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THESIS

EFFECTS OF SECONDARY METABOLITES OF SOME YEASTS ON *Colletotrichum musae* AND ANTHRACNOSE DISEASE ON BANANAS CV. HOM THONG

VEERANEE TONGSRI

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Veeranee Tongsri 2010: Effects of Secondary Metabolites of Some Yeasts on *Colletotrichum musae* and Anthracnose Disease on Bananas cv. Hom Thong. Doctor of Philosophy (Plant Pathology), Major Field: Plant Pathology, Department of Plant Pathology. Thesis Advisor: Associate Professor Somsiri Sangchote, Ph.D. 139 pages.

This study concerns the metabolites from two yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* on controlling and induced resistance to anthracnose disease of bananas. Yeast metabolites had an effect on the reduction of banana anthracnose and capability of induction of diseased resistance. Metabolites from yeasts directly affected on the growth of *Colletotrichum musae* by producing hydrolytic enzymes; ß-1,3-glucanase and chitinase. Volatile metabolites from these yeasts also directly reduced the pathogen growth. Whereas the increase in the activities of defensive enzymes; phenylalanine ammonia-lyase (PAL), ß-1,3-glucanase and chitinase in peel tissues after application with both yeast metabolites was shown indirect effect on the disease development. PAL protein was rapidly accumulated at 0 h after treatment as well as PAL mRNA expression was observed. ß-1,3-glucanase activity was enhanced starting from 48 h after treatment, but mRNA level was expressed at 0 h. Whereas chitinase activity was induced at 72 h after treatment, but the expression of mRNA level was not observed in all duration of storage time. An antifungal compound, 3-(2-hydroxyethyl)-indole or tryptophol, which contained in yeast culture filtrates also directly inhibited *C. musae* growth, and probably acted as signal molecule for defense mechanisms in banana tissues. Thus, the ability of two yeasts to produce antifungal compounds, cell wall degrading enzymes and volatile secondary metabolites, subsequently to enhance the defense response in banana tissues have contributed the increased control levels of banana anthracnose.

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INTRODUCTION

Banana anthracnose, caused by *Colletotrichum musae* (Berk & Curtis), is recognized as one of the most destructive diseases of banana worldwide. *C. musae* is one of the component of crown rot complex, one of the major diseases of banana fruits. This pathogen is also the causal agent of stem-end rot and blossom-end rot of banana (Dadzie and Orchard, 1997). *C. musae* infects immature banana in the field but symptoms appear on the fruit peel at the ripening stage. The presence of antifungal compounds in the unripe banana fruits has been related to the latency of *C. musae* in the fruits (Barkai-Golan, 2001). This pathogen causes high losses in banana both in terms of quantity and quality especially in countries with warmer climates (Sanchez-Serrano *et al.,* 1993).

Biological control have been used in postharvest disease management on banana and a number of postharvest commodities. Several microorganisms including fungi, bacteria and yeasts have emerged as a promising antagonist to be used and replaced synthetic fungicides which harmful to human health and environment (De Costa and Erabadupitiya, 2005). Up to present, five antagonistic microorganisms, three yeasts (*Candida oliophila, Cryptococcus albidus*, *Metschnikowia fructicola*) and two strains of bacterium *Pseudomonas syringae* are commercially available to control a wide range of postharvest pathogens (Droby *et al.,* 2002; Janisiewicz and Korsten, 2002; Chanchaichaovivat *et al.,* 2008). Yeasts are the most popular microorganisms that have been used in postharvest disease management as biological control agents. Antagonistic yeasts have been selected mainly because they do not damage the host cells and produce toxic metabolites which could have a negative effect on environment, animal and human health. Mechanisms have been reported in their activities including nutrient and space competition, production of cell wall degrading

enzymes, and activation of host plant resistance to pathogen (Chanchaichovivat *et al.,* 2008; Jiang *et al*., 2009).

Several researches have been reported antagonistic yeasts are capable to induce resistance in the host tissue (Ippolito *et al.,* 2000; Droby *et al.,* 2002; Saligkarias *et al.,* 2002b; Castoria *et al.,* 2003; El Ghaouth *et al.,* 2003; Vivekananthan *et al.,* 2004). Yeast antagonists induced several defense responses including the accumulation of the phytoalexins, scoparone and scopoletin in citrus peel (Kim *et al.,* 1991) and increased the defensive and antioxidant enzymes in the host cells such as phynylalanine ammonia-lyase (PAL), chitinase, ß-1,3-glucanase, supperoxide dismutase (SOD), polyphenol oxidase (PPO) and catalase (CAT) in tomato, apple, jujube and sweet cherry fruits (El Ghaouth *et al.,* 2003; Chan and Tian, 2006; Tian *et al.,* 2007; You-sheng and Shi-ping, 2008; Zhao *et al.,* 2008). Yeasts also induced cell wall accumulation in apple fruit (El Ghaouth *et al.,* 1998).

Although antagonistic yeasts have shown the good results in controlling postharvest diseases in many commodities, these yeasts cells might cause microbial contaminants on produces. The demand for bananas is increasing worldwide, and banana for export must be free from diseases, insects, fungal toxins and chemical residues. Additionally, living cells of microorganisms that used as biological control agents on banana probably cause contamination on the fruits. Therefore, in this research, culture filtrates without living cells of yeasts will be used as substances for direct control and induced resistance against anthracnose disease of banana fruit cultivar Hom Thong (*Musa acuminata*, AAA Group) has been reported.

OBJECTIVES

The objectives of this research were:

1. To study antifungal properties of metabolites from yeasts on the growth of *Colletotrichum musae* and the development of anthracnose disease on banana fruits.

2. To determine the changes of defensive enzymes including phenylalanine ammonia-lyase, ß-1,3-glucanase and chitinase induced by metabolites from yeasts against *C. musae* on banana fruits.

3. To investigate the RNA expression on banana fruit peels in response to stimulation by metabolites from yeasts.

4. To identify secondary metabolites from yeasts that provide inhibition of *C. musae* growth.

LITERATURE REVIEW

1. Banana and its postharvest diseases

Banana (*Musa acuminata* L.) is a widely grown fruit crop in tropical countries. It is also the main fruit in the international trade because of high consumer demand worldwide. In 2009, Office of Agricultural Economics, Thailand reported in terms of volume banana is export fruit by 9,000 tons and in terms of value by 135 million baht. Bananas are mainly exported to Japan and China. UNCTAD (2008) reported world total exports of banana accounted for 15.9 million tons.

Being a highly perishable fruit, postharvest losses of banana are highly expected. Banana postharvest diseases can cause serious losses of fruits both in terms of quantity and quality such as crown rot, fruit rot and anthracnose. Crown rot is the most important disease. It is a disease complex caused by several fungi including *Lasiodiplodia theobromae, Fusarium roseum, F. semitectum, F. pallidoroseum* and *C. musae.* Other species, *Cephalosporium* sp., *Verticillium theobromae, Ceratocystis paradoxa* and *Phomopsis* sp. have been reported as involved in crown rot complex (Dadzie and Orchard, 1997; Abayasekara *et al.,* 1998). In the case of *L. theobromae*, it invades wound on the fruit skin and cause finger rot (Dadzie and Orchard, 1997).

2. Banana anthracnose

Anthracnose of banana is caused by *C. musae* which survives on debris in its asexual stage, as conidia located in an acervulus. Spores are liberated by splashing rain and dispersed by air to floral parts or young fruits (Dadzie and Orchard, 1997). *C. musae* is also found as an endophytic fungus which isolated from some parts of banana plant, such as leaves, midrib, petiole and pseudostem (Photita *et al.,* 2001). When spore germination under moist conditions, the spores produce appressoria which remain quiescent on the fruit skin. Two types of appressoria, hyaline and dark appressoria are produced by this fungus. Hyaline appressoria produce subcuticular hyphae and penetrate the epidermal cells of green banana and cause hypersensitive

reaction in adjacent cells or necrosis of epidermal cells, so this hyphae do not develop further and cause lesions in ripening fruits. Meanwhile, dark appressoria produce penetration hyphae and colonize host tissues, and produce anthracnose lesions on the peel in ripening fruits (Muirhead and Deverall, 1981), and subsequently penetrate into the fruit pulp (Swinburne and Brown, 1983). Sometimes, on wounded fruits, rot develops before the fruit ripens and lesions expand quickly (Meredith, 1960). The infection process of *C. musae* including several stages was shown in Figure 1, according to Jeffries *et al.* (1990).

An infection causes the formation of typical brown spot on ripe fruits. The spots are at first water-soaked, irregular in shape. The spots enlarge and turn dark brown to black with a water-soaked yellowish margin. Several spots may coalesce and affect large areas of the finger. Orange spore masses develop at the center of the spots under moist conditions. The spot symptoms commonly occur on injured and wounded peel during handling. Long storage and fluctuations to high temperatures favour anthracnose development (Dadzie and Orchard, 1997). The small spots enlarge to form sunken dark brown to black lesions on banana fruits. Daundasekera *et al.* (2008) reported that pathogenicity of banana anthracnose has associated with ethylene production by banana fruit tissues. Although *C. musae* is capable of producing ethylene *in vitro* on methionine-supplemented basal medium, it could not utilize methionine in the host tissue for ethylene production. Thus, pathogen-produced ethylene does not appear to play a role in disease development on banana fruits.

In France, Chillet *et al.* (2000) reported that bananas grown in lowland areas are more susceptible to anthracnose disease than those grown in highland zones. Chillet *et al.* (2006; 2007) found that the physiological age of bananas is a critical factor affecting the susceptibility of bananas to anthracnose pathogen. That means early harvest of fruits can reduce banana susceptibility to *C. musae* and increased the green life of fruits.

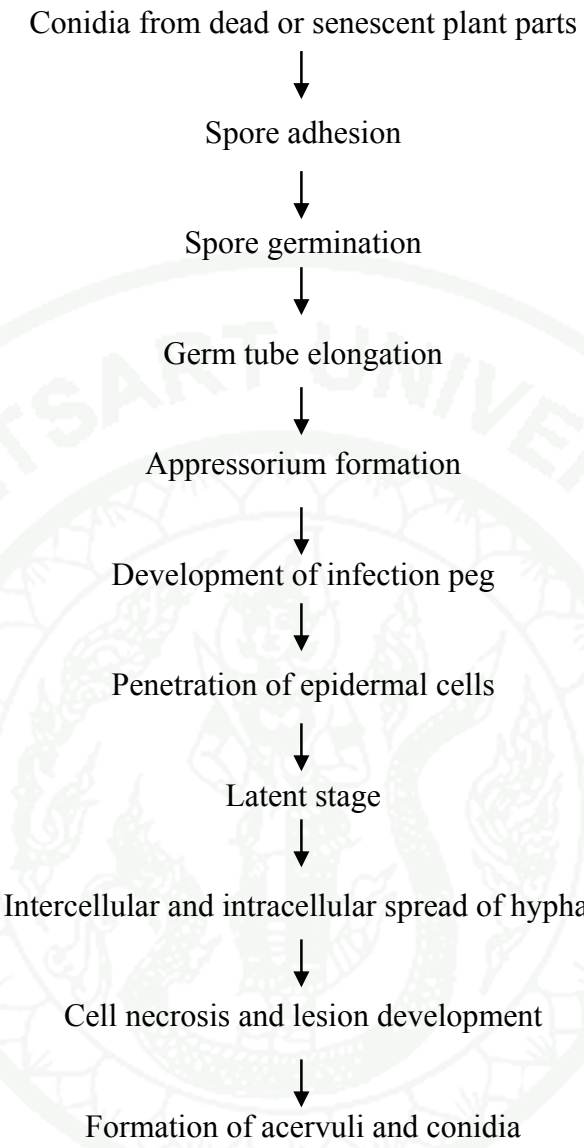


Figure 1 Morphological events in the infection process of *Colletotrichum musae.*

Source: modified from Jeffries *et al.* (1990)

3. Control of banana postharvest diseases

Control of postharvest diseases of banana depends on several steps and different ways including cultural method, physical method, fungicide application, natural and organic chemical control, and biological control.

3.1 Cultural method

The fruit should be harvested at the correct stage of maturity and handled carefully to avoid injury. All fruits are deflowered at an early stage of bunch development in order to reduce fungal inoculum. Fallen leaves and flower bracts should be also removed in the plantation and the packing station to reduce the inoculum. The washing water in the tank should be also changed frequently to minimize the inoculum level (Nelson, 2008). Additionally, Chillet *et al.* (2007) also reported that banana plantations in highland areas leading the fruits are naturally more resistant to anthracnose development since the accumulation of degree days is decreased.

3.2 Physical method

Due to the highly perishable nature of banana fruit, the chain of operations has to be well organized at all steps. Rapid cooling of fruit is a main pre-requisite for effective crown rot and anthracnose control. Hot water treatment is an effective method of postharvest disease control of bananas. Hot water treatment at the temperature of 55˚C for 2 min has been successfully used for controlling anthracnose and crown rot diseases on Santa Catarina Prada and Williums bananas (Reyes *et al.,* 1998) as well as at 50±2˚C for 5 min on Cavendish banana (*Musa sapientum*) (Hassan *et al.,* 2004). Hot water at 50˚C for 3 min and 55˚C for 1 min can also reduce anthracnose and crown rot severity on bananas varieties Embon (AAA type) and Kolikuttu (AAB type) (De Costa and Erabadupitiya, 2005). Kyu Kyu Win, *et al.* (2007) also reported the efficacy of hot water (45˚C for 20 min) combined with cinnamon extract as a good treatment for controlling crown rot disease of bananas cv. Hom Thong (*Musa acuminatum*, AAA Group).

3.3 Fungicide application

Krauss and Johanson (2000) reported that seven fungicides registered for use in banana industry in Eastern Caribbean, but only two fungicides; thiabendazole

and imazalil are designated for postharvest treatment. The mode of action of thiabendazole is the same as benomyl which used in preventive and curative routine sprays against leaf spot caused by *Mycosphaerella musicola* in banana. Additionally, the banana fruits treated with prochoraz at the concentration of 250 ppm also reduced disease incidence of anthracnose (Hassan *et al.,* 2004). However, the resistance to thiabendazole and benomyl have founded in some *C. musae* strains (Johanson and Blazquez, 1992; Khan *et al.,* 2001). Khan *et al.* (2001) also reported that *C. musae* has developed resistance to imazalil. Furthermore, fungicides that have been used in banana production, can cause ecological effects as shown in the report of Chaves *et al.* (2007). They have found that the use of fungicide chlorothalonil, non-systemic foliar fungicide, in the banana plantation in Costa Rica showed the residues in soil, sediment, water and banana leaves. However, Khan *et al.* (2001) have reported that using fungicides combined with some organic chemicals allowing lower concentrations of fungicides to be used. They also have shown that some antioxidant compounds including butylated hydroxyanisole (BHA), propyl paraben, propyl gallate and benzoic acid which are the food-grade chemicals, might have the potential to enhance the activity of fungicides at lower concentration currently used to control *C. musae* on bananas. The best result has been found that combining low concentrations of BHA and imazalil gives a synergic effect to prevent mycelial growth and spore germination of *C. musae*.

3.4 Natural and organic chemicals as alternatives to synthetic fungicides

The presence of fungicide residues in food and the environment increases public concern and has encouraged banana production to use alternative methods to replace fungicides for disease control. Different kinds of plant extracts and organic chemicals have been used in controlling postharvest diseases of banana. Demerutis *et al.* (2008) have shown the efficacy of extract of citrus seed in combination with the wax-based adjuvant to control crown rot of banana. Other plant extracts including cinnamon extract, piper extract and garlic extract have been shown as antifungal agents against crown rot pathogens such as *L. theobromae, C. musae, Thielaviopsis paradoxa* and *Fusarium verticillioides*. Banana fruits with chitosan coating also

delayed crown rot disease severity (Kyu Kyu Win *et al.,* 2007). Ranasinghe *et al.* (2005) also reported that the use of essential oil from cinnamon combined with modified atmosphere packaging can be recommended as the method for controlling crown rot disease and extending the storage life of banana. Some inorganic salts including sodium hypochlorite, sodium bicarbonate and calcium chloride have been used to suppress spore germination of crown rot pathogens (Alvindia and Natsuaki, 2007). When adding surfactant, it enhanced the protective value of salts to inhibit the spore germination of pathogens.

3.5 Biological control

Naturally occurring antagonists on host surfaces are a promising component of biological disease protection. Generally, effectiveness of biological control as the sole method of controlling postharvest pathogens is lower than synthetic fungicides (De Costa and Erabadupitiya, 2005). Efficacy of biological control can be improved by manipulation of the environment and integration with other methods such as combining with low level of synthetic fungicides (De Costa and Erabadupitiya, 2005; Sangchote, 2007; Sharma *et al.,* 2009), combining with sodium bicarbonate (Karabulut *et al.,* 2005) and combining with hot water (De Costa and Erabadupitiya, 2005; Eshel *et al.,* 2009). Many antagonists such as fungi, bacteria and yeasts for the control of pathogens on banana fruits has been reported (Mortuza and Ilag, 1999; Sangwanich, 2004; De Costa and Erabadupitiya, 2005). The antagonistic fungus *Trichoderma viride* had been used for control of crown rot disease, this disease was reduced by 65% (Mortuza and Ilag, 1999). De Costa and Erabadupitiya (2005) reported that the antagonistic bacterium, *Burkholderia cepacia* isolated from banana fruit, combining with hot water gave more effective control of anthracnose, crown rot and blossom end rot than each treatment. De Costa *et al.* (2008) showed that *Burkholderia spinosa* reduced anthracnose, blossom end rot and crown rot of banana by 95, 82 and 98%, respectively. Other antagonistic bacteria including *Bacillus amyloliquefaciens* DGA14 and *Pseudomonas syringae* ECS-11 had been used to control anthracnose and crown rot of banana (Williamson *et al.,* 2008; Alvindia and Natsuaki, 2009). In addition, antagonistic yeast *Endomycopsis fibuligera* has been

introduced to control of banana crown rot. This yeast reduced germ tube growth of crown rot pathogen, *Lasiodiplodia theobromae*. Application of this yeast in combination with hot water, crown rot disease was completely inhibited (Sangwanich, 2004). Spadaro and Gullino (2004) also reported an effective biological control in combination with other methods or non-competitive antagonists gave a better control than one microorganism.

4. Mechanisms of yeasts in controlling postharvest diseases

Recently, yeasts have been used as antagonists to control a number of postharvest pathogens on a variety of vegetables and fruits. The mechanisms of biological control on postharvest diseases by antagonistic yeasts have been described on various mechanisms including parasitism, antibiosis, production of lytic enzymes, competition for nutrients and space, and induced disease resistance as shown in diagram in Figure 2.

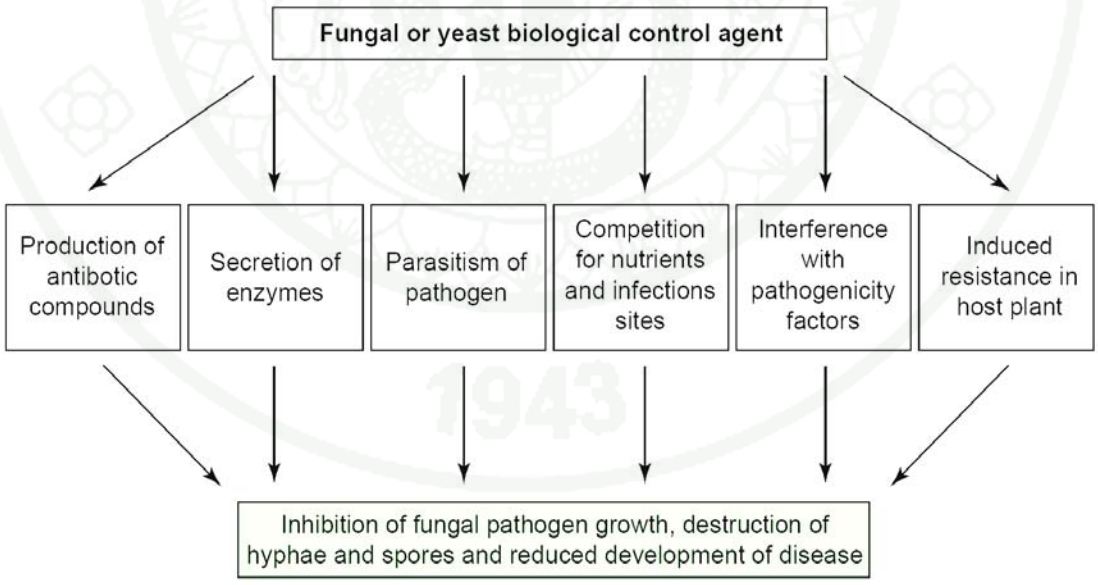


Figure 2 Mechanisms by fungi and yeasts reduce the growth of plant pathogens and diseases.

Source: Punja and Utkhede (2003)

The main mode of action of antagonistic yeasts was competition for nutrient and space (Droby and Chalutz, 1994). The attachment of antagonists to pathogen hyphae had been acted as an important factor in competition for nutrients between the antagonistic yeast *Pichia guilliermondii* and pathogen, *Penicillium italicum* on citrus fruit (Arras *et al.,* 1998). Additionally, yeast *Metschnikowia pulcherrima* against postharvest pathogens of apple fruit including *Botrytis cinerea, Penicillium expansum, Alternaria* sp. and *Monilinia* sp. were also principally acted by competition for space and/or nutrients but not on the production of antibiotics or other secondary toxic metabolites (Droby and Chalutz, 1994; Spadaro *et al.,* 2002). The antagonistic activity of yeasts *Cryptococcus laurentii* and *Candida famata* could be due to the nutrient competition and direct parasitism against the blue mold caused by *P. expansum* and the bitter rot caused by *Glomerella cingulata* on apple fruits (Blum *et al.,* 2005), and green mold caused by *P. digitatum* on orange fruits (Arras, 1996). Additional mode of action on yeast *Cryptococcus humicola* also had mechanism of nutrient competition and parasitism against *Botrytis cinerea* on apple fruit (Filonow *et al.,* 1996). The yeast *Pichia guilliermondii* competed with *Botrytis cinnerea* for glucose, sucrose, adenine, histidine and folic acid and its non-volatile compounds played a crucial role in suppression of pathogen (Guesky *et al.,* 2002).

Most antagonistic yeasts have more than one mode of actions against postharvest pathogens. Several yeasts produce different kinds of extracellular enzymes are able to use as biological control agents against postharvest diseases (El Ghaouth, 1998; Chi *et al.,* 2009). Castoria *et al.* (1997) reported that the yeast *Cryptococcus laurentii* acted against *P. expansum* and *B. cinerea* through nutrient competition and degradation of the pathogen cell wall by ß-1,3-glucanase. Saligkarias *et al.* (2002a, 2002b) also reported that two strains of antagonistic yeasts, *Candida guilliermondii* strain 101 and US 7, and *Candida oleophila* strain I-182 showed an efficacy to control *B. cinerea* on tomato by competition of nutrients and secretion of hydrolytic enzymes such as ß-1,3-exoglucanase and chitinase which play an important role in degradation of pathogen cell wall. Wisniewski *et al.* (1991) found that yeast cells which could produce lytic enzymes had the potential to enhance the attachment to pathogen hyphae. In addition, secretion of toxic metabolites by yeasts

Metschnikowia pulcherrima and *Rhodotorula glutinis* such as rhodotorulic acid and siderophores also reduced apple rots and the growth of *B. cinerea* and *P. expansum* (Calvente *et al.,* 1999; Spadaro *et al.,* 2002). Whereas the yeast *Candida oliophila,* the base of the commercial product Aspire, its modes of action include nutrient competition, site exclusion and direct mycoparasitism, caused the reduction on the growth of *P. digitatum* in grapefruit (Droby *et al.,* 2002). Moreover, the competition for space and nutrients of two antagonistic yeasts *Rhodotorula glutinis* LS11 and *Aureobasidium pullulans* LS30 affected *P. expansum* on infected apple fruits as well as to pathogen, *Aureobasidium carbonarius* on infected wine-grape berries, caused the reduction of pathogen growth, and reduced toxins, patulin and ochratoxin A which contaminated in apple fruit and wine (Castoria *et al.,* 2005a, 2005b).

Additional modes of action, antagonistic yeasts have been shown to elicit defense mechanisms on postharvest commodities (Figure 3). Droby *et al.* (1992) showed that the yeast *Pichia guilliermondii* had an ability to stimulate wound healing or to induce other defense mechanisms in the host plants. This yeast also stimulated ethylene production and raised the level of the enzyme phenylalanine ammonia-lyase (PAL) on *Pichia* sp. inoculated fruits (Barkai-Goland, 2001). Ippolito *et al.* (2000) found that the inoculation of yeast *Aureobasidium pullulans* on wounded apple fruits caused an increasing ß-1,3-glucanase, chitinase and peroxidase in host tissues. Stimulation of these enzymes was also triggered by wounding but the level of increase was lower than that on inoculated fruit. ß-1,3-glucanase and chitinase were involved in host defense mechanisms and capable of hydrolyzing fungal cell wall and therefore, inhibited fungal growth (Schlumbaum *et al.,* 1986). Whereas peroxidases were involved in the information of structural barriers includimg the formation of hemispherical protuberances along host cell wall against pathogen invasion when yeast *Candida saitoana* had been applied on apple fruits (El Ghaouth *et al.,* 1998).

