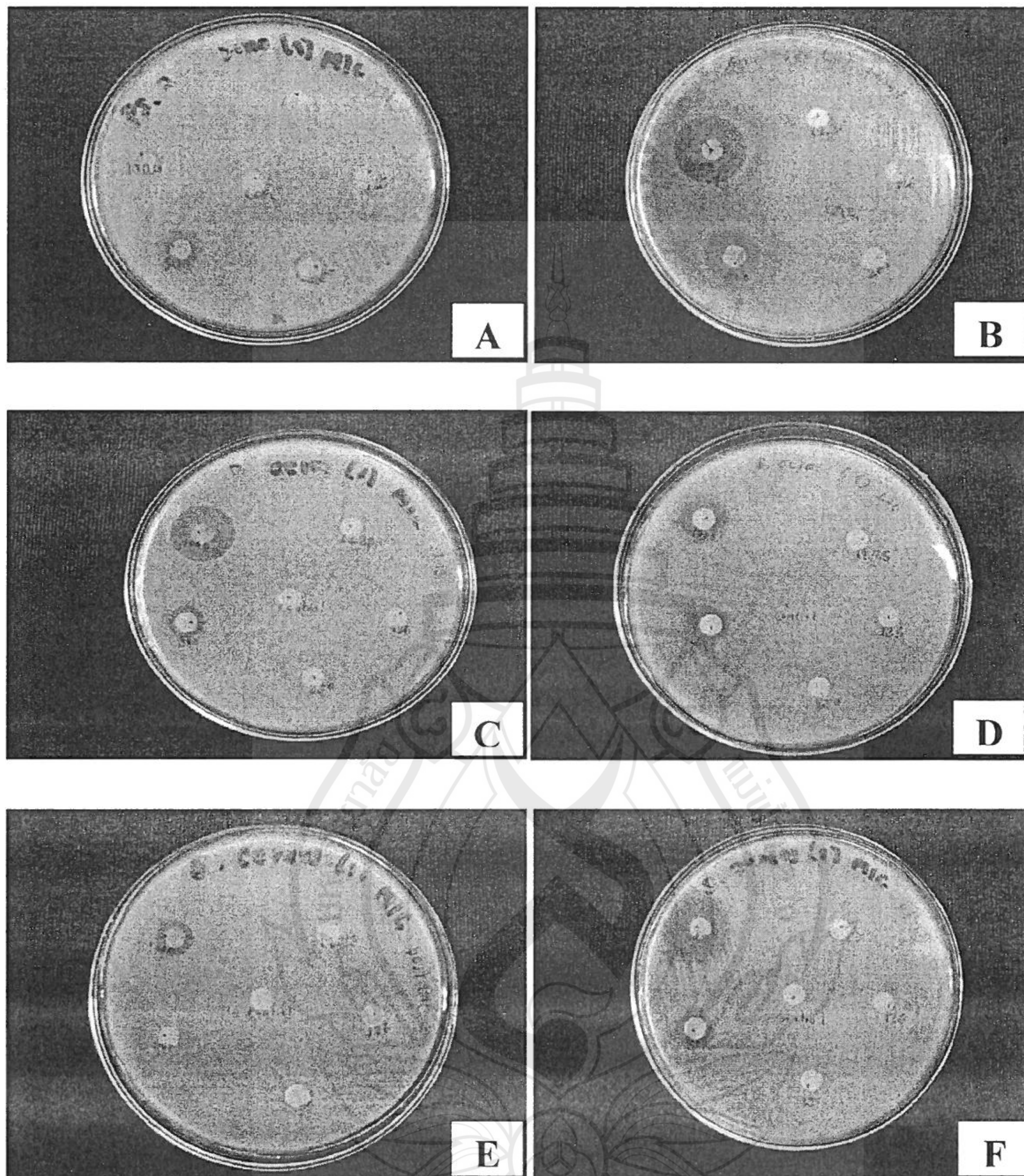


Figure 5 Antibacterial activity of *A. crassna* essential oils with various concentrations; *B. subtilis* strain TN-51 inhibits *P. aeruginosa* (A), *S. cerevisiae* inhibits *E. aerogenes* (B), *B. subtilis* strain S1-13 inhibits *B. cereus* (C), *L. bulgaricus* inhibits *B. cereus* (D), *B. subtilis* strain Best-195 inhibits *B. cereus* (E) and *B. subtilis* strain S1-13 inhibits *S. aureus* (F)