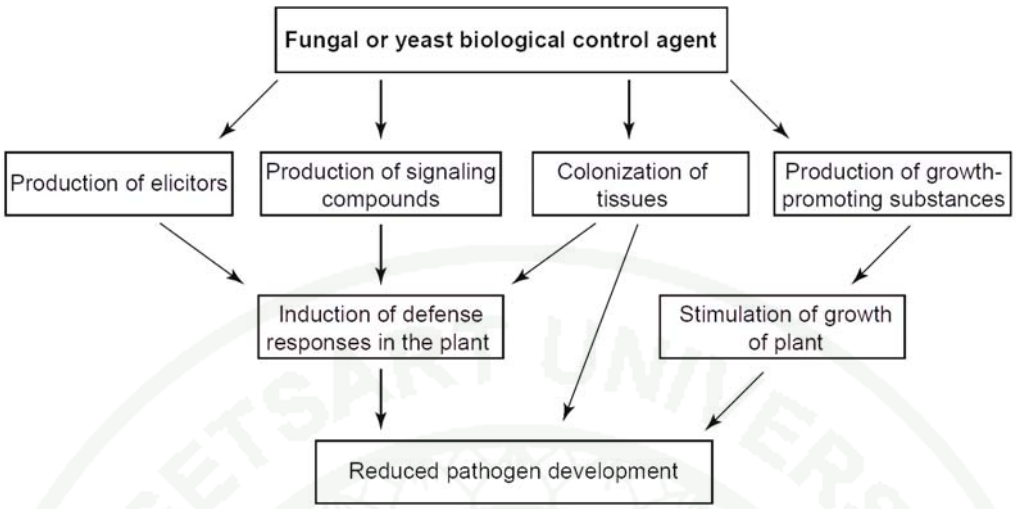


Figure 3 Induction of plant host responses by fungal and yeast biological control agents that can lead to biological control of disease.

Source: Punja and Utkhede (2003)

Furthermore, yeast extract also has been used as elicitor to activate defense mechanisms in plant tissues and postharvest produces. Hrazdina (2003) found that application of yeast extract on apple fruit showed the accumulation of phenolic compounds such as *p*-coumaric acid, phloridzin and phloretin against apple scab caused by *Venturia inaequalis.* In addition, crude extract from yeast *Saccharomyces cerevisiae* was also shown as elicitor to activate reactive oxygen species such as superoxide (O^{-}_{2}) and hydrogen peroxide (H_2O_2) of *Catharanthus roseus* which can be own signal molecules and probably activate other signal molecules like jasmonic acid to stimulate gene expression in host cells leading secondary metabolite production against pathogen attack (Pauw *et al.,* 2004). The model of these strategies have been shown in Figure 4.

However, the mixture of various antagonistic yeasts or combined treatments of yeasts with organic/inorganic compounds were exhibited greater biocontrol activity against postharvest diseases than yeast applied alone (Janisiewicz *et al.,* 2008).

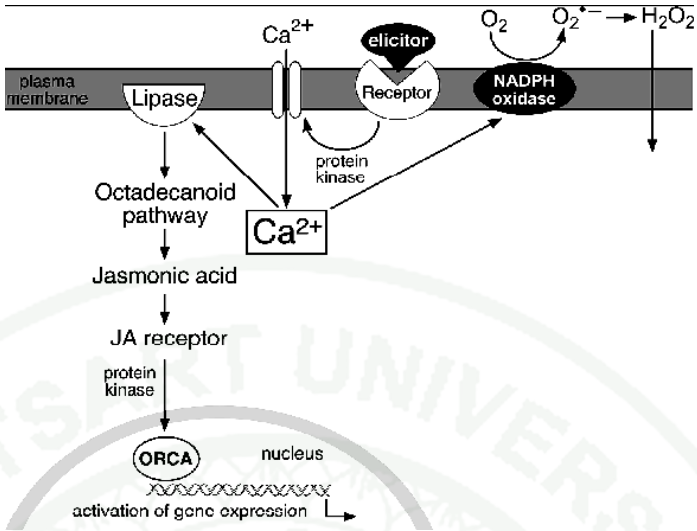


Figure 4 Model for elicitor signaling in plant cells by yeast extract activated reactive oxygen species (ROS) production and biosynthetic gene expression leading to stimulation of secondary metabolites against pathogen.

Source: Pauw *et al.* (2004)

5. The use of antagonistic yeasts for controlling postharvest diseases in Thailand

Yeast *Candida inconspicua* isolate CiKul had been reported in controlling mango anthracnose caused by *C. gloeosporioides*. This study revealed that the yeast played an important role in competition for nutrients and spaces (Chormduang, 1999). Sangwanich (2004) had shown that the yeast *Endomycopsis fibuligera* was the most effective antagonist. It reduced germ tube elongation of *L. theobromae* by 42% and crown rot severity by 92% on wounded banana fruits. Moreover, when bananas were treated with yeast *Endomycopsis fibuligera* in combination with 150 ppm thiabendazole or dipped in hot water (50˚C for 20 min), the disease was completed inhibited. The use of antagonistic yeasts in controlling green mold of citrus fruits was reported by Niamjang (2006) and Sangchote (2006). Green mold rot was completely suppressed when application of yeast *Candida utilis* for 12 and 24 h prior to inoculate with pathogen, *Penicillium digitatum*. Yeast *Candida utilis* also reduced green mold

of citrus fruits when combining with 2% sodium bicarbonate. Additionally, Chantrasri (2006) had also reported that yeast *Candida famata* which isolated from the surface of mango leaves was a promising antagonist to control anthracnose disease on mango fruits. This antagonistic yeast also stimulated defense responses in mango tissues by increasing ß-1,3-glucanase, chitinase and peroxidase activities. However, combination of yeast *Candida famata* and 0.5% chitosan offered better control of anthracnose in mango fruit than yeast or chitosan alone.

6. Metabolites from microorganisms in controlling postharvest pathogens and diseases

There are various microorganisms which can produced metabolites against plant pathogens. Metabolites produced by *Trichoderma virens* was shown an inhibitory activity on the growth of *Rhizoctonia solani* (Mischke, 1997). Additionally, metabolites produced by several fungi such as *Clonostachys byssicola, Curvularia pallescens* and *Trichoderma harzianum* significantly inhibited mycelial growth and spore germination of banana crown rot pathogens including *L. theobromae, C. musae, Thielaviopsis paradoxa* and *Fusarium verticillioides* (Alvindia and Natsuaki, 2008). Cell-free supernatant and volatile metabolites from bacterium *Bacilus subtilis* also inhibited mycelial growth and spore germination of green mlold pathogen, *Penicillium digitatum* on citrus fruit (Leelasuphakul *et al.,* 2008). Alvindia and Natsuaki (2009) also reported that *Bacillus subtilis* DGA14 produced a diffusible metabolites which inhibited crown rot pathogens of banana. Volatile and non-volatile compounds produced by *Bacillus mycoides* inhibited the growth of *Botrytis cinerea* on strawberry and also activated defense system of the host (Guetsky *et al.,* 2002). A range of volatile organic compounds produced by three yeasts, *Debaryomyces melissophilus, Rhodotorula glutinis* and *Cryptococcus laurentii* which isolated from strawberries including acetone, ethyl acetate, ethanol, isopropyl acetate, ethyl butyrate, etc. Their biosynthetic pathway was proposed by glucose catabolism as shown in Figure 5 (Ragaert *et al.,* 2006).

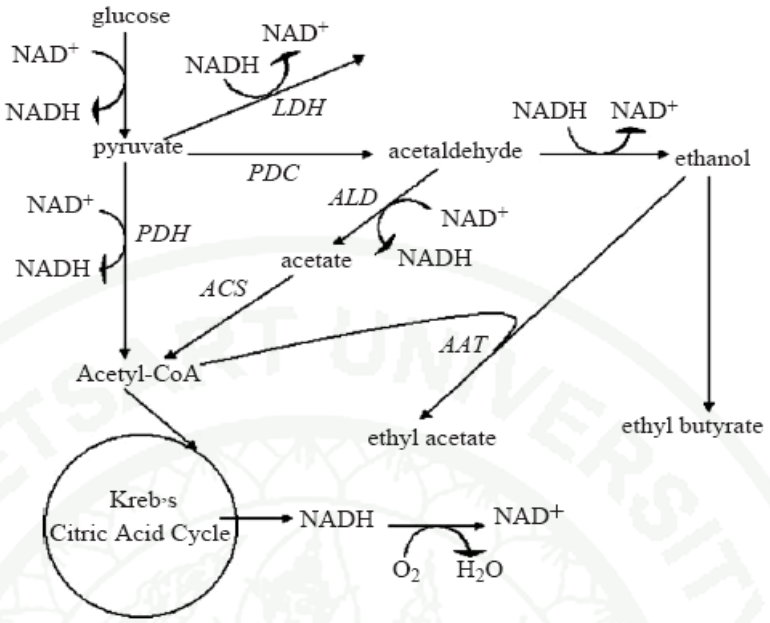


Figure 5 Biosynthetic pathway of volatile metabolites by yeasts. Abbreviations: LDH: lactate dehydrogenase, PDC: pyruvate decarboxylase, PDH: pyruvate dehydrogenase, ADH: alcohol dehydrogenase, ALD: acetaldehyde dehydrogenase, ACS: acetyl-CoA synthetase, AAT: alcohol acetyltransferase.

Source: Ragaert *et al*. (2006)

6.1 Production of antibiotic compounds by antagonistic microorganisms

The production of antibiotic compounds is characteristic of many effective fungal and yeast biocontrol agents. Species of *Trichoderma* was known to produce several secondary metabolites with broad-spectrum antimicrobial activity such as gliotoxin and gliovirin (Howell, 2003). The antibiotics produced by *Pseudozyma flocculosa* were the mixture of fatty acid-containing derivatives that affected membrane permeability of the target organisms, thereby inhibiting their growth (Avis and Belanger, 2001). Another antifungal compound, Aureobasidin A, which isolated from liquid culture medium of yeast *Aureobasidium pullulans* (Ikai *et*

al., 1991), was highly active *in vitro* against many pathogenic fungi with mode of action on inhibiting fungal cell wall ß-1,3-glucan synthesis (Takesako *et al.,* 1993). The research by Endo *et al.* (1997) also reported that fungicidal action of Aureobasidin A against *Saccharomyces cerevisiae* were inhibiting the normal budding process and destruction of membrane integrity leading to cell death. Another report had shown that metabolites produced by four fungi including *Clonostachys byssicola, Curvularia pallescens, Penicillium oxalicum* and *Trichoderma harzianum* which isolated from the surface of banana fruits were also effective as antibiotic against crown rot pathogens, *Thielaviopsis paradoxa, C. musae* and *Fusarium verticillioides* (Alvindia and Natsuaki, 2008). Additionally, Zhang *et al.* (2006) found that antimicrobial peptide ABP-CM4 produced by yeast *Pichia pastoris* was a promising candidate for a new antibiotic which had an antifungal effect on pathogenic fungi including *Aspergillus niger, Trichoderma viride, Gibberella saubinetii* and *Penicillium chrysogenum.* Whereas yeast *Rhodotorula glutinis* had an efficacy to produce rhodotorulic acid that enhanced biological control activities against blue mold caused by *Penicillium expansum* in harvested apples (Calvente *et al.,* 1999).

6.2 Role of extracellular enzymes produced by antagonistic microorganisms

Secretion of hydrolytic enzymes, particularly chitinase and glucanase, is a feature common of many effective biological control agents. Punja and Utkhede (2003) showed that mutant strain of *Trichoderma* with disrupted activity of *ech 42,* a chitinase encoding gene, were shown less effective against *Rhizoctonia solani* and *Botrytis cinerea* compared with wild type strain. In addition, *Pichia guilliermondii* produced hydrolytic enzymes including ß-1,3-glucanase and chitinase against anthracnose pathogen of chilli, *Colletotrichum capsici.* The activity of these enzymes was higher when this yeast was grown in media supplemented with hyphal cell wall of *C. capsici* than larminarin or glucose as carbon source (Chanchaichaovivat *et al.,* 2008). Whereas the report by Saligkarias *et al.* (2002b) found that the secretion of hydrolytic enzymes, ß-1,3-exoglucanase and chitinase by yeasts *Candida guilliermondii* strains 101 and US7 and *Candida oleophila* strain I-182 against *Botrytis cinerea* in tomato was shown the highest amount of these enzymes in the

culture amended with glucose as carbon source. In addition, Jijakli and Lepoivre (1998) have shown that enzyme ß-1,3-glucanase was produced in wounded apple treated an antagonistic yeast *Pichia amomala*. This strengthens the hypothesis that exo-ß-1,3-glucanase production was involved in the biological control of gray mold against *Botrytis cinerea* by this antagonist. Furthermore, apple which were treated with antagonistic yeast *Candida saitoana* showed increasing activity of chitinase and ß-1,3-glucanase, and induced systemic resistance against *Botrytis cinerea* (El Ghaouth *et al.,* 2003). Another report, higher ß-1,3-glucanase and chitinase activity was also detected in wounded apple treated with yeast *Aureobasidium pullulans* (Ippolito *et al.,* 2000). Castoria *et al.* (2001) also reported that *Aureobasidium pullulans* LS-30 against important postharvest pathogens such as *Botrytis cinerea, Penicillium expansum, Aspergillus niger* and *Rhizopus stolonifer* on table grapes and apple fruits. It played an important role in competition for nutrients. Extracellular exochitinase and ß-1,3-glucanase activities were also detected both *in vitro* and in apple wounds, suggesting that these enzymes might actually be involved in the antagonistic activity of this microorganism.

MATERIALS AND METHODS

1. Screening for antagonistic yeasts against anthracnose pathogen and anthracnose disease on banana fruits

Ten yeasts including *Candida utilis*, *Candida tropicalis*, *Pichia membranaefaciens*, *Cryptococcus humicola* BCC 7701, *Candida guilliermondii* BCC 5389, *Candida sake* TISTR 5143, *Debaryomyces hansenii* TISTR 5155, *Aureobasidium pullulans* TISTR 3389, *Saccharomycopsis fibuligera* TISTR 5033 and *Rhodotorula glutinis* TISTR 5159 were used in this study. The first three yeasts were obtained from Department of Microbiology, Faculty of Science, Kasetsart University. Other seven yeasts were obtained from National Science and Technology Development Agency (NSTDA) and Thailand Institute of Scientific and Technological Research (TISTR)**.** All yeasts were maintained in 10 ml vial containing 4 ml of 20% glycerol which mixed with liquid medium, yeast extract malt extract broth (YMB- 3 g of yeast extract, 5 g of malt extract, 5 g of peptone and 10 g of glucose per liter of distilled water), exception for *A. pullulans* TISTR 3389 was maintained in 20% glycerol in potato dextrose broth (PDB- 200 g of potato and 20 g of glucose per 1 liter of distilled water) and stored at -20˚C. For further experiments, these yeast containers were left in warm water (37˚C) for 10 min. Thereafter, each yeast cell suspension was streaked on either yeast extract malt extract agar (YMB added 15 g of agar per liter of distilled water) or potato dextrose agar (PDB added 15 g of agar per liter of distilled water), and incubated for 48 h at room temperature (28-32˚C). Single colony of each yeast was transferred to YMA or PDA slants and used for trials.

1.1 Antagonistic effects of yeasts against *Colletotrichum musae in vitro*

The interaction between individual yeast and pathogen was accessed for direct parasitism by dual culture method on PDA. The individual yeast was striped on the medium in 9 cm diameter Petri dish, 1.5 cm from the border. Another side, a 5 mm mycelium disc of *C. musae* was placed at position of 1.5 cm from the border. The

radial growth of pathogen towards the yeast strip was measured after incubation for 6 days at room temperature. Three Petri dishes per treatment were used. The results were compared with the radial growth of control (pathogen alone) and expressed as a percentage of radial growth inhibition. Radial growth inhibition was determined from the following equation (modified from the method of Pacheco *et al.*, 2008). Yeasts showed no inhibition zones were selected for further studies.

$$RI\ (\%) = \frac{[R1 - R2]}{R1} \times 100$$ where

RI = the radial growth inhibition

R1 = the average of radial colony of pathogen in the control Petri dish

R2 = the average of radial colony of pathogen in dual culture Petri dish

1.2 Antagonistic effects of yeasts in suppressing anthracnose lesions on banana fruits

1.2.1 Preparation of yeast cell suspensions

All tested yeasts were streaked on YMA and incubated for 48 h at room temperature (28-32˚C). A liquid culture was made by adding a loop full of yeast cells from single colony to a 250 ml Erlenmeyer flask containing 100 ml YMB medium and shaken at 120 rpm for 48 h at room temperature. Each yeast cell suspension was collected and adjusted to the concentration of $1x10^8$ cells/ml by haemacytometer.

1.2.2 Preparation of *Colletotrichum musae* inoculum on banana fruits

Pinkish spore masses of *C. musae* that naturally appeared on the over-ripening banana fruits were isolated for pure culture on PDA and then, it was multiplied in the same medium for 7 days at room temperature. Spore suspension was prepared by flooding the Petri dishes with sterile distilled water and rubbing the surface of the culture with a glass rod to dislodge spores. Mycelial fragments were

removed from suspension by filtration through 2-3 layers of sterile cheesecloth and spore suspension was used for inoculation.

Banana fruits were washed in tap water and dried in air. Fruits were sprayed using an airbrush (BADGER AIR-BRUSH™, U.S.A.) with *C. musae* spore suspension at the concentration of $1x10^5$ spores/mm^2. Inoculated fruits were incubated in high moisture condition in the plastic bag at room temperature for 10 days. When spore masses appeared on the ripening fruit skins, spore suspension was prepared from pinkish spore masses obtained from diseased fruit by soaking that fruit in a beaker containing sterile distilled water. Spore suspension was filtered by 2-3 layers of cheesecloth and adjusted to the concentration of $1x10^5$ spores/mm^2 by haemacytometer.

1.2.3 Inoculation of yeast cell suspensions and pathogen inoculum on wounded banana fruit

Green bananas at commercial stage which obtained from wholesale market were cut into the single fruit. Fruit surface was washed in tap water and then air dried. Fruits were gently wounded (3 mm deep) at 3 sites with a sterile needle. A 20 µl of each yeast cell suspension at concentration of $1x10^8$ cells/ml was applied on 5 mm diameter disks of Whatman No.1 paper on the wound site. Treated fruits were placed on plastic trays that enclosed with polyethylene bag under high moisture condition. Banana fruits were stored at room temperature for 24 h and then each treatment was inoculated with 20 µl of *C. musae* spore suspension at the concentration of $1x10^5$ spores/ml to each wound site and incubated at the same condition for 24 h, and then polyethylene bag was removed. Lesion diameters were measured at 7 days after inoculation. Each treatment consisted ten fruits and repeated three times. Percentage of lesion size reduction was determined from the following equation and the best performing yeasts were selected for further experiments.

$$LR\ (\%) = \frac{[L1 - L2]}{L1} \times 100$$ where

LR = the lesion size reduction

L1 = the average of lesion size in YMB treated fruits (control)

L2 = the average of lesion size in yeast cell suspension treated fruits

2. Preliminary screening of metabolites from yeasts against anthracnose disease on banana fruits

2.1 Yeast metabolite preparation

The promising antagonistic yeasts from 1.2.3 were used in this study. Selected yeasts were streaked on either YMA or PDA medium and incubated for 48 h at room temperature (28-32˚C). Single colony of individual yeast was transferred to a 500 ml Erlenmeyer flask containing 300 ml YMB or PDB and then shaken at 120 rpm for 48 h at room temperature. The yeast cells were separated by centrifugation at 9000 rpm for 10 min, and the supernatant was filtered using a 0.45 μm glass fiber filter disc. Culture filtrates were collected and used immediately as metabolites.

2.2 Yeast metabolite treatments on anthracnose disease of banana fruits

Green banana hands were cut into the single fruit. Fruits were surface-disinfected with 5% sodium hypochlorite solution for 5 min, washed with tap water and dried in air. Ten fruits were individually sprayed with culture filtrate from each yeast using an airbrush (BADGER AIR-BRUSH™, U.S.A.). After spraying, banana fruits were stored in plastic tray and enclosed with plastic bag with high humidity at room temperature for 24 h. Banana fruits were then inoculated with spore suspension of *C. musae* at the concentration of $1x10^5$ spores/mm^2 using an airbrush. Pathogen spore suspension was obtained from diseased fruits as described in 1.2.2. After inoculation, inoculated fruits were incubated at the same condition for 24 h. After 7 days, anthracnose disease development on banana fruits was measured as percentage of disease severity reduction. Disease severity (%) was evaluated from total area of anthracnose symptom development on the fruit skin. Each treatment consisted ten fruits and repeated three times. Metabolites from yeasts which showed the best in controlling anthracnose disease on banana fruits were selected for further studies. Percentage of disease severity reduction was determined from the following equation.

$$DR\ (\%) = \frac{[D1 - D2]}{D1} \times 100$$ where

DR = the disease severity reduction

D1 = the average of disease severity in YMB treated fruits (control)

D2 = the average of disease severity in yeast metabolite treated fruits

3. Determination of growth curve and cell viability of antagonistic yeasts for secondary metabolite production

3.1 Growth curve measurements

Selected yeasts from 2.2 which gave the good results in controlling anthracnose disease of banana fruits by using their metabolites were streaked on YMA medium and incubated for 48 h at room temperature. A loop full of yeast cells from single colony was transferred to a 500 ml Erlenmeyer flask containing 300 ml YMB medium and incubated at room temperature and shaken at 120 rpm. Five milliliters of each yeast cell suspension was collected every 3 h for 14 days for absorbance measurement and viable cell evaluation. Optical density (OD) was measured at 600 nm on a UV-visible spectrophotometer (CECIL CE7200 Series 7000). The OD measurement was repeated twice.

3.2 Yeast cell viability

Cell viability of yeasts was evaluated by plate counting technique according to the method of Chanchaichaovivat *et al.* (2007). One milliliter of yeast cell suspension was suspended in 9 ml of sterile distilled water and shaken vigorously for a few minutes. Serial dilution of yeast cell suspension were made in sterile distilled water. An aliquot of 0.1 ml of suspension was spread on YMA medium, consequently incubated at room temperature (28-32˚C) for 48 h. All colonies of individual yeast were counted. Three Petri dishes per concentration were used.

4. Study of metabolites from yeasts on *Colletotrichum musae* and anthracnose disease on banana fruits

4.1 Preparation of metabolites from yeasts

A single colony of 48 hours old cultures of selected yeasts on YMA media were transferred to Erlenmeyer flask containing 300 ml YMB medium and shaken at 120 rpm for 5 days at room temperature. Culture filtrates of yeasts were made as described previously and used as metabolites for further studies.

4.2 Metabolites from yeasts on the *in vitro* growth of *Colletotrichum musae*

4.2.1 Metabolites from yeasts on mycelial growth of pathogen

C. musae was grown on PDA for 7 days at room temperature. The growing mycelium was cut with a 5 mm-diameter cork borer at the edge of colony and transferred to an empty 9 cm-diameter Petri dish. Agar surface with mycelium was faced up and placed at the middle of Petri dish. Twenty milliliters of culture filtrate from each yeast were then added to a Petri dish and incubated at room temperature for 5 days. Consequently, culture filtrate was removed by filtration using a Whatman No.1 filter paper. Then, mycelial mats were dried in the oven at 80˚C for 6 h and weighed immediately. Mycelial agar plug with YMB medium served as control. Ten Petri dishes per treatment were used.

4.2.2 Metabolites from yeasts on spore germination and germ tube elongation of pathogen

Spore suspension was obtained from 7 days old culture of *C. musae* by flooding the dishes with sterile 0.85% sodium chloride solution and rubbing the surface of the colony with a glass rod to dislodge spores and filtered through 2-3 layers of cheesecloth. Then, spore suspension was centrifuged at 3000 rpm for 5 min. Pellet at the bottom of centrifuge tube was used for spore germination test. Briefly, a

loop full of spore masses of *C. musae* was transferred to vial containing 10 ml of each yeast culture filtrate and mixed. All vials were stored at 4°C for 12 h. Subsequently, 100 µl of spore suspension was pipetted to the surface of water agar, spread with triangle glass rod, and incubated at room temperature. The germination of spores was checked at 6 h after incubation. Germ tube elongation was measured using micrometer. Each treatment consisted three replications. Total spores were randomly selected 400 spores per replication.

4.2.3 Determination of fifty percent inhibition concentration (IC_{50}) of metabolites from yeasts on *Colletotrichum musae*

The IC_{50} values of metabolites from yeasts on the mycelial growth and spore germination of pathogen were also determined. Yeast metabolites were diluted with sterile YMB medium at various concentrations of 0, 25, 50, 75 and 100%. Each percentage of *A. pullulans* metabolite concentrations was calculated into 0, 50, 100, 150 and 200 mg/L, respectively, while each percentage of *C. utilis* metabolite concentrations was calculated into 0, 55, 110, 165 and 220 mg/L, respectively, depending on dry weight of *A. pullulans* metabolite residues (200 mg/L) and *C. utilis* metabolite residues (220 mg/L). After incubation at 5 days for mycelial growth and 6 h for spore germination, mycelial mats were dried in hot air oven at 80˚C for 6 h and weighed immediately, as well as germinated spores were recorded as previously described in 4.2.1 and 4.2.2.