Table 6 Antioxidant activity (IC_{50}) of the essential oil of *A. crassna* obtained from different microbial treatments. Values represent averages ± standard deviations for triplicate experiments. BHT; butyl hydroxyl toluene

Essential oil	IC_{50} (μg/ml)
no microbial	184.71±0.90
S. cerevisiae (yeast)	107.36±1.21
TN-51	98.69±0.85
S1-13	144.99±0.89
Best-195	154.47±1.22
L. bulgaricus (yogurt)	134.24±0.62
BHT	30.37±0.17
α-tocopherol	50.27±0.16

Antioxidant activities (IC_{50}) of the essential oil of *A. crassna* obtained from different microbial treatments. Values represent averages ± standard deviations for triplicate experiments are demonstrated in Table 6 where BHT is butyl hydroxyl toluene. It was found that antioxidant activity of all essential oils was not closed to that of phenolic compounds. Antioxidant activity of various essential oils of *A. crassna* obtained from microbial treatment is not related to major compounds such as agarospirol, β-agarofuran and p-anisaldehyde, neo-dihydro carveol acetate, cubenol and jinkoh-eremol.

CHAPTER 4
CONCLUSION

Essential oils of *A. crassna* obtained from Rayong province with microbial treatment and without microbial treatment extracted using a modified Likens-Nickerson apparatus appeared as yellow viscous liquids with percentage yields of 0.03-0.21 w/w. Highest yield was detected in essential oil obtained from TN-51 followed by Best-195, S1-13, *S. cerevisiae* and *L. bulgaricus*, respectively when compared with essential oil of agarwood without microbial treatment (control). All essential oils extracted by hydrodistillation method comprise different color and odor. It seems that agarospirol, jinkoh-eremol and β-agarofuran play important role in the special odor of *A. crassna* essential oil. Increased number of components might be also affected from enzymatic production of microbes which enzymatic activities produced by microbes, such as protease, amylase and galactosidase improved odor volatile components during fermentation. Furthermore, various isolated *B. subtilis* could produce several extracellular enzymes with the same function, such as nattokinase, protease, amylase, phytase, lipases and glutamyl hydrolase. Enzymatic degradation products might be generated further complex odorous compounds through other reactions. Culture supernatants of *S. cerevisiae* (yeast) and *B. subtilis* TN-51 strains improved the high yield of essential oil with their effectiveness on increasing of volatile components. As the result, the antibacterial activity of each essential oil depends on each microbe used for fermentation because enzyme produced from each microbe provides different ability to breakdown cell of agarwood oil resulting various percent yield and chemical composition. The different inhibition among these essential oils may be due to the different major compound in these essential oils which greater inhibition was found in the *B. subtilis* strain TN-51 followed by yeast (*S. cerevisiae*), S1-13, *L. bulgaricus* from yogurt and Best-195, respectively. The essential oils of *A. crassna* obtained from different microbial treatment exhibited lower antioxidant activity than the synthetic antioxidant

α-tocopherol and BHT. Although the essential oil contained different monoterpenes and sesquiterpenes, poor antioxidant activity was detected among all essential oils. It was shown that these terpene hydrocarbons, whose antioxidant activity was not closed to that of phenolic compounds. Antioxidant activity of various essential oils of *A. crassna* obtained from microbial treatment is not related to major compounds such as agarospirol, β-agarofuran and p-anisaldehyde, neo-dihydro carveol acetate, cubenol and jinkoh-eremol.

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