4.3 Volatile metabolites from yeasts on the *in vitro* growth of *Colletotrichum musae*

4.3.1 Volatile metabolites from yeasts on mycelial growth of pathogen

The effect of volatile metabolites on pathogen was investigated following the procedure of Alvindia and Natsuaki (2008) with some modifications. Five cm-diameter Petri dish containing PDA was placed inside of 9 cm-diameter Petri dish containing YMA. Selected yeasts were separately streaked on the surface of

YMA media and incubated for 48 h. Then, 5 mm-diameter agar plug of 7 days old culture of *C. musae* was transferred to the middle of PDA Petri dish. Petri dish was sealed with paraffin film and incubated for 4 days. Colony diameter and mycelial density of pathogen were measured comparing with the control which did not contain the yeast antagonists. Mycelial density scores were recoded as 1 = slight mycelial growth, 2 = moderated mycelial growth and 3 = thick mycelial growth (Figure 6).

1 = slight mycelial growth | 2 = moderated mycelial growth | 3 = thick mycelial growth

Figure 6 Mycelial density scores of *Colletotrichum musae* growth

4.3.2 Volatile metabolites from yeasts on spore germination of pathogen

Spore germination test on volatile metabolites was done following the method as described in 4.3.1. PDA medium was replaced by water agar in 5 cm-diameter Petri dish. *C. musae* spore suspension at the concentration of $1x10^5$ spores/ml was spread on the water agar surface, then sealed the Petri dish with paraffin film and incubated for 6 h. Germinated spores were counted under the microscope compared with the control using 400 spores per replication. Each treatment consisted three replications.

4.4 Metabolites from yeasts on disease severity of banana fruits

Banana fruits were prepared as described in 2.2. Culture filtrate from each yeast was divided into two groups. The first group, banana fruits were sprayed with non-heated culture filtrate. The second one, banana fruits were treated with heated culture filtrate at 90˚C for 20 min. Ten fruits were individually sprayed with each culture filtrate using an airbrush (BADGER AIR-BRUSH™, U.S.A.). After

metabolite treatments, banana fruits were stored in plastic bag for 24 h. Subsequently, the fruits were inoculated with spore suspension of *C. musae* at the concentration of $1x10^5$ spores/mm^2. Spore suspension was prepared from diseased fruits as described previously in 1.2.2. Inoculated fruits were stored in high moisture for 24 h. After 7 days, disease severity (%) was measured at the ripening stage of the fruit, comparing with the fruit treated with 0.5 mM salicylic acid which used as inducer (Qin *et al.,* 2003) for investigation of induced resistance in banana fruits and YMB medium which used as no metabolites. Disease severity (%) was evaluated from total area of anthracnose symptom development on the fruit skin. Percentage of disease severity reduction was determined from the equation as described in 2.2.

Salicylic acid at different concentrations of 0.5 and 2 mM were also used for checking the ability of inhibition of *C. musae* mycelial growth and spore germination on PDA Petri dishes. The mycelial growth inhibition was evaluated with five replications per treatment. The spore germination inhibition was investigated with three replications using 400 spores each.

5. Cell wall degrading enzymes produced by yeasts against *Colletotrichum musae*

Culture filtrates of selected yeasts were obtained from 5 days old YMB culture which incubated on shaker at 120 rpm at room temperature (28-32˚C).

5.1 ß-1,3-glucanase production

ß-1,3-glucanase activity from yeast metabolites was assayed immediately after obtaining culture filtrates from yeast cultures, according to the method of Burner (1964) and Chanchaichaovivat *et al.* (2008) with some modifications and used laminarin as the substrate. Two hundred and sixty micro litter of 2 mg/ml larminarin in 0.05 M sodium acetate buffer, pH 5.0 was added to 1 ml of each yeast culture filtrate. The reaction mixture was incubated at 35˚C in water bath for 30 min, and the reaction was terminated by placing the mixture in boiling water

for 10 min. 580 µl of 3,5-dinitrosalicylic acid (DNS) solution and 2.16 ml of 0.05 M sodium acetate buffer, pH 5.0 were added, and subsequently heated for 5 min. After boiling, the reaction tubes were left at room temperature to cooling down, 1.58 ml of distilled water was then added prior to reading absorbance at 540 nm. Enzyme activity was determined by quantifying the glucose released from laminarin comparing with the standard curve of glucose. Enzyme activity was expressed as micrograms per milliliter of culture filtrate.

5.2 Chitinase production

Chitinase activity was determined according to the method of Reissig *et al.* (1955) with some modifications using swollen chitin as the substrate. Assay of enzyme activity was performed immediately after obtaining culture filtrates from yeast cultures. The 1% swollen chitin in 0.05 M citrate-phosphate buffer, pH 6.6, was incubated with 1 ml of each yeast culture filtrate for 1 h at 50˚C. The reaction was stopped by boiling for 5 min. The reaction mixture was left for precipitation at room temperature. Supernatant was collected for N-acetylglucosamine (NAG) analysis. A 0.5 milliliter of supernatant mixed with 100 µl of 0.8 M $K_2B_4O_7$, subsequently heated for 3 min. After cooling down, 3 ml of dimethylaminobenzaldehyde (DMAB) was added and allowed to stand in water bath at 37˚C for 20 min. When the mixture cooled down, absorbance was measured at 585 nm within 10 min. Chitinase was assayed by measuring the concentration of NAG and calculated comparing with the standard curve of NAG. Enzyme activity was expressed as micrograms per milliliter of culture filtrate.

6. Study on crude extracts of yeast metabolites

6.1 Preparation of crude extracts from yeast metabolites

Culture filtrates from selected yeasts were obtained from 5 days old culture of yeasts in YMB media with shaking at 120 rpm at room temperature. The culture filtrate was partitioned with AR-grade dichloromethane in a separating funnel

(2:1 v/v), modified from the method of Chirawut (2005). The upper aqueous layer was partitioned with same solvent again. The lower dichloromethane layer was collected and dried in a rotary evaporator (Buchi, Rotavapor R110) at 40˚C. Subsequently, the residues were resuspended in 5 ml of absolute methanol. Crude extracts were stored in the dark vials at -20˚C and used for further study on antifungal activity. Identification of antifungal compounds was conducted by Thin Layer Chromatography (TLC) or High Performance Liquid Chromatography (HPLC) or Nuclear Magnetic Resonance (NMR) spectroscopy.

6.2 Antifungal activity of crude extracts from yeast metabolites on paper discs

The minimum inhibitory concentration (MIC) and 50 percent inhibition concentration (IC_{50}) of crude extracts from yeast metabolites were carried out following the method of Zainuri (2006) with slight modifications. The minimal concentration that significantly inhibited spore germination of *C. musae* comparing with the spore germination in control (at the 5% level, DMRT) was recorded as MIC (Leelasuphakul *et al.*, 2006). Crude extracts of yeast metabolites which obtained from 6.1 were serially diluted with absolute methanol. Ten microliters of each concentration of crude extracts were applied on a 5 mm-diameter sterile disc of Whatman No.1 filter paper and each drop was allowed to dry before application of the next drop. Each paper disc was applied for 4 drops (40 µl). The paper discs were placed into the concave microscope slides and left at room temperature for dismissing toxic solvent for 3 h before application of pathogen spore suspension. Subsequently, 10 µl droplet of spore suspension of *C. musae* ($1x10^5$ spores/ml) which obtained from 7 days old culture on PDA Petri dish was pipetted onto filter paper discs and incubated in moist condition. After 8 h, a small droplet of lactophenol cotton blue was added to terminate any further germination and growth. The numbers of germinated spores were counted under the light microscope (200x magnification).

6.3 Bioassay of antifungal compounds on the TLC plates

A TLC method was used to separate compounds which contained in yeast metabolite extracts. Separation occurred when the mobile phase flowed over the surface of TLC plate containing the extracts and compounds in the extracts were eluted across the surface at different rates (Randerath, 1966). TLC development was carried out using aluminium plates (20 x 20 cm) coated with 0.2 mm-thick silica gel 60 F_{254} (MERK, Germany), according to the method of Droby *et al.* (1986).

6.3.1 Preparation of the TLC plates and sample loading

The TLC plates which used for separation of compounds in metabolites from both yeasts, were prewashed in dicholoromethane : methanol (49 : 1 v/v) that used as running solvent, and then air dried. From the lower edge of the plates, 2 cm, they were marked on the front side with pencil. These marks served as the application points for the sample loading. The crude extracts were carefully loaded using micro haematocrit tubes (Vitrex Medical AIS, Denmark) with 100 µl of samples onto the mark position on each plate. Spots were completely dried before TLC plate development.

6.3.2 TLC development

The TLC plates with loading by samples, were placed into a glass tank (27 cm x 25 cm x 7 cm) containing 100 ml of the running solvent, dichloromethane : methanol (98 : 2 v/v, modified from the method of Peret-Almeida *et al.,* 2005). The plates were run in one dimension for approximately 1 h and the run was determinated when solvent reached 1.5 cm from the top edge of the plates. The plates were then removed from the tank and dried in air around 3 h for removing toxic solvents. The developed plates were observed under a Spectroline UV-Lamp at wavelength of 365 nm for determining visible active compounds.

6.3.3 Determination of antifungal compounds on the TLC plates

The TLC plates were sprayed with spore suspension of either *C. musae* or *Cladosporium cladosporioides* in 1.7% malt extract broth using an airbrush (BADGER AIR-BRUSH™, U.S.A.). The plates were then incubated in a 24 x 24 x 1.5 cm moist plastic chamber at room temperature for 3 days. Inhibition zones of fungal growth were observed and the retention factors (R_f) values (Figure 7) of the active compounds were calculated as following equation.

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

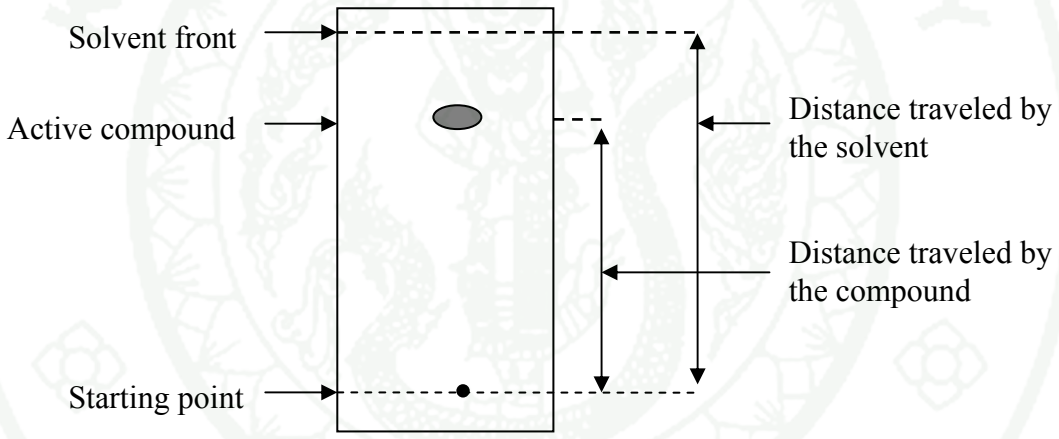


Figure 7 The method for determining the retention factor (R_f) of an antifungal compound.

6.4 Separation of antifungal compounds by column chromatography (CC)

Column chromatography was used as a method for fractionation of crude extracts based on the different polarity of the compounds. The crude extracts were applied to a glass column (length 72 cm, diameter 2 cm) filled with silica gel (Silica gel 60, 0.2-0.5 mm, MERCK). The column was washed with acetone and left silica gel to dry, and eluted with organic solvents of increasing polarity (Table 1). One hundred milliliters (100 ml) of each solvent mixture were applied to the column and

50 ml of each fraction were collected. The fractions were dried by evaporation of the solvents and weighted, and further analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and/or nuclear magnetic resonance (NMR).

Table 1 Organic solvent mixtures for column chromatography

Fraction No.	Hexane	Ethyl acetate	Methanol
I	100	-	-
II	95	5	-
III	90	10	-
IV	75	25	-
V	50	50	-
VI	25	75	-
VII	-	100	-
VIII	-	95	5
IX	-	90	10
X	-	75	25
XI	-	50	50
XII	-	-	100

Source: Khewkhom (2006)

6.5 Identification of antifungal compounds

6.5.1 Analysis by thin layer chromatography (TLC)

The fractions were loaded onto TLC plates as previously described in 6.3.1. After TLC plate development, the plates were observed under a Spectroline UV-Lamp at wavelength of 365 nm. Subsequently, the developed plates were sprayed with either Anisaldehyde reagent to determine the presence of

terpenoids, polyacetylenes and isocoumarins or Dragendorff reagent to determine the presence of alkaloids.

Anisaldehyde reagent (Merck, 1980):

Mixed 0.5% Anisaldehyde in sulfuric acid : glacial acetic acid : methanol 5 : 10 : 85 v/v/v. After spraying of Anisaldehyde reagent, TLC plates were activated at 100˚C for 10 min to visualize UV-invisible compounds in the extracts. Anisaldehyde reagent was stored at 4˚C. Terpenoids would be violet or pink in colour. Polyacetylenes would be brown or gray. Isocoumarins would be green.

Dragendorff reagent (Houghton and Raman, 1998):

Solution A contained 0.85 g of bismuth subnitrate in glacial acetic acid : distilled water 10 : 40 v/v.

Solution B contained 8 g of potassium iodine in 20 ml of distilled water.

Mixed solutions A and B, and diluted to 100 ml with 20% glacial acetic acid. Alkaloides would give orange zones on yellow background.

6.5.2 Analysis by high performance liquid chromatography (HPLC)

1) Removal of active compounds from the TLC plates

Extracts were loaded onto a line mark with pencil (18 cm long) at 2 cm high from the lower edge of the glass TLC plates (Silica gel 60 F_{254}, 0.5 mm). The plates were developed in running solvents as described previously. After drying in air, the silica containing antifungal substances were removed from the TLC plates by gentle scraping with a spatula. The powdered silica was suspended in 50 ml of absolute methanol and then was filtered through a 0.45 µm millipore membrane filter. The filtrates were concentrated to 2 ml using a rotary evaporator at 40˚C and kept at -20˚C for further HPLC analysis.

2) HPLC analysis

Ten microlitters of each fraction sample was injected into the HPLC. Total time for each run was 35 min with a flow rate of 1 ml/min. The retention

time of the peaks from each sample were matched with the retention time of the peaks of known compounds in database to confirm the identity of the compounds.

The conditions for HPLC were as follows:

- HPLC: Agilent 1100 Series with UV/VIS DAD at 230 nm
- Column: Agilent Hypersil BDS C18, 250x4.6mm, 5µm
- Solvents: MeOH (gradient grade) and phosphate buffer (0.015mol/l ortho-phosphoric acid and 0.0015mol/l tetrabutylammoniumhydroxide)
- Start: 20% MeOH, 22 min 80% MeOH, 26 min 100% MeOH, 35 min 100% MeOH

6.5.3 Analysis by nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were measured at the Institute of Inorganic Chemistry, University of Vienna, Austria (Prof. Lothar Brecker).

1) NMR sample preparation

The compound was dissolved in $CDCl_3$ (99.9% D; Sigma-Aldrich; ~5.0 mg in 0.7 ml) and transferred into 5 mm a high precision NMR glass sample tube (Wilmad 507 PP 8'', RototecSpintec GmbH, Bibesheim, Germany).

2) NMR spectroscopic measurements

All spectra were recorded on a DRX-400 AVANCE spectrometer (Bruker, Rheinstetten, Germany), equipped with a *z*-gradient inverse probe and processed using the topspin 2.1 software. ^{1}H and ^{13}C irradiation and measurement frequencies were 400.13 MHz and 100.61 MHz, respectively, and the sample temperature was 300.15 +/-0.1 K. $CHCl_3$ was used as internal standard for ^{1}H (δ_H 7.24) and ^{13}C (δ_C 77.1) measurements. The 1D spectra were recorded with sufficient number of scans (^{1}H: 32 scans; ^{13}C: 1,200 scans) by acquisition of 32k data points, in order to reach a good signal to noise ratio. After zero filling to 64k data points and Fourier transformation, the spectra were performed with a range of 7,200

Hz (^{1}H) and 20,000 Hz (^{13}C). To determine the 2D DQF-COSY, TOCSY, NOESY, HMQC, and HMBC spectra, 128 experiments in *F1* dimension were recorded, each with 1,024 data points in *F2* dimension. After linear forward prediction to 256 data points in the *F1* dimension as well as appropriate sinusoidal multiplication in both dimensions, a Fourier transformation led to 2D-spectra with a range of 4,000 Hz and 20,000 Hz for ^{1}H and ^{13}C, respectively. Mixing times of TOCSY and NOESY were 100 ms and 800 ms, respectively.

7. Enzyme activity assays

7.1 Banana treatments

Unripe banana fruits were cut from banana hands, washed with tap water, and dried in air. Ten fruits from various banana hands were sprayed with 0.5 mM salicylic acid solution for checking the induction of defensive enzymes such as phenylalanine ammonia-lyase (PAL), ß-1,3-glucanase and chitinase in banana fruit peels within 24 h. After spraying, banana fruits were stored at 23˚C and the fruit peels were collected at 0, 6, 12, 18 and 24 h. Banana fruit peels treated with yeast metabolites were also investigated. If the investigation of enzyme activity within 24 h, salicylic acid solution and yeast metabolites had the capability on the activation of PAL, ß-1,3-glucanase and chitinase in banana fruit peels, the induction of enzyme activity by metabolites from yeasts would be investigated for longer time. *C. musae* inoculation on the fruit which had been treated with yeast metabolites was also determined on the change of enzyme activity.

Ten combined fruits from various banana hands were sprayed with individual culture filtrate of yeast and then kept in plastic bags at 23˚C. The fruit peels were collected at 0, 24, 48, 72 and 96 h after spraying. YMB medium sprayed fruit and untreated fruit were used as controls. All treatments were compared with the fruit which had been sprayed with 0.5 mM salicylic acid. Fruit peels were removed from the middle part of fruit (3 cm length), cut into small pieces (0.5-1 cm), packed in a

plastic bag, and then immediately transferred to storage at -20˚C until enzyme extraction.

7.2 Banana fruit peel extraction

7.2.1 Extraction for phenylalanine ammonia-lyase (PAL)

Fruit peel extraction was carried out following the method of Ramamoorthy *et al.* (2002) with some modifications. A 5 g of frozen peel samples was ground using a pre-cooled mortar and pestle in 15 ml of cold 0.1 M sodium borate buffer, pH 7.0, containing 1.4 mM of 2-mercaptoethanol, 0.45 g of polyvinylpyrrolidone (PVP) and 150 µl of 100 mM phenylmethylsulfonylfluoride (PMSF). The extract was filtered through 2-3 layers of cheesecloth, and then centrifuged at 12,000 rpm for 15 min at 4˚C. The supernatant was collected for enzyme testing.

7.2.2 Extraction for ß-1,3-glucanase and chitinase

Fruit peel samples were extracted using a modification of the method by Velasquez and Hammerschmidt (2004). A 5 g of banana fruit peel samples was ground in 15 ml of 0.05 M sodium acetate buffer, pH 5.0 using a pre-cooled mortar and pestle (15 ml of 0.05 M sodium acetate buffer contained 150 µl of 100 mM PMSF and 0.45 g of PVP). The homogenates were filtered through 2-3 layers of cheesecloth, then centrifuged at 12,000 rpm for 15 min at 4˚C. The supernatant was collected for enzyme testing.

7.3 Enzyme analysis

7.3.1 PAL analysis

PAL activity was determined using L-phenylalanine as the substrate by the method of Silva *et al.* (2004) with some modifications. The reaction

mixture contained 1 ml of crude extract, 1 ml of 0.1 M borate buffer, pH 8.8 and 1ml of 12 mM L-phenylalanine in 0.1 M borate buffer, pH 8.8 and incubated for 30 min at 30°C. Conversion of L-phenylalanine to cinnamic acid was calculated by measuring absorbance at 290 nm compared with standard curve of cinnamic acid solution (Appendix Figure A1). The activity was expressed as micrograms per gram of fresh weight.

7.3.2 ß-1,3-glucanase analysis

Enzyme activity was assayed by using larminarin as the substrate according to the method of Burner (1964) with some modifications. The reaction was determined by measuring the amount of reducing sugars released from the substrate. The reaction tubes were set up with 1 ml of crude extract and 260 µl of 2 mg/ml larminarin in 0.05 M sodium acetate buffer, pH 5.0. The mixture was immersed in water bath at 35˚C for 30 min, then heated for 2-3 min to denature the enzyme. Five hundreds and eighty microlitters (580 µl) of 3,5-dinitrosalicylic acid (DNS) solution and 2.16 ml of 0.05 M sodium acetate buffer, pH 5.0 were added, and subsequently heated for 5 min. After boiling, the reaction tubes were left at room temperature to cooling down, then 1.58 ml of distilled water was added prior to reading absorbance at 540 nm. The reducing sugar concentration was calculated by comparing with the standard curve of glucose (Appendix Figure A2). Enzyme activity was expressed as micrograms per gram of fresh weight.

7.3.3 Chitinase analysis

Chitinase assay was determined using 1% swollen chitin as the substrate. The reaction mixture tube contained 1 ml of crude extract and 1 ml of 1% swollen chitin. The mixture tube was incubated at 50˚C for 60 min, then heated for 5 min to denature the enzyme. The mixture was left for precipitation at room temperature. Supernatant was collected for N-acetylglucosamine (NAG) analysis. Determination of NAG was assayed by using 0.5 ml of supernatant mixed with 100 µl of 0.8 M $K_2B_4O_7$, then heated for 3 min. After cooling down, 3 ml of

dimethylaminobenzaldehyde (DMAB) was added, mixed and allowed to stand in water bath at 37˚C for 20 min. When the mixture cooled down, absorbance was measured at 585 nm within 10 min. Chitinase was assayed by measuring the concentration of NAG and calculated by comparing with the standard curve of NAG (Appendix Figure A3) following the method of Reissig *et al.* (1955) with some modifications. Enzyme activity was expressed as micrograms per gram of fresh weight.

8. RNA expression on the banana fruit peels

8.1 Sample preparation

Green banana hands were separated into individual finger, washed with tap water, and dried in air. Banana fingers were divided into five sample groups of ten fingers each. The group of banana sample was sprayed with yeast culture filtrate and then stored in plastic bag at 23˚C. The samples were collected at 0, 24, 48, 72 and 96 h after spraying by removing the middle part of fruit peels in 3 cm long. YMB medium sprayed fruit and untreated fruit were used as negative controls. Salicylic acid solution at the concentration of 0.5 mM sprayed fruits were used as positive control. The fruit peels were cut into small pieces (0.5-1 cm) and packed in plastic bag, then immediately kept at -70˚C until RNA extraction.

8.2 Extraction of total RNA

The sample of banana fruit peel was ground to a powder in liquid nitrogen using a mortar and pestle at room temperature. Ground plant material was stored at -70˚C prior to extraction with Plant RNA Reagent (Concert ^{TM} Plant RNA Reagent kit; Invitrogen, 25-0432 061301). The frozen ground tissue (0.1 g) was transferred to cool RNase-free microcentrifuge tubes in dry ice and added 0.5 ml of cold Plant RNA Reagent and then, mixed by using vortex around 1 min. The tube was incubated at room temperature for 5 min. Subsequently, the suspension was centrifuged at 12,000 x g at room temperature. The supernatant was transferred to a

new Rnase-free tube and added 0.1 ml of 5 M NaCl to the clarified extract, and mixed. 0.3 ml of chloroform was added and mixed thoroughly. The sample was centrifuged at 4˚C for 10 min at 12,000 x g to separate the phase. The top layer, aqueous phase, was transferred to an Rnase-free tube and added an equal volume of isopropyl alcohol. After mixing, the sample was left at room temperature for 10 min and then centrifuged at 4˚C for 10 min at 12,000 x g. The supernatant was discarded. The pellet was washed with 75% ethanol and centrifuged at room temperature for 1 min at 12,000 x g. The supernatant was decanted. The RNA pellet was dissolved by 30 µl of Rnase-free water. The supernatant was transferred to a fresh tube and stored at -70˚C. The concentration of RNA (30 µg/µl) was determined by measuring the absorbance at 260 nm.

8.3 Primers for Reverse transcriptase-Polymerase chain reaction (RT-PCR)

The forward and reverse primers were designed using Primer 3 Input (version 0.4.0) for 20 bps. The primers corresponded to the PAL (EU856394.1, Appendix Figure B1), ß-1,3-glucanase (AF001523.2, Appendix Figure B2) and chitinase (AY507150.1, Appendix Figure B3) genes of *Musa acuminata* were employed. The housekeeping gene actin (ACT1, EF672732.1, Appendix Figure B4) of *M. acuminata* was used as an internal control during RT-PCR. The properties of primers were shown in Table 2.

Table 2 Sequences and properties of the forward and reverse primers using Primer 3 Input (version 0.4.0) designed from *Musa acuminata* AAA Group genes.

Genes		Length	Tm	% GC	Sequence 5'-----3'	bp
Chitinase I (AY507150.1)	Forward	20	60	50	GGGGTTGACCTGCTAAACAA	530
Chitinase I (AY507150.1)	Reverse	20	60	50	GTATGTTTGGCATGGTGCTG	

Table 2 (Continued)

Genes		Length	Tm	% GC	Sequence 5'-----3'	bp
PAL (EU856394.1)	Forward	20	59.96	55	TGTGGAGGTTGGGTTAGGAG	
PAL (EU856394.1)	Reverse	20	60	55	AGAGTGGTTGTTGGGAGTGC	400
ß -1,3-glucananse (AF001523.2)	Forward	20	59.84	55	CCAGCGTCTCCTTTCGATAC	
ß -1,3-glucananse (AF001523.2)	Reverse	20	59.88	50	TCCGTTACTCGCCAAGAACT	200
Actin (EF672732.1)	Forward	20	60	55	AGCACCCTGTATTGCTCACC	
Actin (EF672732.1)	Reverse	20	60	50	CGTAGGCAAGCTTTTCCTTG	390

8.4 Reverse transcriptase-Polymerase chain reaction (RT-PCR)

To determine the expression pattern of the ß-1,3-glucanase, chitinase and PAL genes in banana fruit peel, semi-quantitative expression analysis using RT-PCR approach was applied in this study. Messenger RNA (mRNA) was transcribed into cDNA with the help of reverse transcriptase.

The reverse transcription reaction mix was assembled on ice to contain the components of the ImProm-II™ Reverse Transcription System (Table 3). The mixture allowed 15 µl for each cDNA synthesis reaction to be performed and was gently vortexed and kept on ice prior to dispensing into the reaction tubes. 5 µl of total RNA and primer mix was added to each reaction for a final reaction volume of 20 µl. Experimental RNA was combined with the experimental primer, or an aliquot of the positive control. RNA was combined with oligo(dT)15. The primer/template mixture was thermally denatured at 70˚C for 5 min and chilled on ice. In experimental systems, the addition of 1 unit/µl of Recombinant RNasin® Ribonuclease Inhibitor

was recommended but optional. As a final step, the template-primer combination was added to the reaction mixture on ice. Following an initial annealing at 25˚C for 5 min, the reaction was incubated at 42˚C for up to one hour. The cDNA synthesis, the product was directly added to amplification reactions.

PCR reactions were carried out in 20 µl volumes with 5 unit/µl of i-Taq DNA polymerase (Table 4). The PCR conditions were performed with pre-denaturation at 94˚C for 2 min, denaturation at 94˚C for 45 sec, annealing at 55˚C for 45 sec, extension at 72˚C for 1 min and final extension at 75˚C for 5 min. PCR cycle numbers at 25, 30, 35 and 40 were applied to check the differential expression of interested genes. Fragments of gene-specific primers used were shown in Table 2. The amplified DNA were electrophored on 1.0% agarose gel with Tris-Borate-EDTA (TBE) buffer (0.445 M Tris, 0.445 M Boric acid and 0.01 M EDTA) at 75 volts for 1.5 h and stained with ethidium bromide (0.5 µg/ml) for 5 min. Agarose gel was pictured using Gel Documentation.

Table 3 The components of the ImProm-II™ Reverse Transcription System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase Reaction	
Experimental reaction	
ImProm-II™ 5X Reaction buffer	4.0 µl
$MgCl_2$ (8.0 mM)	6.4 µl
dNTP Mix (0.5 mM each dNTP)	1.0 µl
Recombinant RNasin Ribonuclease Inhibitor (optional)	20 unit
ImProm-II™ Reverse Transcriptase	1.0 µl
Nuclease-Free Water	2.6 µl
Negative (No-Reverase Transcriptase) Control	
ImProm-II™ 5X Reaction buffer	4.0 µl
$MgCl_2$ (8.0 mM)	6.4 µl
dNTP Mix (0.5 mM each dNTP)	1.0 µl
Recombinant RNasin Ribonuclease Inhibitor (optional)	20 unit
Nuclease-Free Water	2.6 µl

Table 3 (Continued)

Reverse Transcriptase Reaction	
Positive Control	
ImProm-II™ 5X Reaction buffer	4.0 µl
$MgCl_2$ (6.0 mM)	6.4 µl
dNTP Mix (0.5 mM each dNTP)	1.0 µl
Recombinant RNasin Ribonuclease Inhibitor (optional)	20 unit
ImProm-II™ Reverse Transcriptase	1.0 µl
Nuclease-Free Water	2.6 µl

Table 4 The components of PCR

PCR components	
ddH_2O	9.8 µl
dNTP (0.2 mM)	1.0 µl
10x Buffer	2.0 µl
Primer Forward (5 µM)	2.0 µl
Primer Reward (5 µM)	2.0 µl
i-Taq DNA polymerase (5 unit/µl)	0.2 µl
cDNA	3.0 µl

RESULTS

1. Screening for antagonistic yeasts against anthracnose pathogen and anthracnose disease on banana fruits

1.3 Antagonistic effects of yeasts against *Colletotrichum musae in vitro*

The determination of dual culture tests between each yeast and anthracnose pathogen, *Colletotrichum muasae* was evaluated on potato dextrose agar Petri dish and incubated for 6 days at room temperature (28-32˚C). The results showed that yeast *Aureobasidium pullulans* TISTR 3389 gave the highest inhibition of pathogen growth by 40.6%, following by six yeasts including *Candida utilis*, *Candida tropicalis, Pichia membranaefaciens, Cryptococcus humicola* BCC 7701, *Candida sake* TISTR 5143 and *Saccharomycopsis fibuligera* TISTR 5033, could reduce pathogen growth by 27.4-32.1%. Whereas two yeasts, *Candida guilliermondii* BCC 5389 and *Rhodotorula glutinis* TISTR 5159 showed the lowest inhibition by 1.4 and 6.6%, respectively (Table 5). All ten antagonistic yeasts did not produce inhibition zones on PDA, but they had rapid growth on the medium. The strips of all yeasts were not covered by the mycelium growth of the pathogen (Figure 8).

Table 5 Inhibition of radial growth (%) of *Colletotrichum musae* by ten yeasts which were tested by dual culture on potato dextrose agar plate, incubated for 6 days at room temperature (28-32˚C).

Yeast strains	Inhibition of radial growth (%) [1]
Aureobasidium pullulans TISTR 3389	40.6 a
Candida guilliermondii BCC 5389	1.4 d
Candida tropicalis	30.7 b
Candida sake TISTR 5143	27.4 bc
Candida utilis	32.1 b
Cryptococcus humicola BCC 7701	30.7 b
Debaryomyces hansenii TISTR 5155	21.7 c
Pichia membranaefaciens	27.4 bc
Rhodotorula glutinis TISTR 5159	6.6 d
Saccharomycopsis fibuligera TISTR 5033	30.7 b

[1] Means followed by different letters are significant difference (P=0.05), according to Duncan's Multiple Range Test

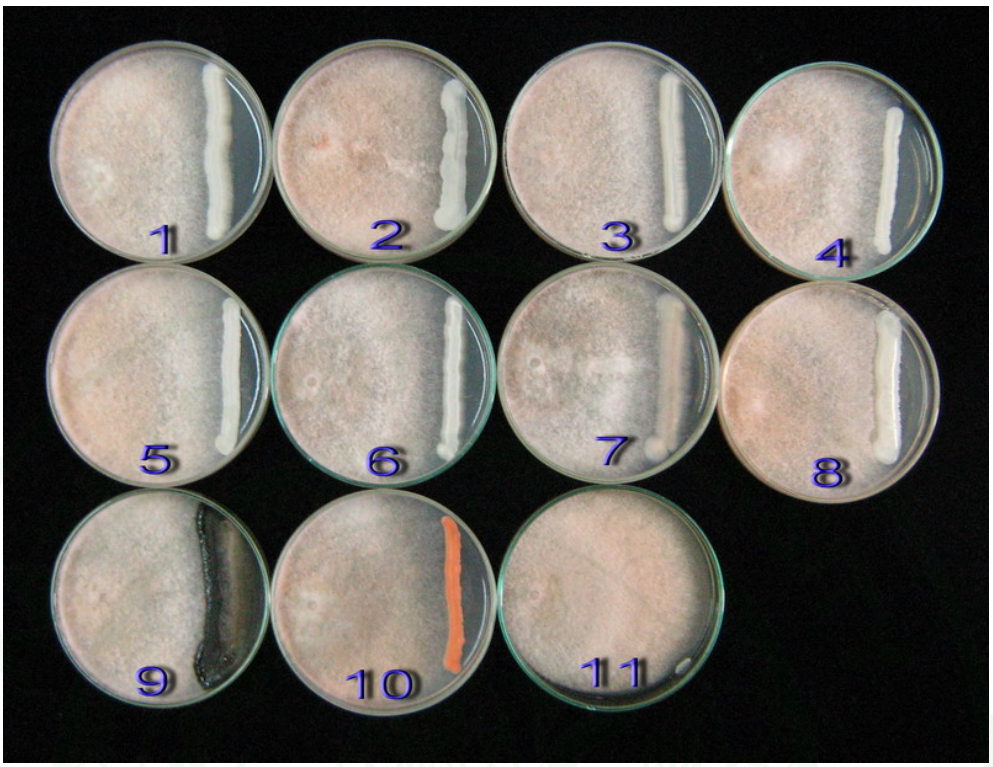


Figure 8 Dual culture tests between ten yeasts and *Colletotrichum musae* on potato dextrose agar, incubated for 6 days at room temperature (28-32˚C).

1 = *Candida guilliermondii* BCC 5389
2 = *Candida utilis*
3 = *Candida sake* TISTR 5143
4 = *Saccharomycopsis fibuligera* TISTR 5033
5 = *Pichia membranaefaciens*
6 = *Candida tropicalis*
7 = *Debaryomyces hansenii*
8 = *Cryptococcus humicola* BCC 7701
9 = *Aureobasidium pullulans* TISTR 3389
10 = *Rodotorula glutinis* TISTR 5159
11 = *Colletotrichum musae* (Control)

1.4 Antagonistic effects of yeasts in suppressing anthracnose lesions on banana fruits

Banana fruits were applied with each yeast cell suspension ($1x10^8$ cells/ml) at the wounded parts and incubated at room temperature (28-32˚C) for 24 h in moist condition, subsequently the fruits were inoculated with *C. musae* spore suspension ($1x10^5$ spores/ml) and further incubated at the same condition for 24 h. The lesion sizes on the fruits were measured at 6 days. The results revealed that yeast *Debaryomyces hansenii* TISTR 5155 showed the highest inhibition of lesion development on wounded banana fruits by 54.4%, but no significant difference from two yeasts, *Candida sake* TISTR 5143 and *Candida utilis* which lesion sizes were reduced by 43.7 and 38.0%, respectively. Whereas yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida tropicalis* showed the second on the reduction of lesion development by 26.4 and 31.6%, respectively (Table 6).

Five antagonistic yeasts including *Debaryomyces hansenii* TISTR 5155, *Candida sake* TISTR 5143, *Candida utilis*, *Aureobasidium pullulans* TISTR 3389 and *Candida tropicalis* which showed the good results on the reduction of lesion development on banana fruits were re-tested their efficacy in controlling the disease development on banana fruits again. The results showed that four yeasts; *Debaryomyces hansenii* TISTR 5155, *Candida sake* TISTR 5143, *Candida utilis* and *Aureobasidium pullulans* TISTR 3389 showed the good reduction of lesion development by 26.2 - 31.6% (Table 7 and Figure 9).

Table 6 Lesion diameters on the banana fruits which treated with ten yeast cell suspensions and incubated for 24 h before inoculating with *Colletotrichum musae*. Inoculated fruits were incubated at room temperature (28-32˚C) for 6 days.

Yeast strains	Lesion diameter (mm) [1]	Reduction of lesion development (%)
Debaryomyces hansenii TISTR 5155	6.99 a	54.4
Candida sake TISTR 5143	8.63 ab	43.7
Candida utilis	9.50 ab	38.0
Aureobasidium pullulans TISTR 3389	10.48 bc	31.6
Candida tropicalis	11.27 bc	26.4
Rhodotorula glutinis TISTR 5159	12.80 cde	16.4
Pichia membranaefaciens	14.18 de	7.4
Cryptococcus humicola BCC 7701	14.22 de	7.2
Candida guilliermondii BCC 5389	14.60 e	4.7
Saccharomycopsis fibuligera TISTR 5033	15.93 e	0.0
Yeast extract malt extract broth (Control)	15.32 e	-

[1] Means followed by different letters are significant difference (P=0.05), according to Duncan's Multiple Range Test

Table 7 Lesion diameters on the banana fruits which treated with five yeast cell suspensions and incubated for 24 h before inoculation with *Colletotrichum musae*. Inoculated fruits were incubated at room temperature (28-32˚C) for 6 days.

Yeast strains	Lesion diameter (mm) [1]	Reduction of lesion development (%)
Debaryomyces hansenii TISTR 5155	10.33 ab	26.2
Candida sake TISTR 5143	9.37 a	33.0
Candida utilis	9.57 a	31.6
Aureobasidium pullulans TISTR 3389	9.57 a	31.6
Candida tropicalis	10.93 b	21.9
Yeast extract malt extract broth (Control)	13.99 c	-

[1] Means followed by different letters are significant difference (P=0.05), according to Duncan's Multiple Range Test

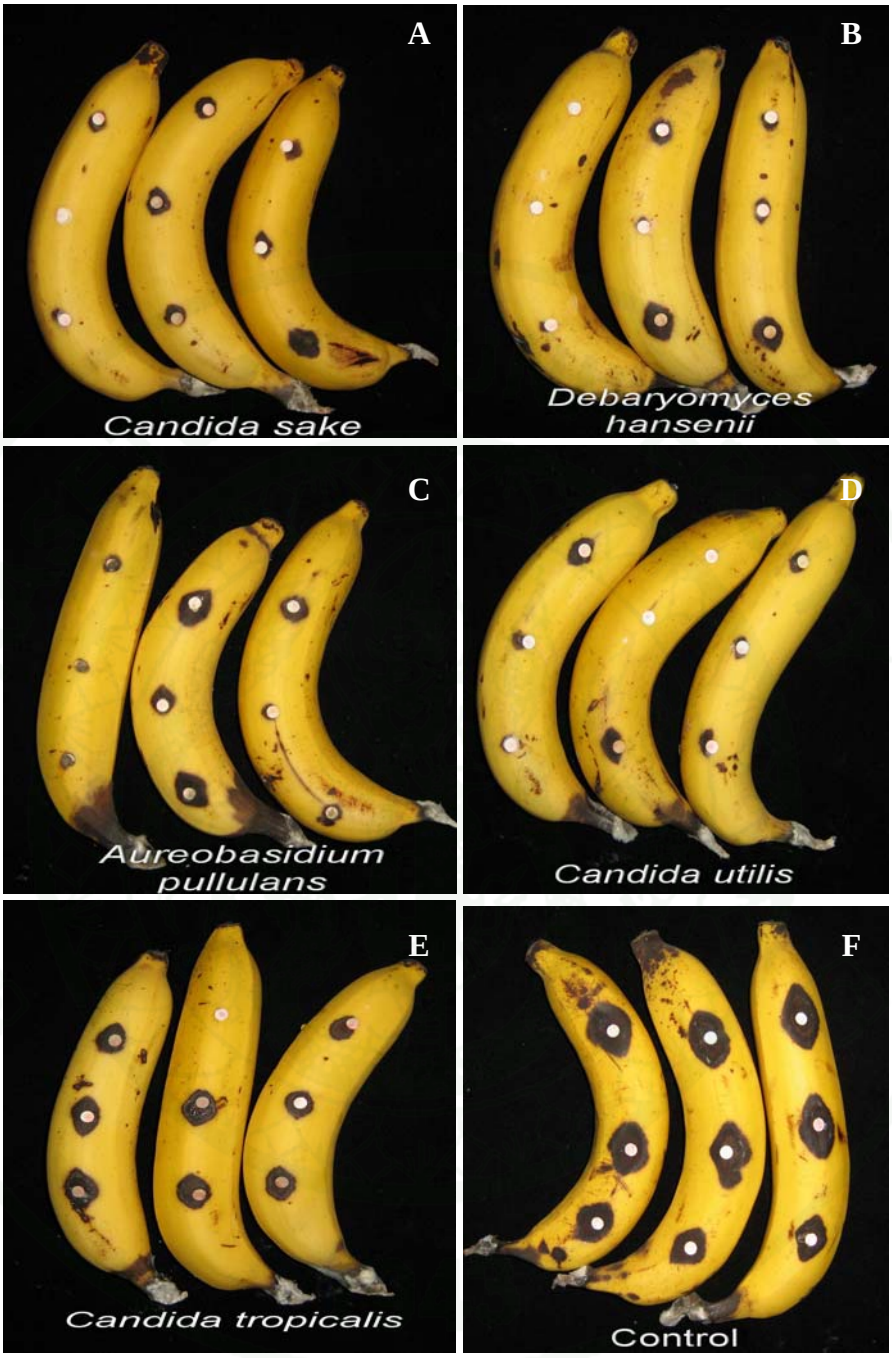


Figure 9 Lesion sizes of disease development on banana fruits were treated with yeast cell suspensions of *Candida sake* (A), *Debaryomyces hansenii* (B), *Aureobasidium pullulans* (C), *Candida utilis* (D) and *Candida tropicalis* (E), and incubated for 24 h before inoculation with *Colletotrichum musae.* Banana fruits treated with yeast extract malt broth following *C. musae* inoculation served as control (F). Inoculated fruits were incubated at room temperature (28-32˚C) for 6 days.

2. Preliminary screening of metabolites from yeasts against anthracnose disease on banana fruits

From the results in 1.2, four yeast cell suspensions including *Debaryomyces hansenii* TISTR 5155, *Candida sake* TISTR 5143, *Candida utilis* and *Aureobasidium pullulans* TISTR 3389 which showed good potential to control anthracnose lesions on banana fruits were selected for this experiment. Culture filtrates of four yeasts which obtained from the cultures of these yeasts in potato dextrose broth (PDB) and yeast extract malt extract broth (YMB) at 48 h were used as yeast metabolites. The application of PDB metabolites of *Candida utilis* prior challenge with *C. musae* on banana fruits for 24 h showed a good reduction of disease severity by 44.9%, compared with the fruit untreated with metabolites (Table 8 and Figure 10). Whereas YMB metabolites from two yeasts, *Candida utilis* and *Aureobasidium pullulans* showed the reduction of disease severity by 71.6 and 61.9%, respectively (Table 9 and Figure 11). However, metabolites of all yeasts produced in YMB medium showed higher reduction of disease severity on banana fruits than in PDB.

Table 8 Anthracnose disease severity on banana fruits were treated with yeast metabolites obtained from potato dextrose broth (PDB) and incubated for 24 h before inoculation with *Colletotrichum musae.* Inoculated fruits were incubated at room temperature (28-32˚C) for 7 days.

Yeast metabolites	Disease severity (%) [1]	Reduction of disease severity (%)
Debaryomyces hansenii TISTR 5155	74.0 c	15.9
Candida sake TISTR 5143	65.2 b	25.9
Candida utilis	48.5 a	44.9
Aureobasidium pullulans TISTR 3389	78.5 cd	10.8
No metabolites	88.0 d	-

[1] Means followed by different letters are significant difference (P=0.05), according to Duncan's Multiple Range Test

Table 9 Anthracnose disease severity on banana fruits were treated with yeast metabolites obtained from yeast extract malt extract broth (YMB) and incubated for 24 h before inoculation with *Colletotrichum musae.* Inoculated fruits were incubated at room temperature (28-32˚C) for 7 days.

Yeast metabolites	Disease severity (%) [1]	Reduction of disease severity (%)
Debaryomyces hansenii TISTR 5155	61.0 c	31.8
Candida sake TISTR 5143	41.0 b	53.4
Candida utilis	25.0 a	71.6
Aureobasidium pullulans TISTR 3389	33.5 ab	61.9
No metabolites	88.0 d	-

[1] Means followed by different letters are significant difference (P=0.05), according to Duncan's Multiple Range Test

Figure 10 Disease severity of anthracnose on banana fruits were treated with yeast metabolites of *Debaryomyces hansenii* (A), *Candida sake* (B), *Candida utilis* (C) and *Aureobasidium pullulans* (D) obtained from potato dextrose broth (PDB) and incubated for 24 h before inoculation with *Colletotrichum musae*. Banana fruits treated with PDB following *C. musae* inoculation served as control (E). Banana fruits were incubated at room temperature (28-32˚C) for 7 days.

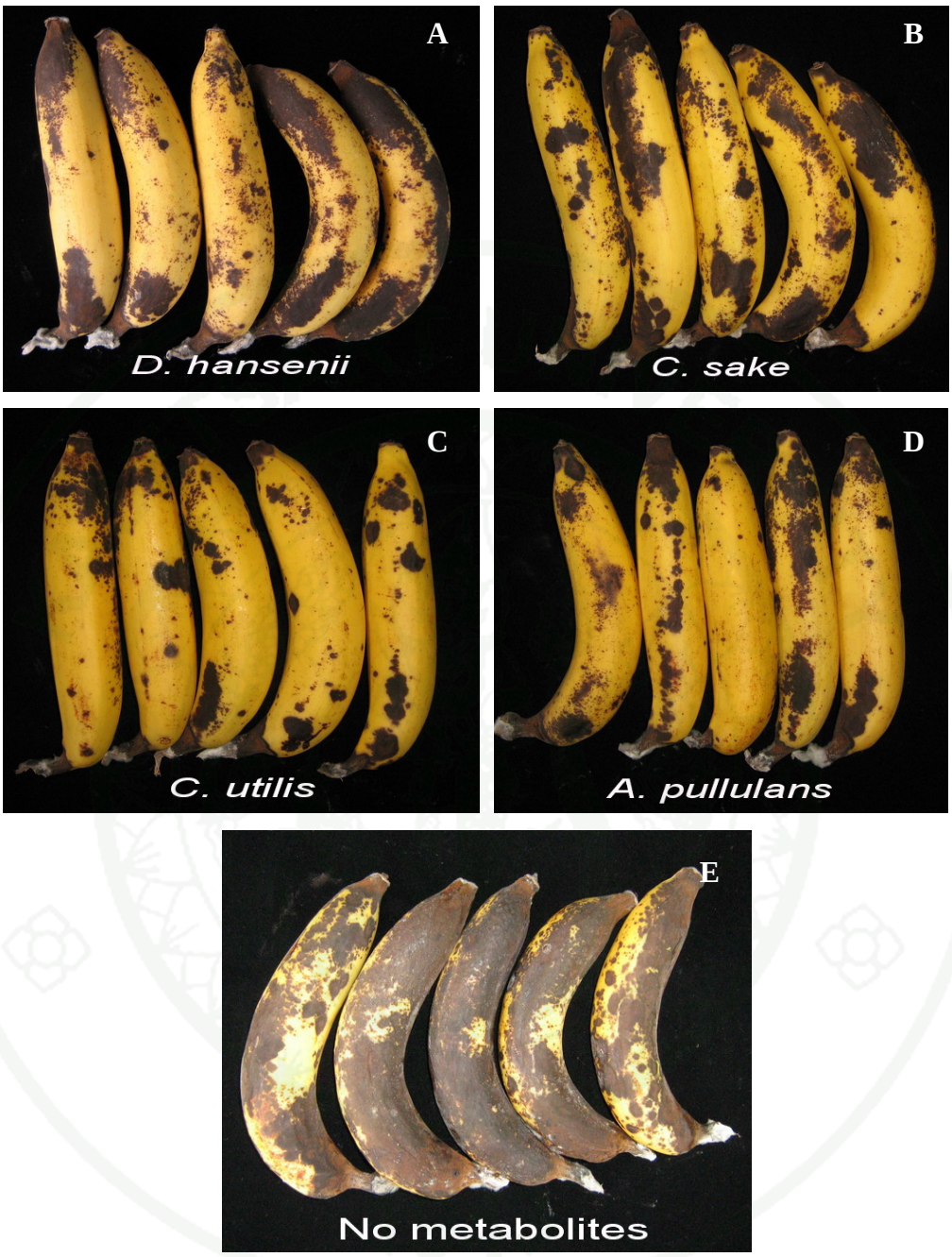


Figure 11 Disease severity of anthracnose on banana fruits were treated with yeast metabolites of *Debaryomyces hansenii* (A), *Candida sake* (B), *Candida utilis* (C) and *Aureobasidium pullulans* (D) obtained from yeast extract malt extract broth (YMB) and incubated for 24 h before inoculation with *Colletotrichum musae.* Banana fruits treated with YMB following *C. musae* inoculation served as control (E).Banana fruits were incubated at room temperature (28-32˚C) for 7 days.

3. Determination of growth curve and cell viability of yeasts for secondary metabolites production

From the results in 2, metabolites from two yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* which obtained from YMB cultures at 48 h showed the best potential to suppress anthracnose disease development on banana fruits. The growth curve of both yeasts was determined by using YMB with shaking at 120 rpm and incubated at room temperature (28-32˚C) for 14 days for finding the appropriate incubation period of secondary metabolite production. The results showed that yeast *C. utilis* increased population in culture media faster than *A. pullulans* according to lag phase of *C. utilis* had a short period, 18 h, whereas lag phase of *A. pullulans* had a longer period for 24 h (Figure 12a, c). Additionally, the exponential phase was occurred at higher rate in yeast *C. utilis* than *A. pullulans* (Figure 12a, c). After incubation for 72 h, the growth of yeast *A. pullulans* was stable at the stationary phase, while the growth of *C. utilis* had rapidly shown the period of this growth phase starting from 30 h (Figure 12a, c).

From this experiment, the growth phase of both yeasts could not determined the dead phase by OD measurement throughout 14 days of incubation period. Therefore, plate counting assay had been introduced to check viable cells of yeasts in YMA. The results showed that at stationary phase of both yeasts, the highest living cells was shown after incubation for 9 days (Figure 12b, d). Subsequently, cell viability had been decreased until to the end of incubation time at 14 days. This might indicate that the growth of yeasts had no shown dead phase on the curve by OD measurement, mortality of yeast cells was observed as indicated the dead phase.

From the growth curve, stationary phase at incubation time for 5 days which expected to produce enormous secondary metabolites were selected for further studies.

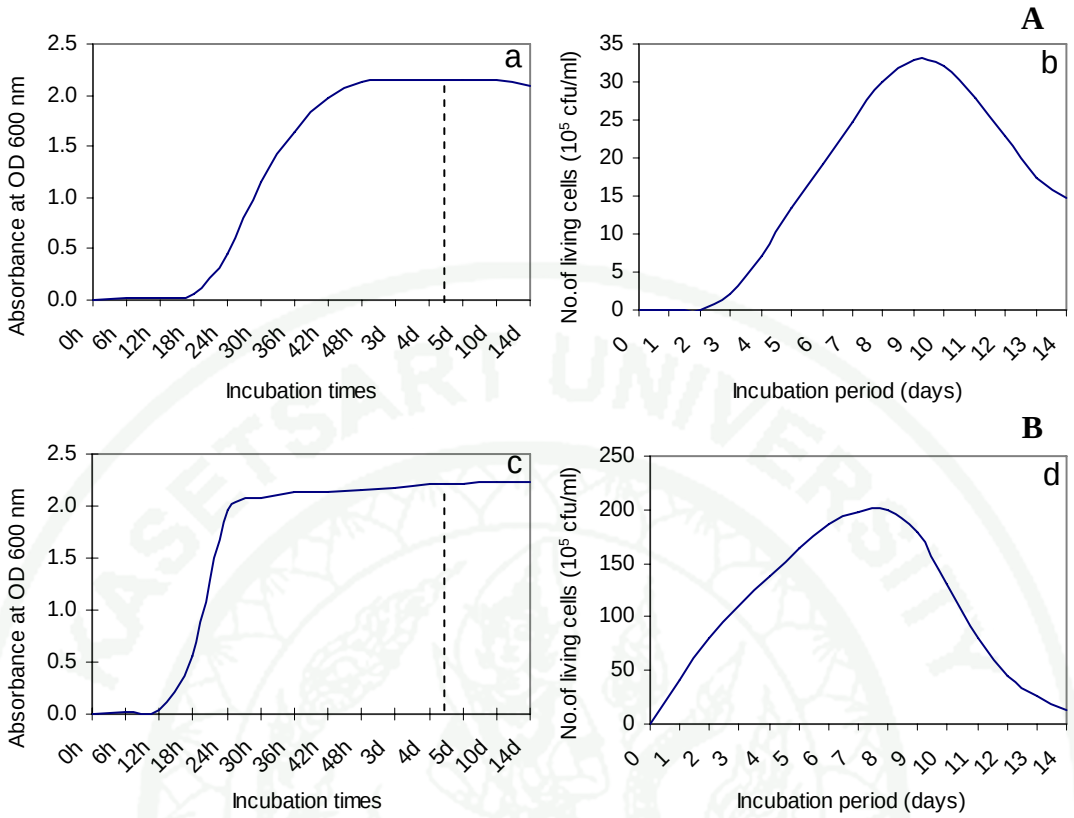


Figure 12 Growth of yeasts *Aureobasidium pullulans* TISTR 3389 (A) and *Candida utilis* (B) in yeast extract malt extract broth (YMB) incubated for 14 days at room temperature (28-32˚C) on shaking at 120 rpm. Growth curve by reading optical density (OD) at 600 nm (a,c) and cell viability (b,d), evaluated by plate counting on yeast extract malt extract agar at 48 h. Dot vertical lines indicated the incubation time at day 5 for collecting yeast secondary metabolites.

4. Study of metabolites from yeasts on *Colletotrichum musae* and anthracnose disease on banana fruits

4.1 Metabolites from yeasts on the *in vitro* growth of *Colletotrichum musae*

Culture filtrates from two yeasts, *C. utilis* and *A. pullulans* greatly reduced mycelial growth of the pathogen, *C. musae* (Table 10 and Figure 13). Dry weight mycelia in *A. pullulans* and *C. utilis* metabolites were reduced by 83.9 and 85.6%, respectively. Similarly, spore germination and germ tube elongation of pathogen in metabolites from both yeasts were also significantly reduced (Table 11 and Figure 14). Metabolites from yeast *A. pullulans* reduced spore germination and germ tube elongation by 20.3 and 23.1%, respectively, whereas yeast *C. utilis* metabolites was lower at 6.4 and 12.9%, respectively.

Table 10 Effect of metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* on dry weight mycelia of *Colletotrichum musae* incubated for 5 days at room temperature (28-32˚C)

Treatments	Dry weight mycelia (g) [1]	Mycelial growth inhibition (%)
A. pullulans metabolites	0.019 a	83.9
C. utilis metabolites	0.017 a	85.6
No metabolites	0.118 b	-

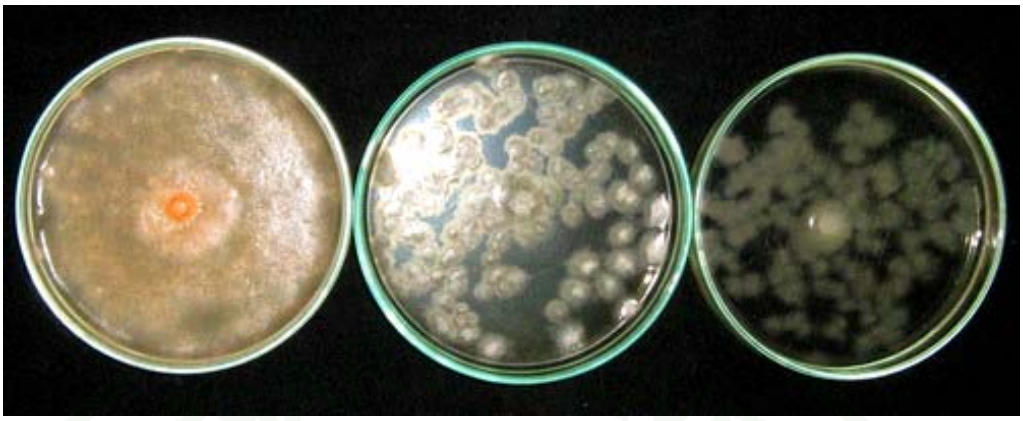
[1] Mean values followed by a same letter indicated no significant different (P=0.05), according to Duncan's Multiple Range Test

Table 11 Effect of metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* on spore germination and germ tube elongation of *Colletotrichum musae* incubated for 6 h on water agar at room temperature (28-32˚C)

Treatments	Percentage of germinated spores [1]	Spore germination inhibition (%)	Germ tube elongation (μm) [1]	Germ tube elongation inhibition (%)
A. pullulans metabolites	75.6 a	20.3	76.2 a	23.1
C. utilis metabolites	88.8 b	6.4	86.2 b	12.9
No metabolites	94.9 c	-	99.0 c	-

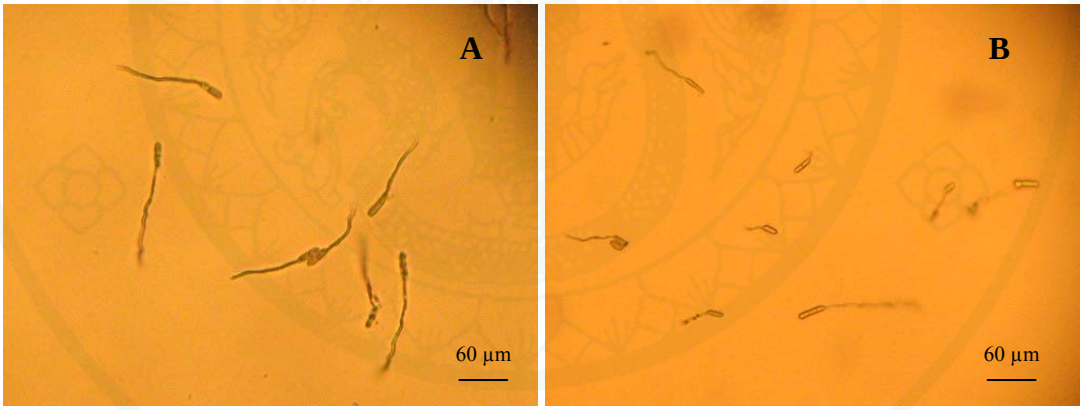
[1] Mean values in each column followed by a different letter indicated significant different (P=0.05), according to Duncan's Multiple Range Test

The 50 percent inhibition concentration (IC_{50}) values for mycelial growth and spore germination inhibition of *A. pullulans* metabolites calculated from the regression equation were 190.4 and 149.0 mg/L, respectively. Whereas the metabolites from yeast *C. utilis* showed the highest growth inhibitors as it had the lowest IC_{50} on mycelial growth at 158.0 mg/L, while spore germination inhibition had the IC_{50} at 150.5 mg/L (Figure 15).



No metabolites | *A. pullulans* metabolites | *C. utilis* metabolites

Figure 13 *Colletotrichum musae* was grown on yeast *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* metabolites which obtained from 5 days old yeast culture in yeast extract malt extract broth. Pathogen was incubated for 5 days at room temperature (28-32˚C).



No metabolites | *A. pullulans* metabolites

Figure 14 Spore germination (200x) of *Colletotrichum musae* after treated with metabolites from yeast *Aureobasidium pullulans* TISTR 3389, incubated for 6 h on water agar at room temperature (28-32˚C).

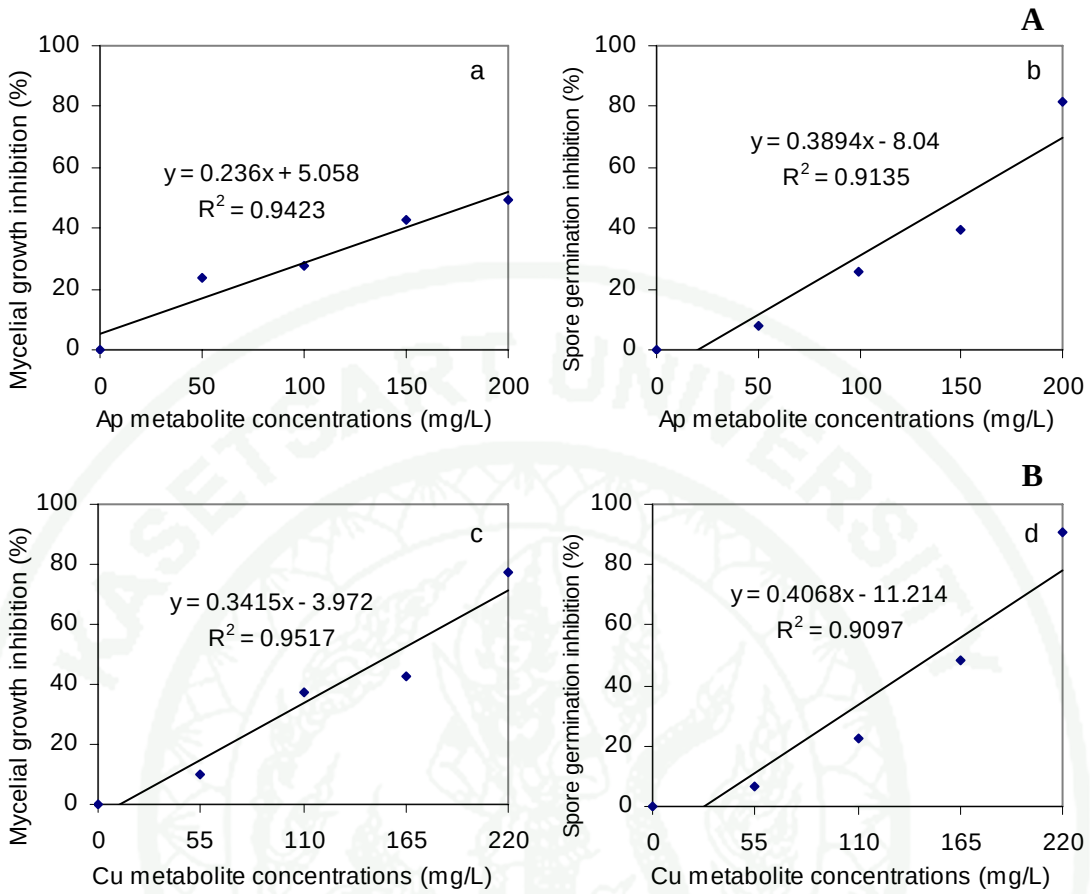


Figure 15 The regression equations of the relationship between various concentrations of metabolites from two yeasts *Aureobasidium pullulans* TISTR 3389 (A) and *Candida utilis* (B), and mycelial growth (a,c) as well as spore germination inhibition (b,d) of *Colletotrichum musae* after incubation for 5 days and 6 h, respectively.

4.2 Volatile metabolites from yeasts on the *in vitro* growth of *Colletotrichum musae*

Volatile metabolites from two yeasts *A. pullulans* and *C. utilis* significantly inhibited mycelial growth of *C. musae* by 30.5 and 29.3%, respectively (Table 12). Additionally, mycelial density of pathogen was also affected by both volatile metabolites compared with the control (Figure 16). Furthermore, the efficacy of volatile metabolites from yeast *A. pullulans* also affected on the inhibition of spore germination and germ tube elongation by 19.7 and 23.1%, respectively, whereas volatile metabolites from *C. utilis* had no effect on the inhibition of spore germination and germ tube elongation with no significant difference from non volatile metabolites treatment (Table 13).

Table 12 Effect of volatile metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* on colony diameter and mycelial density of *Colletotrichum musae* incubated on potato dextrose agar for 4 days at room temperature (28-32˚C).

Volatile metabolites from yeasts	Colony diameter (cm) [1]	Mycelial growth inhibition (%)	Mycelial density [1,2]
A. pullulans	4.17 a	30.5	2 a
C. utilis	4.24 a	29.3	2 a
No metabolites	6.00 b	-	3 b

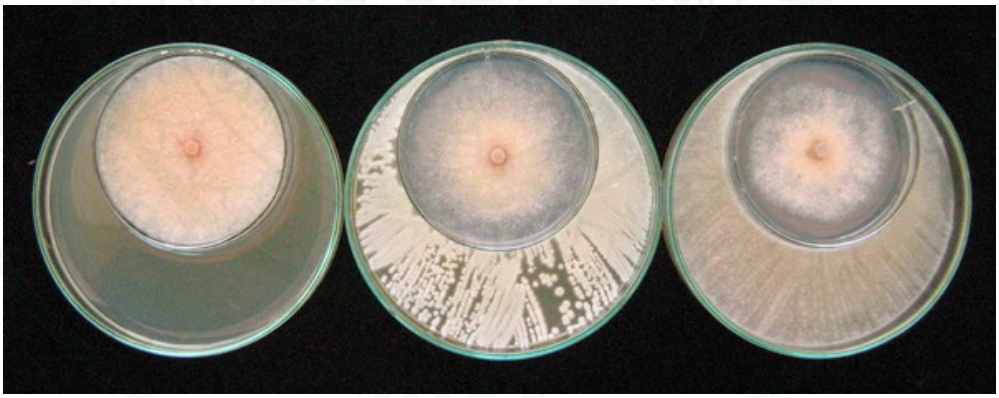
[1] Mean values followed by a same letter indicated no significant different (P=0.05), according to Duncan's Multiple Range Test

[2] 1 = slight mycelial growth, 2 = moderated mycelial growth and 3 = thick mycelial growth.

Table 13 Effect of volatile metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* on spore germination and germ tube elongation of *Colletotrichum musae* incubated on water agar for 6 h at room temperature (28-32˚C).

Volatile metabolites from yeasts	Percentage of germinated spores [1]	Spore germination inhibition (%)	Germ tube elongation (µm) [1]	Germ tube elongation inhibition (%)
A. pullulans	59.5 a	19.7	76.7 a	23.1
C. utilis	72.5 ab	2.2	95.7 ab	4.0
No metabolites	74.1 b	-	99.7 b	-

[1] Mean values in each column followed by a different letter indicated significant different (P=0.05), according to Duncan's Multiple Range Test



No volatile metabolites | *C. utilis* volatile metabolites | *A. pullulans* volatile metabolites

Figure 16 Colony of *Colletotrichum musae* after treated with volatile metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* incubated for 4 days at room temperature (28-32˚C).

4.3 Metabolites from yeasts on disease severity of banana fruits

The application of non-heated and heated metabolites from both yeasts *A. pullulans* and *C. utilis* and salicylic acid solution was tested to control anthracnose on banana fruits. The results showed that all treatments significantly reduced the infection of *C. musae* on banana fruit (Table 14 and Figure 18). This indicated that yeast *A. pullulans* metabolites was the most effective to control this disease, disease severity was reduced by 81.2%, similar to an application of 0.5 mM salicylic acid solution, disease severity was reduced by 65.7%.

Metabolites from *C. utilis* was the second on the reduction of disease severity that was reduced by 56.4%. Whereas an application of heated metabolites from both yeasts was less reduction of anthracnose disease, disease severity was reduced by 33.1%.

Additionally, salicylic acid at the concentration of 0.5 mM which was directly used to inhibit the growth of *C. musae* on PDA (for mycelial growth) and WA (for spore germination) showed no direct inhibition on the growth of pathogen (Figure 17). This indicated that spore germination inhibition of pathogen might affect from other reasons when salicylic acid was applied on the fruits and followed by pathogen inoculation.

Table 14 Anthracnose disease severity (%) on banana fruits were treated with metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* and incubated for 24 h before inoculation with *Colletotrichum musae* compared with the fruits treated with heated metabolites and salicylic acid solution. Inoculated fruits were incubated at room temperature (28-32˚C) for 7 days.

Treatments	Disease severity (%) [1]	Reduction of disease severity (%)
Non-heated *A. pullulans* metabolites	12.5 a	81.2
Non-heated *C. utilis* metabolites	29.0 b	56.4
Heated *A. pullulans* metabolites	44.5 c	33.1
Heated *C. utilis* metabolites	44.5 c	33.1
0.5 mM salicylic acid	22.8 ab	65.7
No metabolites	66.5 d	-

[1] Mean values followed by different letters indicated significant different (P=0.05), according to Duncan's Multiple Range Test

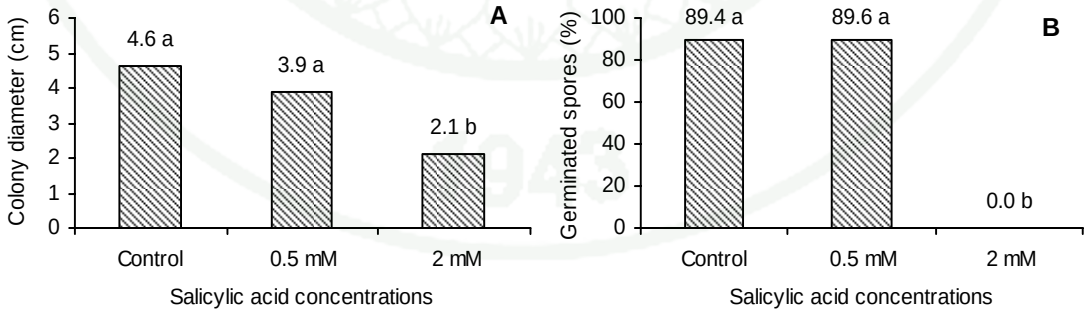


Figure 17 Various concentrations of salicylic acid solution amended in potato dextrose agar on mycelial growth (A) and spore germination (B) of *Colletotrichum musae* incubated at room temperature (28-32˚C) for 7 days.

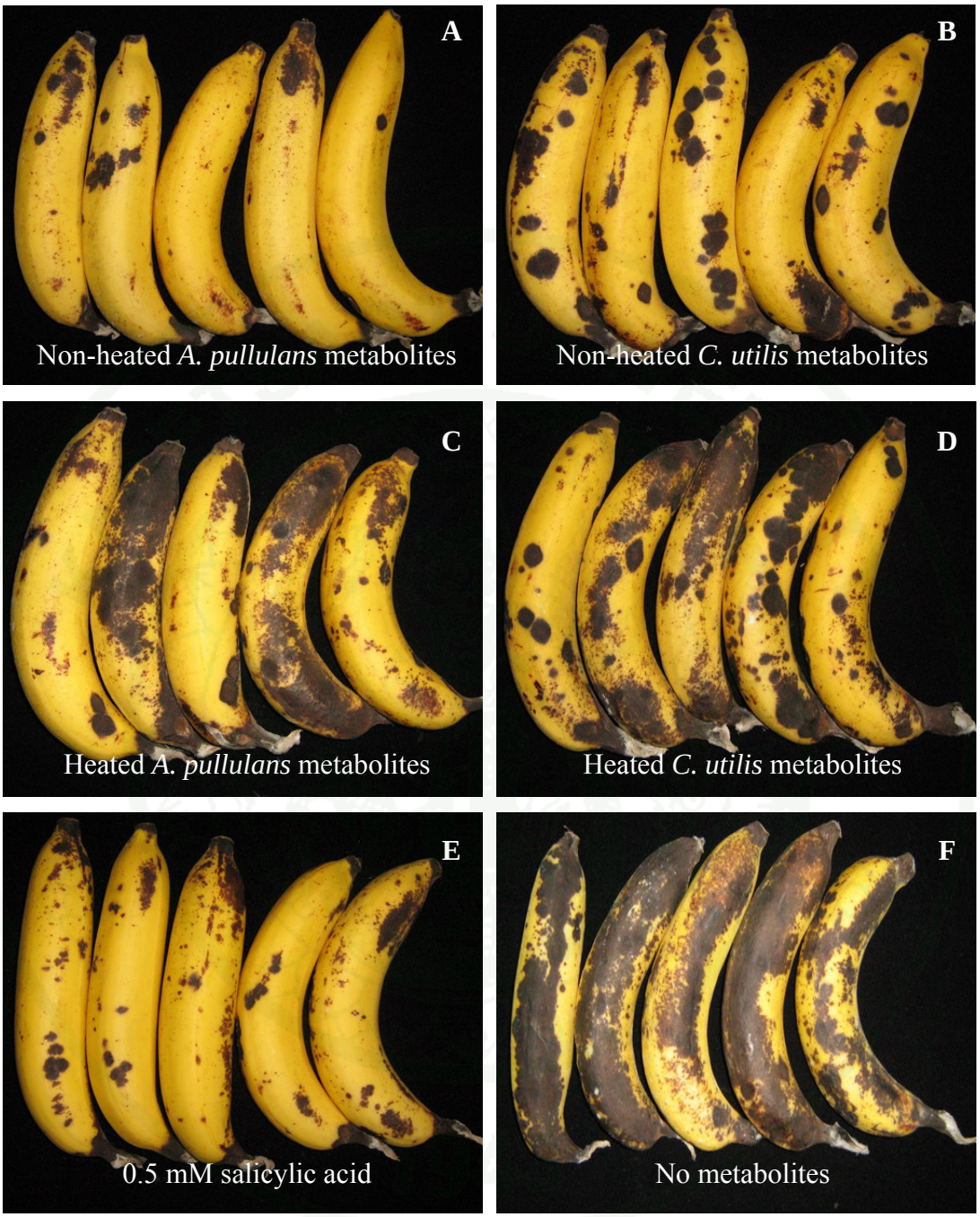


Figure 18 Disease severity of anthracnose on banana fruits were treated with non-heated and heated metabolites from yeasts. The fruits treated with non-heated *Aureobasidium pullulans* metabolites (A), non-heated *Candida utilis* metabolites (B), heated *A. pullulans* metabolites (C) and heated *C. utilis* metabolites (D) incubated for 24 h before inoculation with *Colletotrichum musae* compared with the fruits treated with 0.5 mM salicylic acid (E) and YMB medium (no metabolites) (F). Inoculated fruits were incubated at room temperature (28-32˚C) for 7 days.

5. Cell wall degrading enzymes produced by yeasts against *Colletotrichum musae*

Culture filtrates from two yeasts *A. pullulans* and *C. utilis* which obtained from 5-day-old culture of YMB were investigated for cell wall degrading enzyme production such as ß-1,3-glucanase and chitinase. The results showed that yeast *A. pullulans* had a potential to produce both degrading enzymes, whereas yeast *C. utilis* had only secrete ß-1,3-glucanase into liquid media (Figure 19). These might indicate that hydrolytic enzymes which contained in culture filtrates had the potential to inhibit the growth of *C. musae*.

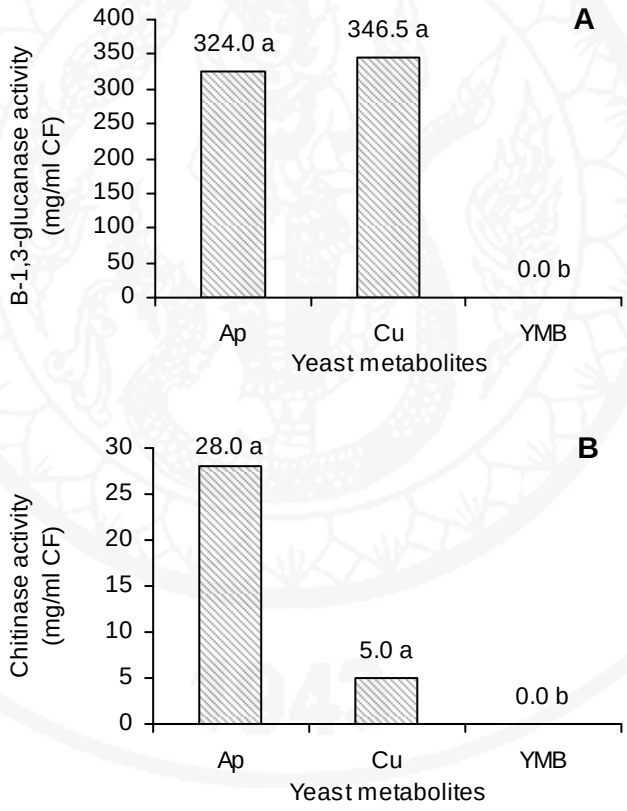


Figure 19 Cell wall degrading enzymes, ß-1,3-glucanase (A) and chitinase (B) produced by yeasts *Aureobasidium pullulans* TISTR 3389 (Ap) and *Candida utilis* (Cu) in 5-day-old liquid culture of yeast extract malt extract broth (YMB), incubated at room temperature (28-32˚C). Mean values in bars followed by a different letter indicated significant difference (P=0.05), according to Duncan's Multiple Range Test.

6. Study on crude extracts of yeast metabolites

6.1 Antifungal activity of crude extracts from yeast metabolites on paper discs

The application of each crude extract from yeast metabolites in various concentrations onto filter paper discs affected the germination of *C. musae* spores. The dilution of crude extract from *A. pullulans* metabolites which significantly inhibited spore germination was starting from 1:16, whereas the dilution of crude extract from *C. utilis* metabolites was starting from 1:32 (Table 15). This indicated that the minimum concentration of crude extracts from *A. pullulans* and *C. utilis* metabolites that could inhibit the growth of pathogen (MIC) was 6.31 and 5.73 mg/mL, respectively, calculated from crude extract stock solutions of *A. pullulans* and *C. utilis* metabolites at concentrations of 100.92 and 183.36 mg/mL, respectively (Table 16).

In addition, the value of inhibition concentration at 50 percent (IC_{50}) for spore germination inhibition of both crude extracts calculated from the regression equation was 4.73 mg/mL for extract of *A. pullulans* metabolites and 6.82 mg/mL for extract of *C. utilis* metabolites (Table 16).

Table 15 Spore germination of *Colletotrichum musae* on filter paper discs which applied with crude extract of metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* and incubated for 8 h at room temperature (28-32˚C).

Ratio of crude extract : MeOH	Germinated spores (%) [1]	
	Crude extract of *A. pullulans* metabolites	Crude extract of *C. utilis* metabolites
1 : 8	6.7 a	6.5 a
1 : 16	24.4 b	8.5 a
1 : 32	65.3 c	22.4 b
1 : 64	73.7 cd	60.4 c
1 : 128	74.9 cd	74.7 de
1 : 256	83.8 d	80.6 e
MeOH alone (Control)	72.7 cd	67.3 cd

[1] Mean values in each column followed by a different letter indicated significant different (P=0.05), according to Duncan's Multiple Range Test

Table 16 The minimum inhibitory concentration (MIC) and 50 percent inhibition concentration (IC_{50}) of crude extracts of metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* on spore germination of *Colletotrichum musae* on filter paper discs incubated for 8 h at room temperature (28-32˚C).

Crude extracts	MIC [1]	IC_{50}	Regression equation	R^2
A. pullulans metabolites	6.31 mg/ml	4.73 mg/ml	y = 6.2213x + 20.566	0.9195
C. utilis metabolites	5.73 mg/ml	6.82 mg/ml	y = 3.2173x + 28.058	0.8889

[1] MIC values were obtained from spore germination inhibition at lowest concentration which significantly differed from spore germination in control (P=0.05), according to Duncan's Multiple Range Test

6.2 Bioassay of antifungal compounds on the TLC plates

Crude extracts of metabolites from both yeasts were loaded on the TLC plates for separation of the antifungal compounds. After spraying with fungal spore suspension of *Cladosporium cladosporioides*, visibility of active antifungal compounds were indicated as the white areas where the tested fungi did not grow. Similar to the white areas on the plate sprayed with *C. musae* were also observed (Figure 20, 21). However, spraying the TLC plates with *Cladosporium cladosporioides* clearly showed the inhibition areas on the plates more than that with *C. musae* according to *Cladosporium cladosporioides* produce dark pigments in spores and mycelia. In all further studies, this fungus was used on TLC plate bioassay.

There were about two inhibition zones of *Cladosporium cladosporioides* growth on *A. pullulans* metabolite extract at R_f values of 0.68 and 0.47. Similarly, two zones of inhibition on *C. musae* was also determined with near the same R_f values (Figure 20). Inhibition of fungal growth at R_f 0.68 was very strong but there was only slight inhibition of fungal growth at R_f 0.47. In the case of *C. utilis* metabolite extract, there were around three zones of inhibition with R_f values of 0.63, 0.43 and 0.23 on TLC plates which sprayed with *Cladosporium cladosporioides* (Figure 21). These active compounds in crude extract also inhibited the growth of *C. musae*. Inhibition of fungal growth at R_f 0.43 was only very strong.

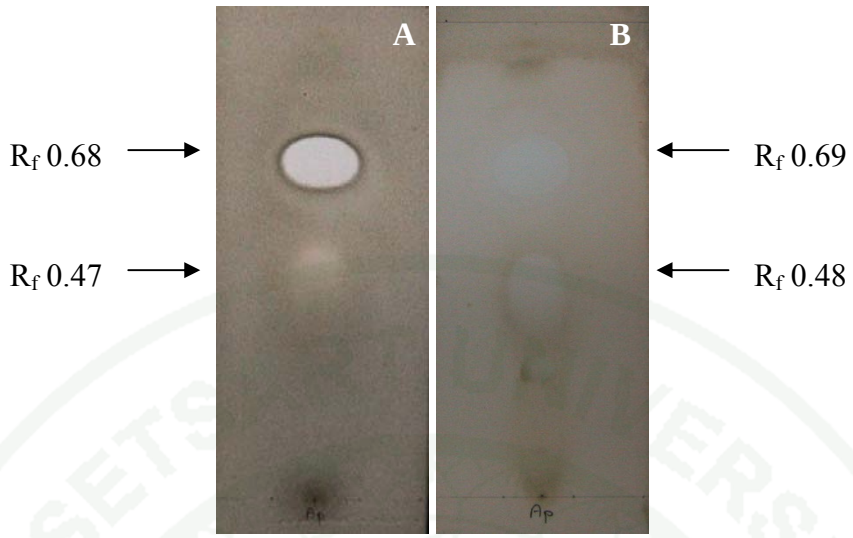


Figure 20 Antifungal activity observed by direct spraying of a spore suspension of *Cldaosporium cladosporioides* (A) and *Colletotrichum musae* (B) on crude extract of yeast *Aureobasidium pullulans* metabolites. Solvent system- dichloromethane : methanol 98 : 2.

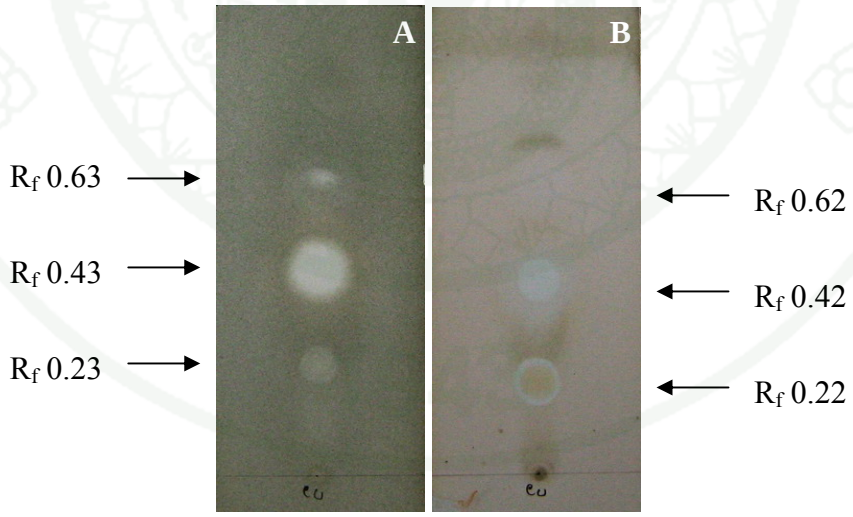


Figure 21 Antifungal activity observed by direct spraying of a spore suspension of *Cldaosporium cladosporioides* (A) and *Colletotrichum musae* (B) on crude extract of yeast *Candida utilis* metabolites. Solvent system- dichloromethane : methanol 98 : 2.

Heat stability of crude extracts from both yeast metabolites on the growth of *Cladosporium cladosporioides* was also investigated on the TLC plates. The results showed that crude extracts (100 µl) obtained from non-heated metabolites of *A. pullulans* showed the inhibition zones at R_f value of 0.69 with 1.5 cm diameter, whereas crude extract from heated metabolites (90˚C, 20 min) showed the inhibition zone at the same R_f value with 1.0 cm diameter (Figure 22A). Similar to crude extract from non-heated *C. utilis* metabolites also showed the inhibition zone at R_f value of 0.68 with 1.2 cm diameter, whereas crude extract from heated metabolites occurred the inhibition zone with 0.7 cm diameter (Figure 22B). Additionally, other positions of inhibition zones were obserbed small or faint areas on TLC plates when loaded with crude extracts from heated metabolites. The inhibition zone at R_f 0.36 was disappeared when crude extract of heated metabolites from *C. utilis* was applied.

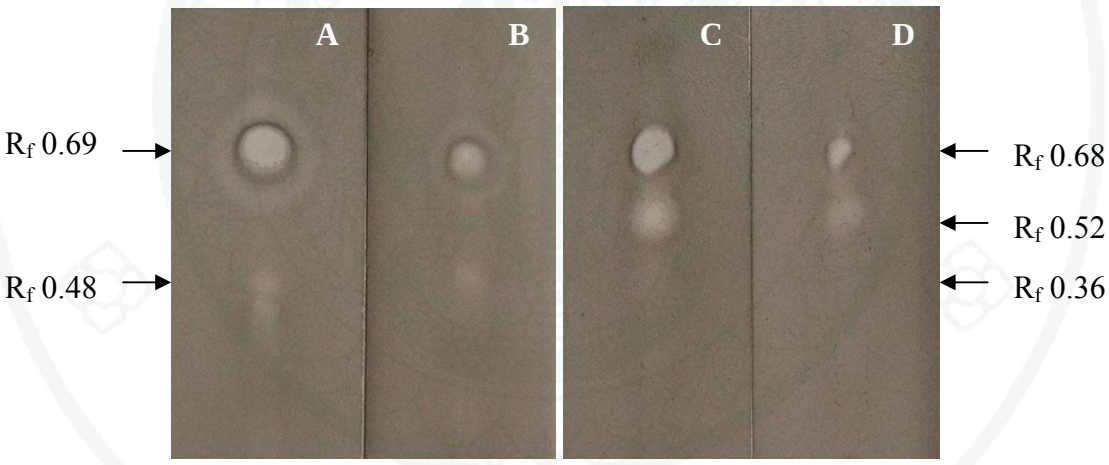


Figure 22 Heat stability testing crude extracts from yeast metabolites were determined on TLC plates after assayed with *Cldaosporium cladosporioides*. Inhibition zones on crude extract of non-heated metabolites (A,C) and heated metabolites (B,D) from yeast *Aureobasidium pullulans*, solvent system- dichloromethane : methanol 98 : 2 and from *Candida utilis*, solvent system- chloroform : ethyl acetate : methanol 70 : 25 : 5. Non-heated metabolites showing larger inhibition areas. Heated metabolites (90˚C, 20 min) showing narrow inhibition areas.

6.3 Separation of antifungal compounds by column chromatography (CC)

Fractionation of crude extracts from yeast metabolites by elution with organic solvent mixtures was investigated for separating the active antifungal compounds into groups of compounds. Twenty four fractions were collected and bioassayed on the TLC plates for screening active compounds against *Cladosporium cladosporioides*. The results showed that active compounds contained in extract of *A. pullulans* metabolites showed the inhibition zones in almost all fractions on TLC plates. Fractions eluted with three organic solvents including hexane : ethyl acetate (25 : 75), absolute ethyl acetate and ethyl acetate : methanol (95 : 5) clearly showed the inhibition zones at R_f values of 0.65, 0.37 and 0.11 with inhibition zone diameters around 2.7, 1.7 and 1.9 cm, respectively (Table 17, Figure 23). Highest concentration of active compounds occurred at the position of R_f 0.65.

In addition, all fractions from extract of *C. utilis* metabolites also showed the inhibition zones on TLC plates. The strong inhibition zones were shown in fractions eluted with four organic solvent mixtures including hexane : ethyl acetate (50 : 50), hexane : ethyl acetate (25 : 75), absolute ethyl acetate and ethyl acetate : methanol (95 : 5) at R_f values of 0.1, 0.35 and 0.13 with inhibition zone diameters about 1.7, 2.6 and 1.8 cm, respectively (Table 17, Figure 24). Inhibition zones at R_f 0.35 showed the biggest area.

The fractions which gave good results on the growth inhibition of *Cladosporium cladosporioides* were further tested on the growth of *C. musae*. The results revealed that active compounds which contained in those fractions also caused inhibition of *C. musae* growth at the same R_f values that had been assayed with *Cladosporium cladosporioides* (Table 18).

Table 17 Bioassay of fractions obtained from crude extracts of metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* eluted with organic solvent mixtures on the growth of *Cladosporium cladosporioides* on TLC plates.

Fraction No.	Inhibition zones of *A. pullulans* metabolite extracts		Inhibition zones of *C. utilis* metabolite extracts	
	R_f values	Band width	R_f values	Band width
I absolute hexane	NI	NI	0.72	++
II hexane:EtOAc 95:5	0.12	++	0.72	+++
	0.76	+++		
III hexane:EtOAc 90:10	0.76	+++	0.72	++
IV hexane:EtOAc 75:25	0.76	+++	0.72	++
V hexane:EtOAc 50:50	0.13	++	0.10	++++
	0.76	+++	0.62	++
			0.72	++
VI hexane:EtOAc 25:75	0.37	+++	0.35	+++++
	0.65	+++++	0.64	++
	0.76	+++	0.72	+
VII absolute EtOAc	0.11	++	0.13	++++
	0.37	++++	0.35	++++
	0.76	++	0.64	++
VIII EtOAc:MeOH 95:5	0.11	++++	0.13	++++
	0.76	+++	0.72	++
IX EtOAc:MeOH 90:10	0.11	++	0.72	+
	0.76	+++		
X EtOAc:MeOH 75:25	0.76	+++	0.72	+++
XI EtOAc:MeOH 50:50	0.76	++	0.72	++
XII absolute MeOH	0.76	++	0.72	+++

NI = No inhibition zones

EtOAc = ethyl acetate, MeOH = mathanol

Band width; + = 0.1-0.5 cm, ++ = 0.6-1.0 cm, +++ = 1.1-1.5 cm, ++++ = 1.6-2.0 cm, +++++ >2.0 cm.

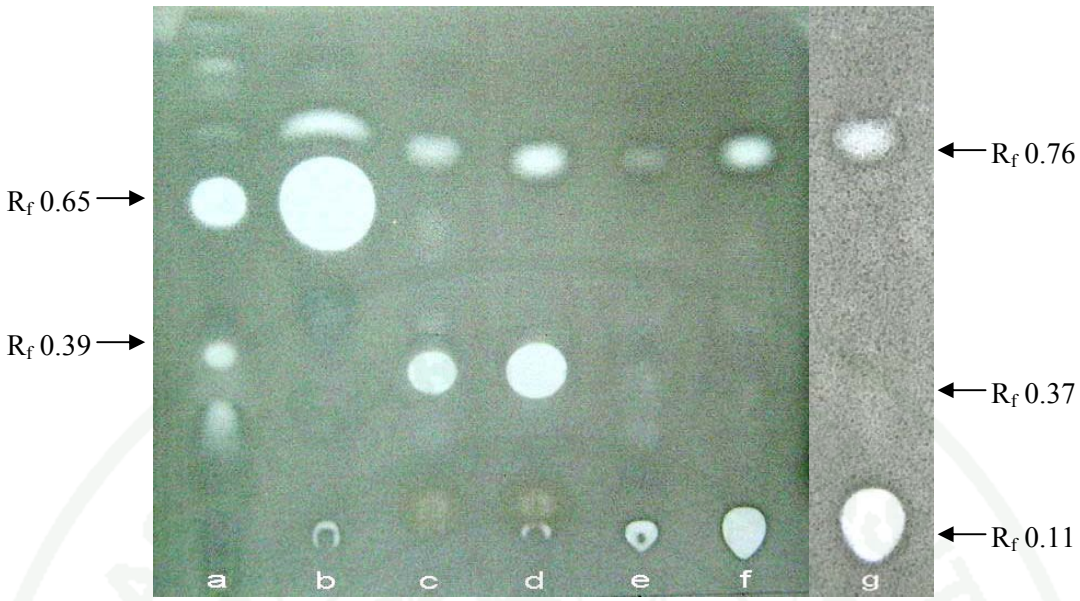


Figure 23 Antifungal activity observed by direct spraying of a spore suspension of *Cldaosporium cladosporioides* on each fraction of metabolite extract from yeast *Aureobasidium pullulans*. Solvent system- dichloromethane : methanol 98 : 2.

Lane a = crude extract
Lanes b-c = fraction eluted with hexane : ethyl acetate 25 : 75
Lanes d-e = fraction eluted with absolute ethyl acetate
Lanes f-g = fraction eluted with ethyl acetate : methanol 95 : 5

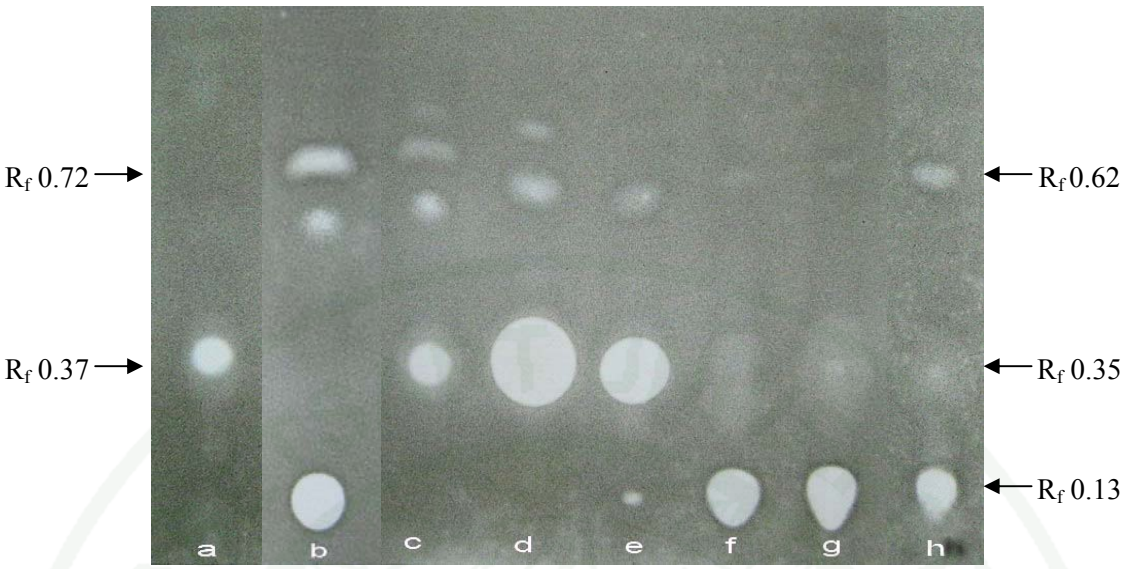


Figure 24 Antifungal activity observed by direct spraying of a spore suspension of *Cldaosporium cladosporioides* on each fraction of metabolite extract from yeast *Candida utilis*. Solvent system- dichloromethane : methanol 98 : 2.

Lane a = crude extract
Lane b = fraction eluted with hexane : ethyl acetate 50 : 50
Lanes c-d = fraction eluted with hexane : ethyl acetate 25 : 75
Lanes e-f = fraction eluted with absolute ethyl acetate
Lanes g-h = fraction eluted with ethyl acetate : methanol 95 : 5

Table 18 Bioassay of fractions obtained from crude extracts of metabolites from yeasts *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* eluted with organic solvent mixtures on the growth of *Colletotrichum musae* on TLC plates.

Fraction No.	R_f values of *A. pullulans* metabolite extracts		R_f values of *C. utilis* metabolite extracts	
	Inhibition zones against *Cladosporium*	Inhibition zones against *C. musae*	Inhibition zones against *Cladosporium*	Inhibition zones against *C. musae*
V hexane:EtOAc 50:50	ND	ND	0.10	✓
			0.62	✓
			0.72	✓
VI hexane:EtOAc 25:75	0.37	✓	0.35	✓
	0.65	✓	0.64	✓
	0.76	✓	0.72	✓
VII absolute EtOAc	0.11	✓	0.13	✓
	0.37	✓	0.35	✓
	0.76	✓		
VIII EtOAc:MeOH 95:5	0.11	✓	0.13	✓
	0.76	✓	0.72	✓

ND = Not determined

✓ = Appearance of inhibition zones

EtOAc = ethyl acetate, MeOH = methanol

6.4 Identification of antifungal compounds

6.4.1 Analysis by thin layer chromatography (TLC)

Developed TLC plates were examined the active compounds which contained in each fraction by expose to UV light at 365 nm. The results showed that all active compounds which represented at all the position of inhibition zones were not visible under UV light.

Spraying reagents including Dragendorff and Anisaldehyde had been used to determine the groups of active compounds. TLC plates sprayed with Dragendorff reagent showed orange colour at the position of inhibition zones at R_f 0.37 and 0.35 which obtained from hexane-ethyl acetate fractions of crude extracts of *A. pullulans* and *C. utilis* metabolites, respectively (Figure 25B, 26B). This colour indicated the compounds belonged to alkaloids. Whereas TLC plates sprayed with Anisaldehyde reagent clearly showed violet colour at the position of the same inhibitory zones in fractions of crude metabolite extracts from both yeasts (Figure 25C, 26C). This violet colour indicated the compounds belonged to terpenoid groups.

Antifungal compounds which showed at the positions of orange and violet colour were further analyzed by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) for identifying the type of compounds.

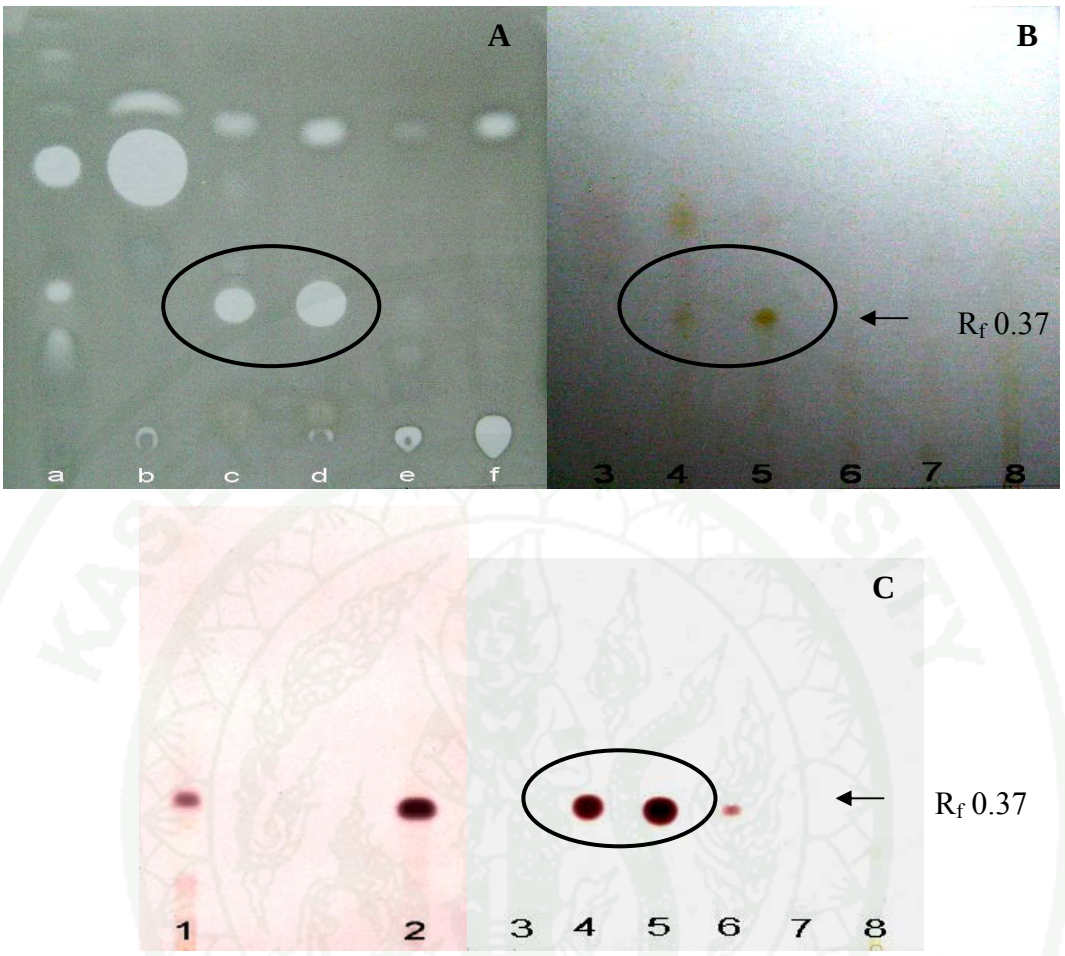


Figure 25 TLC plates showing clear zones where growth of *Cladosporium cladosporioides* were inhibited by active compounds contained in each fraction of metabolite extract from yeast *Aureobasidium pullulans* (A), compared with TLC plates spraying with Dragendorff reagent showed active compounds in orange colour (B), Anisaldehyde reagent showed active compounds in violet colour (C). Lane 1 = crude extract (a), lane 2 = fraction mixture of c and d, lanes 3-4 = fraction eluted with hexane : ethyl acetate 25 : 75 (b,c), lanes 5-6 = fraction eluted with absolute ethyl acetate (d,e), lanes 7-8 = fraction eluted with ethyl acetate : methanol 95 : 5 (f). The position of active antifungal compounds at R_f values of 0.37 in lanes 4-5 and c-d are circled and used for further analysis. Solvent system- dichloromethane : methanol 98 : 2.

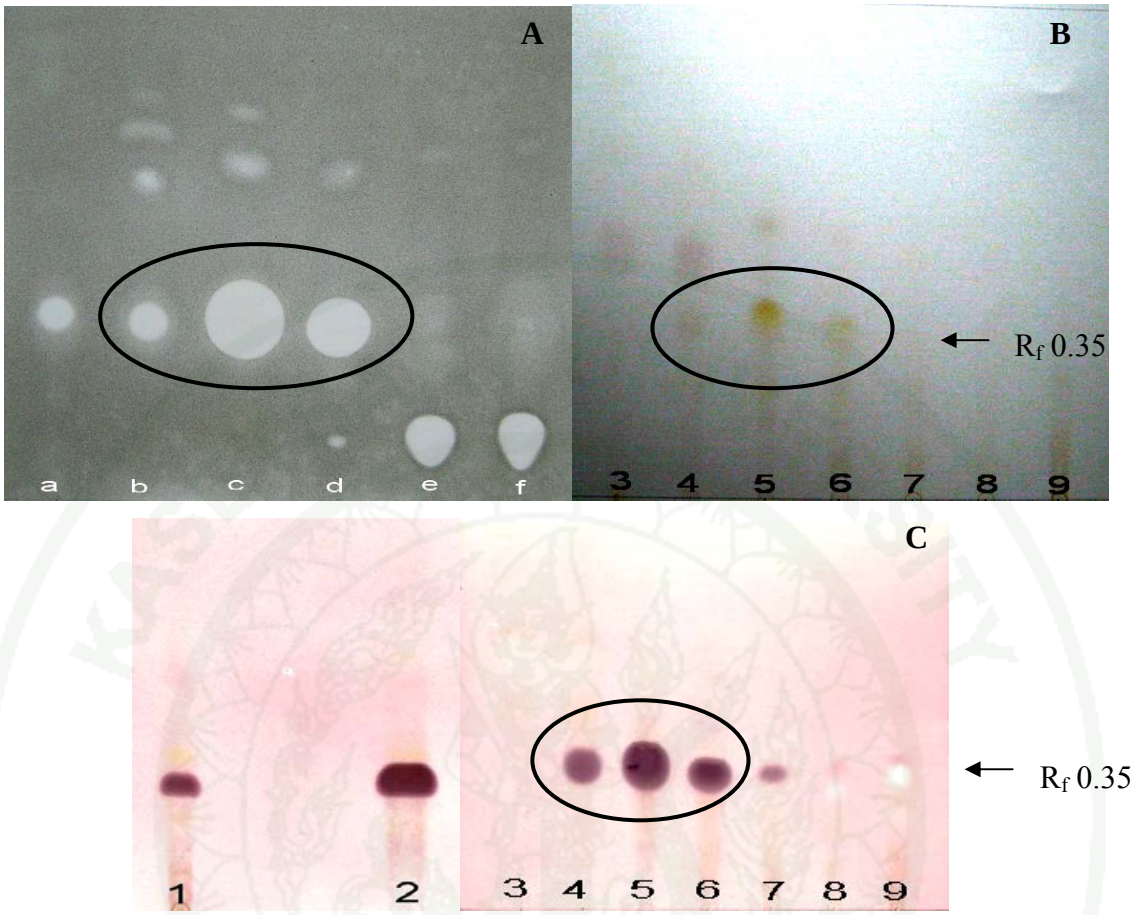


Figure 26 TLC plates showing clear zones where growth of *Cladosporium cladosporioides* were inhibited by active compounds contained in each fraction of metabolite extract from yeast *Candida utilis* (A), compared with TLC plates spraying with Dragendorff reagent showed active compounds in orange colour (B), Anisaldehyde reagent showed active compounds in violet colour (C). Lane 1 = crude extract (a), lane 2 = fraction mixtures of b, c and d, lane 3 = fraction eluted with hexane : ethyl acetate 50 : 50, lanes 4-5 = fraction eluted with hexane : ethyl acetate 25 : 75 (b,c), lanes 6-7 = fraction eluted with absolute ethyl acetate (d,e), lanes 8-9 = fraction eluted with ethyl acetate : methanol 95 : 5 (f). The position of active antifungal compounds at R_f values of 0.35 in lanes 4-6 and b-d are circled and used for further analysis. Solvent system- dichloromethane : methanol 98 : 2.

6.4.2 Analysis by high performance liquid chromatography (HPLC)

The HPLC chromatograms of compounds isolated from *A. pullulans* and *C. utilis* with R_f value of 0.37 (fraction of *A. pullulans*, 2.8 mg) and 0.35 (fraction of *C. utilis*, 11.6 mg) showed one main peak at 14.580 min and 14.569 min, respectively (Figure 27, 28). The UV-spectra of both compounds are identical with maxima at 220 and 280 nm, respectively (Figure 29).

Co-chromatographic comparison from fractions of *A. pullulans* and *C. utilis* metabolites indicated both compounds were identical. UV-spectra of *A. pullulans* and *C. utilis* were not fitted properly with any UV-spectra in database. Therefore, fraction of *C. utilis* was further subjected to NMR analysis for structure elucidation of this bioactive compound.

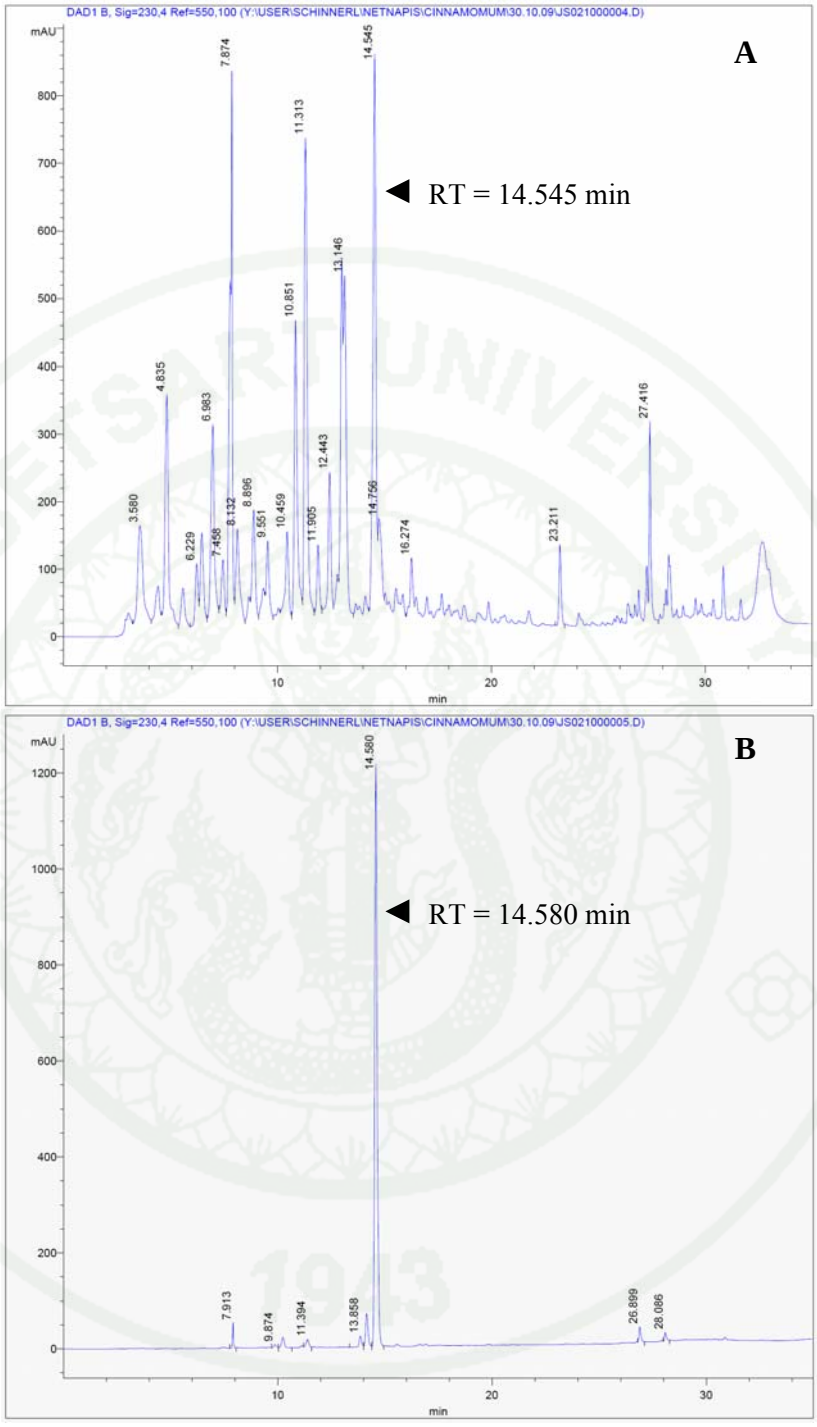


Figure 27 HPLC profiles from crude extract (A) and from the inhibition zone of hexane-ethyl acetate fraction (B) of *Aureobasidium pullulans* metabolite extract.

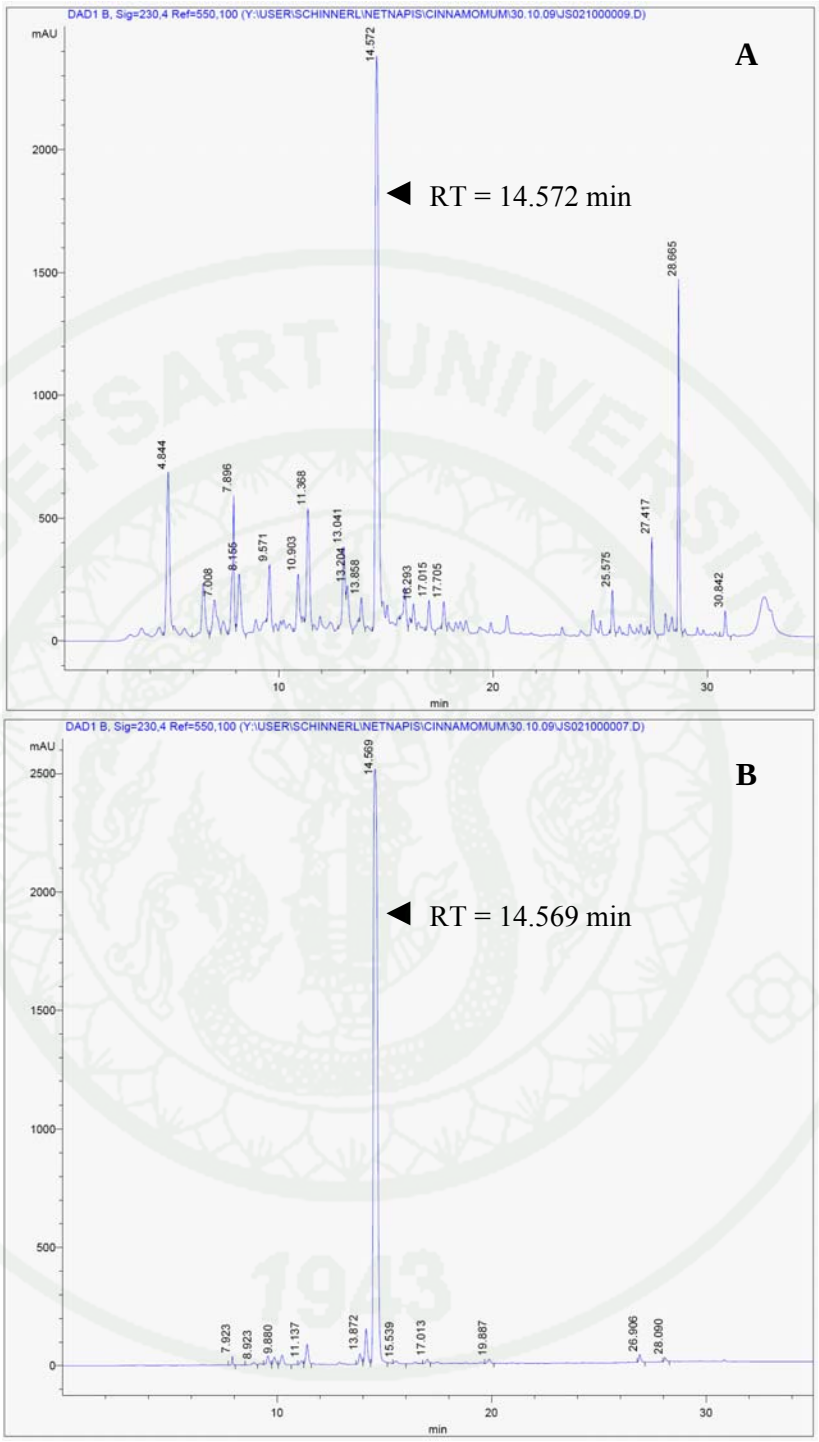


Figure 28 HPLC profiles from crude extract (A) and from the inhibition zone of hexane-ethyl acetate fraction (B) of *Candida utilis* metabolite extract.

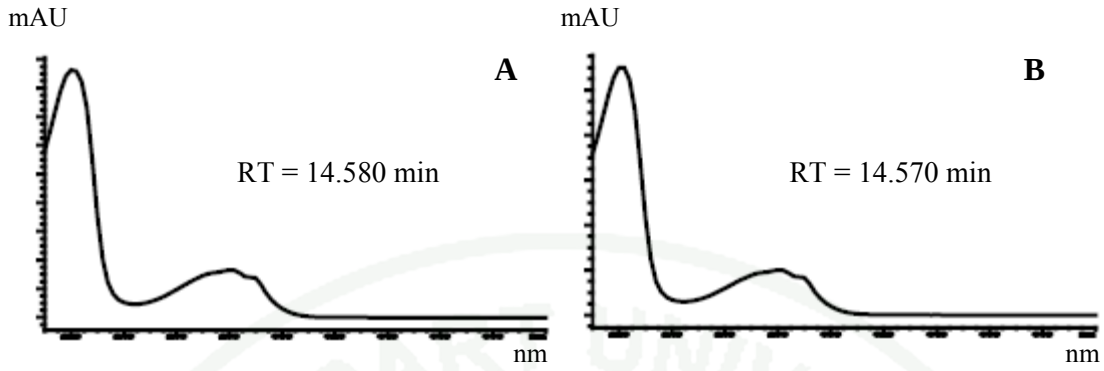


Figure 29 UV-spectra from HPLC profiles indicated the retention time of active compounds obtained from hexane-ethyl acetate fraction of *Aureobasidium pullulans* metabolite extract (A) and *Candida utilis* metabolite extract (B).

6.4.3 Analysis by nuclear magnetic resonance (NMR) spectroscopy

Antifungal compounds which contained in the position of inhibition zones on TLC plates at R_f 0.37 and 0.35 obtained from hexane-ethyl acetate fraction of *A. pullulans* and *C. utilis* metabolite extracts, respectively, were analyzed by NMR. The results showed that the active compounds from both yeast metabolites were the same antifungal compound. The NMR spectra of some active compounds which contained in yeast metabolite extracts completely fitted with the known spectrum of structure units of 3-(2-hydroxyethyl)-indole in database which known in the common name as tryptophol. The other four synonyms of this compound are beta-3-Indolylethanol, 2-(1H-Indol-3-yl)ethan-1-ol, 3-Indoleethanol, and 2-(3-indole) ethanol (Guzman-Lopez *et al.,* 2007; http://www.tryptophol, beta-3-Indolylethanol, 3-(2-Hydroxyethyl)indole, 2-(1H-Indol-3-yl)ethan-1-ol, 526-55-6.htm, 2009). This compound belonged to the alkaloid type of indole which based on the structure of *N*-heterocycle. The molecular structure of this antifungal compound was presented as shown in Figure 30.

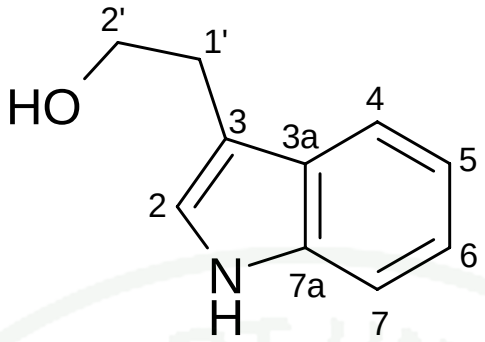


Figure 30 Molecular structure of 3-(2'-Hydroxy-ethyl)-indole

^{1}H NMR spectrum assigns each signal to particular atom of a 3-(2'-Hydroxy-ethyl)-indole molecule. N-heterocycle of indole consisted C-8 with resembled five proton signals at δ 7.08 (m, 1H, CH-2), 7.11 (m, 1H, CH-5), 7.19 (m, 1H CH-6), 7.36 (m, 1H, CH-4) and 7.61 (m, 1H, CH-7). Additionally, ^{1}H NMR signals at δ 1.54 (s, br, 1H, OH), 3.03 (dt, J = 6.4 Hz (t), J = 1.0 Hz (d), 2H, CH_2-1') and 3.90 (t, J = 6.4 Hz, 2H, CH_2-2') indicated ethyl group which was the side chain of molecule (Figure 31, 32).

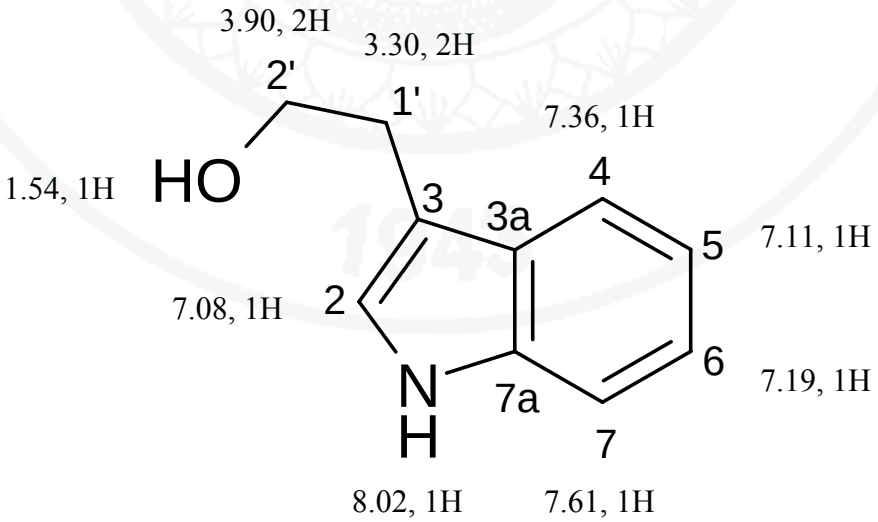


Figure 31 ^{1}H NMR spectrum signals of proton on 3-(2'-Hydroxy-ethyl)-indole

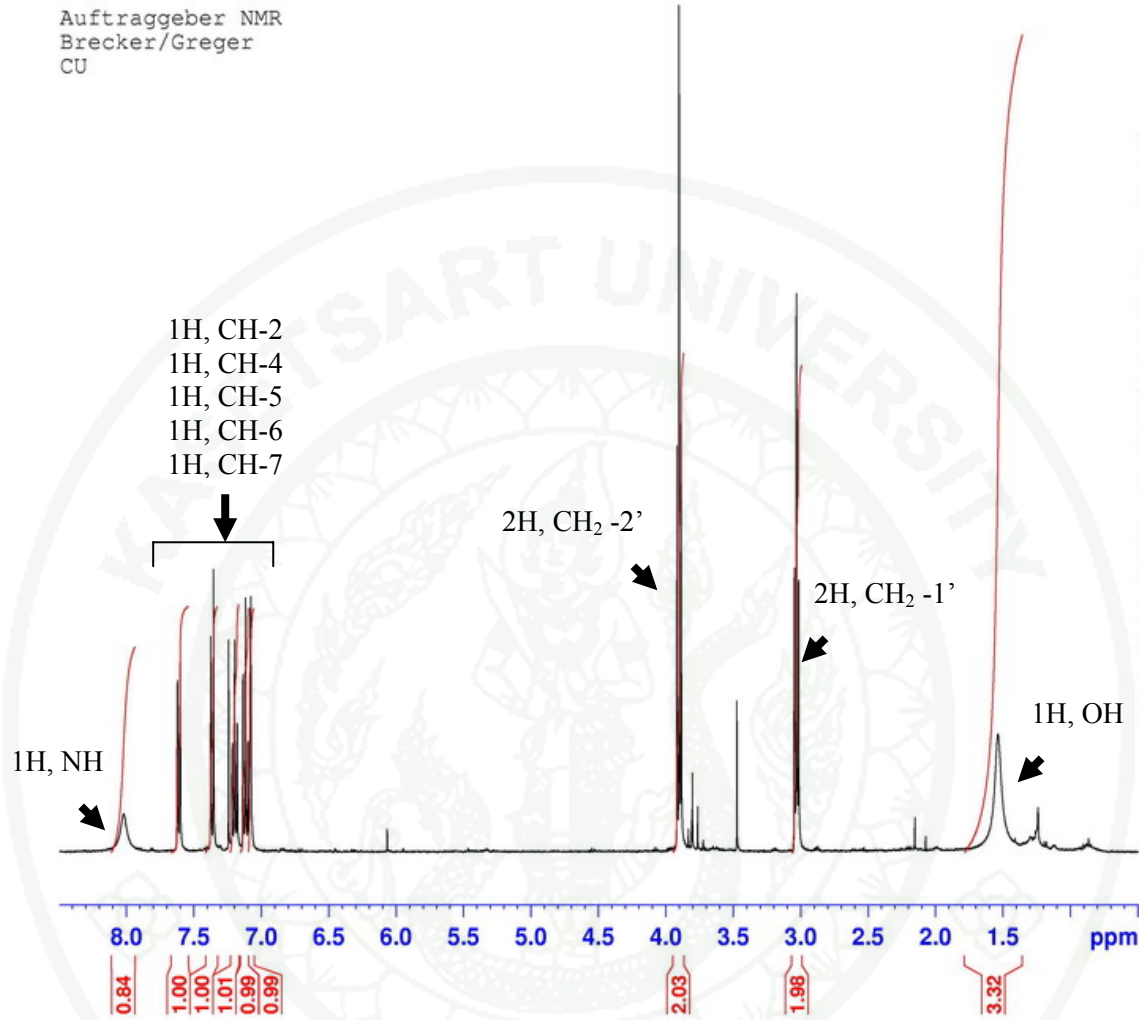


Figure 32 ^{1}H NMR spectrum of 3-(2'-Hydroxy-ethyl)-indole ($CDCl_3$, 400 MH_Z)

7. Enzyme activity assays

7.1 Induction of enzyme activity by salicylic acid and metabolites from yeasts in banana fruit peel during short storage time

Salicylic acid solution at the concentration of 0.5 mM was normally used as inducer for the induction of defensive enzyme activities in banana fruit peel tissues. The results showed that exogenous salicylic acid affected on the accumulation of PAL, ß-1,3-glucanase and chitinase within 24 h comparing with untreated fruits. Salicylic acid stimulated PAL activity during the early storage time. PAL activity exhibited a peak at 18 h after salicylic acid spraying and tended to decrease until 24 h (Figure 33A). Salicylic acid also activated ß-1,3-glucanase activity starting from 6 h after spraying to 24 h. The trend of this enzyme had also shown increasing activity during storage period (Figure 33B). Similarly, chitinase enzyme had shown an increasing activity starting from 12 to 24 h and tended to increase after 24 h. This enzyme was activated by salicylic acid slower than PAL and ß-1,3-glucanase (Figure 33C).

The treatment of metabolites from yeasts *A. pullulans* and *C. utilis* on banana fruits was also investigated the induction of defensive enzymes within 24 h. These yeast metabolites had the potential to stimulate the activity of PAL, ß-1,3-glucanase and chitinase in banana fruit peels as observed on salicylic acid treated fruits. Metabolites from *C. utilis* was the best inducer on stimulation of PAL activity (Figure 34A), whereas metabolites from *A. pullulans* had better on enhancement of ß-1,3-glucanase and chitinase than *C. utilis* (Figure 34B,C) comparing with no metabolite (YMB) treated and untreated fruits.

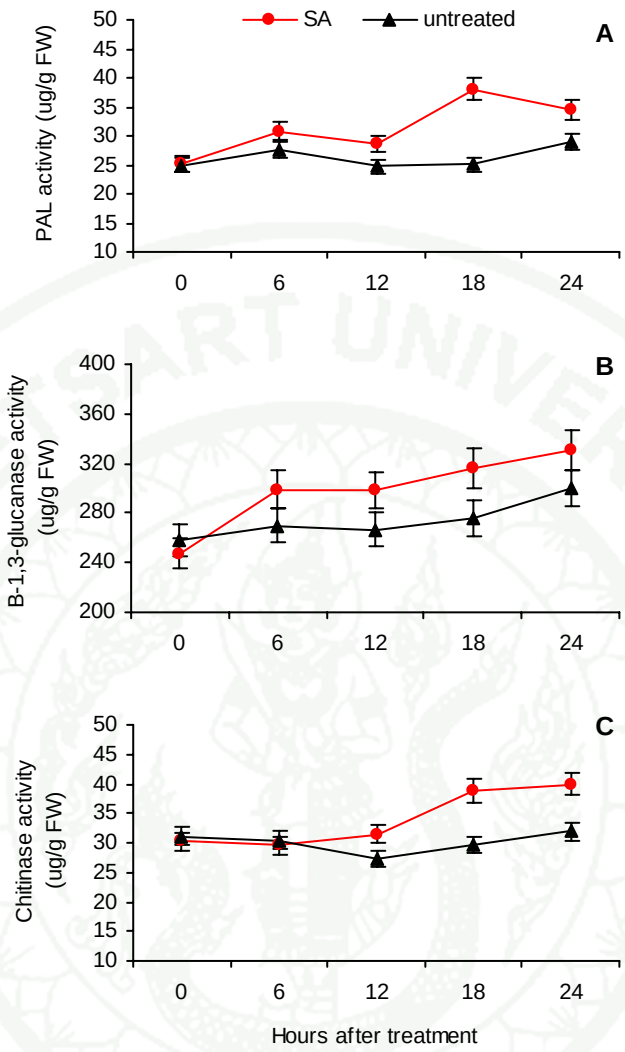


Figure 33 Time cause changes of phenylalanine ammonia-lyase (PAL) (A), ß -1,3-glucanase (B) and chitinase (C) in banana fruit peel at 0, 6, 12, 18 and 24 h after treatment with 0.5 mM salicylic acid solution (SA), compared with untreated fruit. Banana fruits were incubated at 23˚C. Vertical bars indicate 5% error of means.

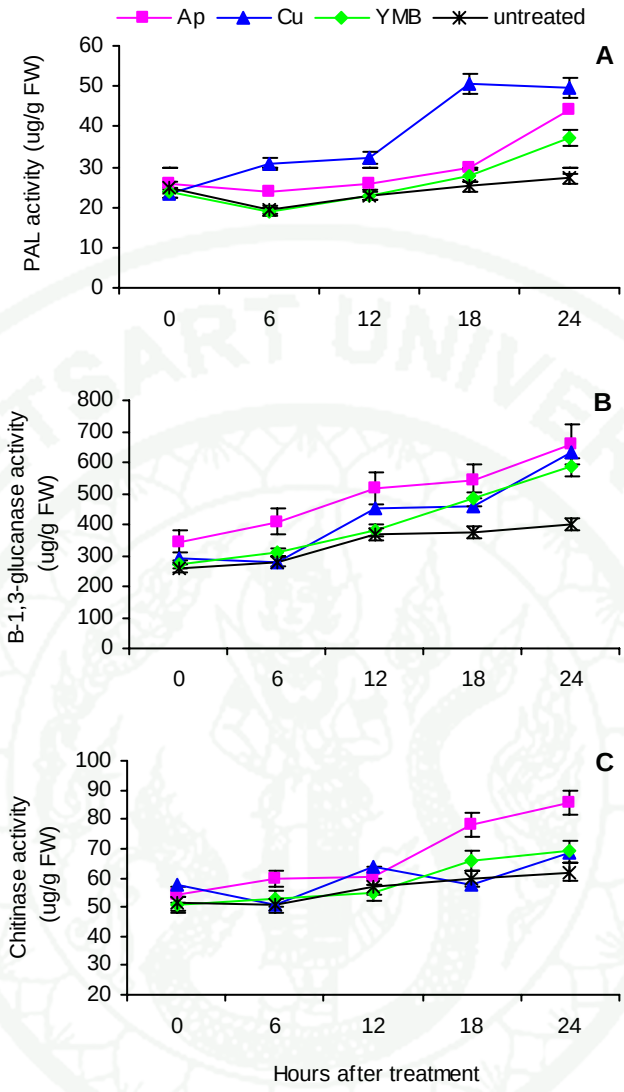


Figure 34 Time cause changes of phenylalanine ammonia-lyase (PAL) (A), ß -1,3-glucanase (B) and chitinase (C) in banana fruit peel at 0, 6, 12, 18 and 24 h after treatment with metabolites from yeast *Aureobasidium pullulans* (Ap) and *Candida utilis* (Cu), compared with non metabolite (YMB) treated and untreated fruit. Banana fruits were incubated at 23˚C. Vertical bars indicate 5% error of means.

7.2 Induction of enzyme activity by salicylic acid and metabolites from yeasts in banana fruit peel during long storage time

7.2.1 Determination of PAL activity

Without pathogen inoculation of banana fruit, PAL activity of peel tissues tended to increase during storage at 23°C in all treatments (Figure 35A). The increase in PAL activity was greater in fruit treated with 0.5 mM salicylic acid solution or metabolites from both yeasts than in fruit from both controls (YMB medium treated and untreated fruit). The activity of PAL on *A. pullulans* metabolite treated fruits rapidly increased over 24 h, but the fruit treated with *C. utilis* metabolites, the activity of PAL was stimulated during the initial storage time and decreased at 24 h. The peak of enzyme activity in these yeast metabolite treated banana fruit peels was at 72 h. It was higher than in peel of fruit from the salicylic acid application. Moreover, PAL activity on both yeast metabolite treatments was significantly higher than non metabolite treated fruit at storage times between 48 and 96 h.

7.2.2 Determination of ß-1,3-glucanase activity

ß-1,3-glucanase activity in all treatments also increased during storage for 96 h (Figure 35B). Banana fruit treated with salicylic acid solution and both yeast metabolites clearly enhanced their activity starting from 48 h until the end of storage at 96 h, and enzyme activity increased to the highest peak at 96 h. *C. utilis* metabolite treated fruit showed the greatest ß-1,3-glucanase activity enhancement. Enzyme activity increased two fold in *C. utilis* metabolite treated fruit at 96 h compared with no metabolite treated fruit.

7.2.3 Determination of chitinase activity

Chitinase activity in all treatments did not significantly increase until 48 h after storage. At 72 h, fruit treated with the metabolites from either yeast

showed elevated activity of this enzyme, but the activity was the greatest in fruit treated with metabolites from *C. utilis,* and still remained elevated at 96 h (Figure 35C). At 96 h after treatement, *A. pullulans* and *C. utilis* metabolites had no significant effect on the stimulation of chitinase activity.

In the case of yeast metabolite treatment prior to challenge with pathogen inoculation on the fruit, the activities of PAL, ß-1,3-glucanase and chitinase were also measured. Changes of these enzyme activities in banana fruit peel were observed at 48 h after treatment with the metabolites from either yeast *A. pullulans* or *C. utilis* or 0.5 mM salicylic acid solution and following *C. musae* inoculation ($1x10^5$ spores/mm^2). The results showed that PAL activity of the fruits treated with metabolites of the yeast *C. utilis* or with salicylic acid was significantly higher than non metabolite treated and untreated fruits. In contrast, metabolites from yeast *A. pullulans* had no effect on PAL activity in peel tissues (Figure 36A).

Assessment of ß-1,3-glucanase activity in the fruit peel showed the greatest separation of treatment effects. The fruit treated with *C. utilis* metabolites resulted in the highest ß-1,3-glucanase activity in the peel from 48 h, but the activity at 96 h was not significantly different from treatment with salicylic acid. Similarly, salicylic acid application before pathogen inoculation activated ß-1,3-glucanase activity in peel tissues particularly at 72 and 96 h. Boosting of ß-1,3-glucanase activity was also evident following treatment with *A. pullulans*, but the increase was lower than the effects of *C. utilis* or salicylic acid (Figure 36B).

In contrast, the application of metabolites from either yeast or salicylic acid solution, or YMB medium treatment of the banana fruit before inoculation with the pathogen did not enhance the activity of chitinase compared with untreated fruit (Figure 36C).

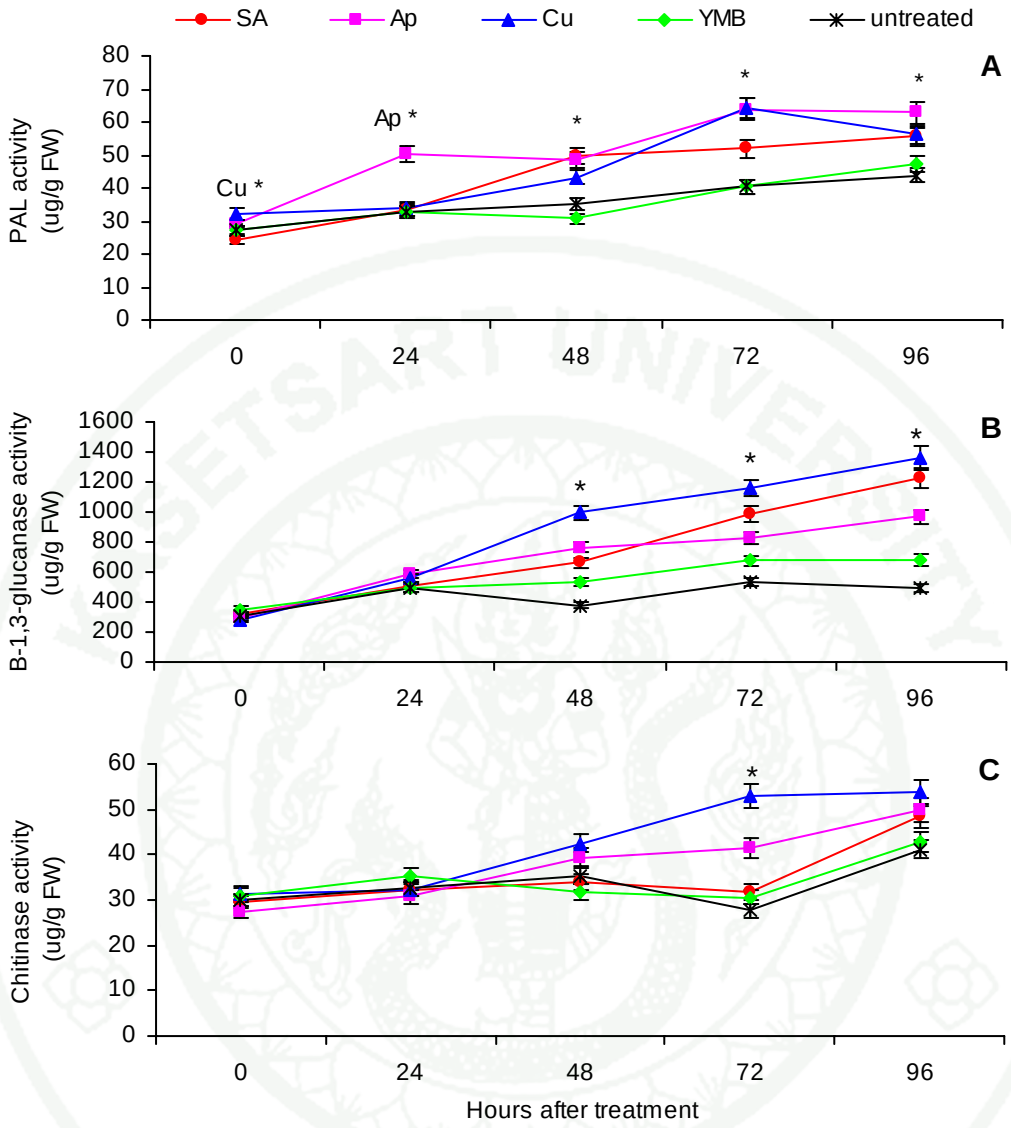


Figure 35 The activities of phenylalanine ammonia-lyase (PAL) (A), ß -1,3-glucanase (B) and chitinase (C) in banana fruit peel at 0, 24, 48, 72 and 96 h after treatment with metabolites from yeast *Aureobasidium pullulans* TISTR 3389 (Ap), *Candida utilis* (Cu) or with 0.5 mM salicylic acid solution (SA), compared with non metabolite (YMB) treated and untreated fruit. Asterisks at any time interval indicate Ap and Cu were significantly different from non metabolite treated and untreated fruit, while Ap* or Cu* indicate only Ap or Cu was significantly different from non metabolite treated and untreated fruit (P=0.05), according to Duncan's Multiple Range Test.

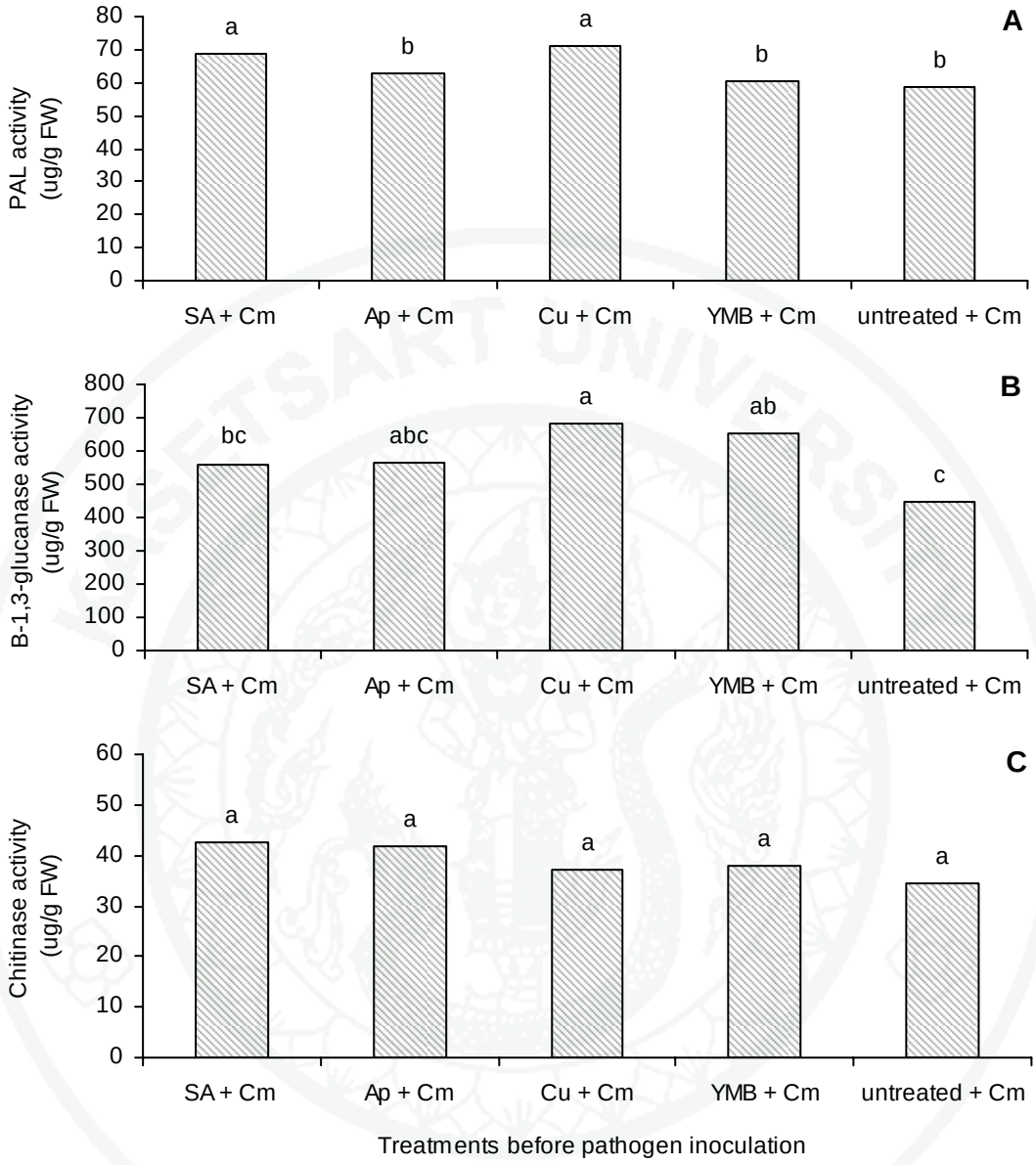


Figure 36 The activities of phenylalanine ammonia-lyase (PAL) (A), ß -1,3-glucanase (B) and chitinase (C) in banana fruit peel after treatment with metabolites from yeast *Aureobasidium pullulans* TISTR 3389 (Ap), *Candida utilis* (Cu) or with 0.5 mM salicylic acid solution (SA), stored for 24 h prior to inoculation with *Colletotrichum musae* (Cm), compared with non metabolite (YMB) treated and untreated fruit. Banana fruit peels were collected for enzyme analysis at 48 h. Different letters above mean values in bars indicate significant differences (P=0.05), according to Duncan's Multiple Range Test.

8. RNA expression on the banana fruit peels

Semi-quantitative expression analysis using RT-PCR approach was studied on RNA expression of ß-1,3-glucanase, chitinase and PAL genes in banana fruit peel. The samples from the peel of *C. utilis* metabolites treated fruit were used in this experiment for RNA extraction at 0, 24, 48 and 96 h after treatments comparing with the fruit treated with 0.5 mM salicylic acid solution and both controls of YMB treated and untreated fruits.

In the expression analysis by semi-quantitative RT-PCR, at the 25th cycle, the amplified bands were faint but at the 30th, 35th and 40th cycle, the bands were clearly visible but differential expression was not observed.

Expression of PAL was observed from 0 until 96 h after treatment in salicylic acid treated and yeast metabolite treated fruits but no expression was observed under YMB treated and untreated fruits. These bands had sizes of 400 bps. The expression level was slightly stronger in salicylic acid treatment than in *C. utilis* metabolite treatment but showed no differential expression (Figure 37).

From expression analysis of ß-1,3-glucanase gene, it was expressed in all four treatments. The intensity of the amplified bands was highest under salicylic acid treatment follow by *C. utilis* metabolite treatment, YMB treatment and untreated treatment respectively. These fragments had sizes of 200 bps. The expression of ß-1,3-glucanase was not observed at 0 and 96 h after treatment of YMB. This result suggested that the expression of ß-1,3-glucanase was induced during the 24 h after the treatment and stable until at least 72 h after treatment and then, disappeared during the 72-96 h after treatment (Figure 37). Whereas the expression level of chitinase which had sizes of bands at 530 bps was not observed in all four treatments (Figure 37).

The expression of actin gene (390 bps) was used as an internal control to confirm the equal amount of RNA use in each RT-PCR reaction (Figure 37).

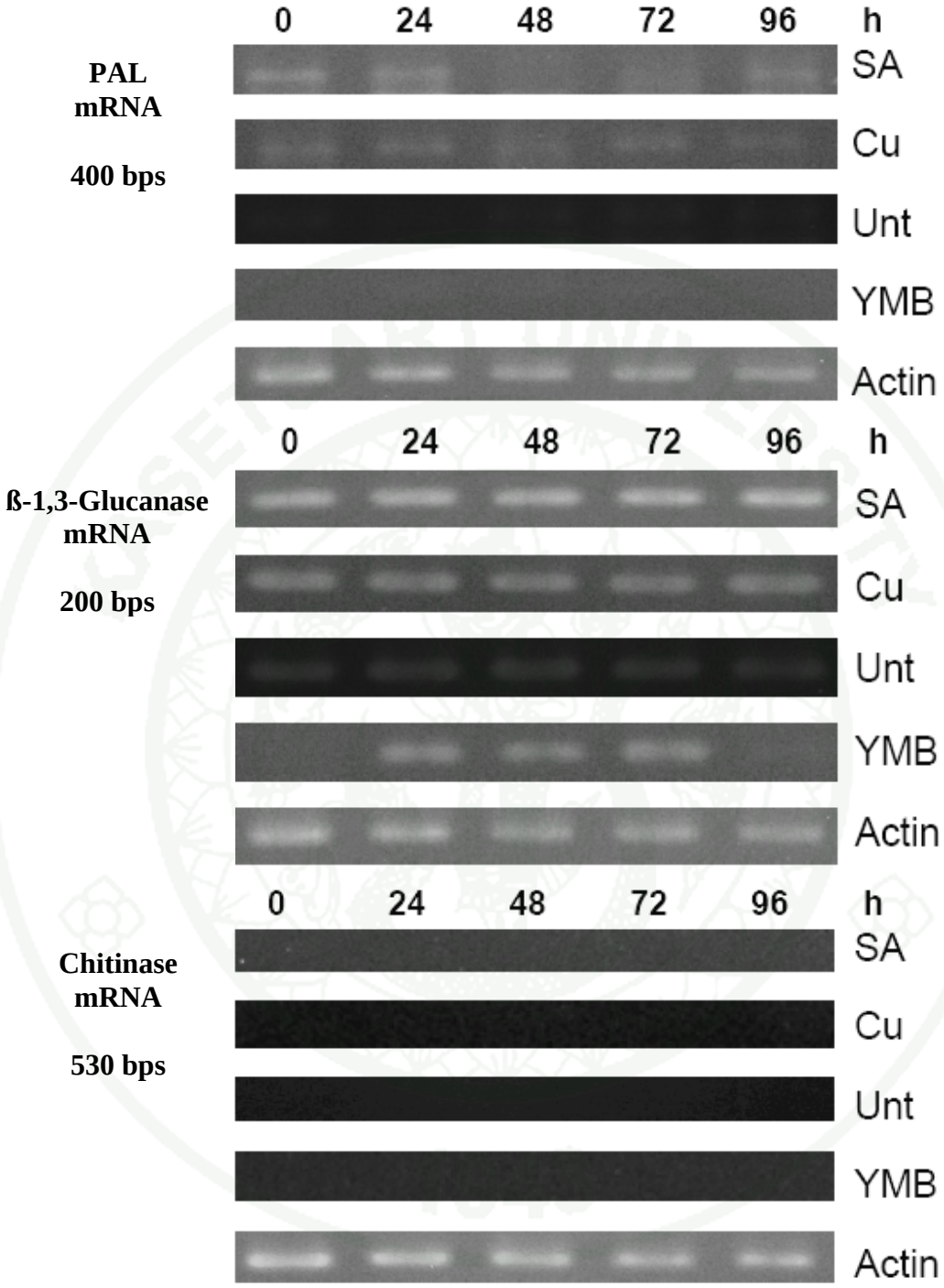


Figure 37 RT-PCR analysis of phenylalanine ammonia-lyase (PAL), ß-1,3-glucanase and chitinase gene expression in banana fruit peel at 0, 24, 48, 72 and 96 h after treatments with 0.5 mM salicylic acid (SA), *Candida utilis* metabolites (Cu) compared with the fruit treated with yeast extract malt extract broth (YMB) and untreated fruit (Unt) were used as controls. PCR cycle number at 40 were applied. Actin gene was used as an internal control.

DISCUSSION

In this study, two strains of antagonistic yeasts, *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* had the good potential to inhibit the growth of *C. musae* on PDA dual culture and their cell suspension also gave the good results in controlling anthracnose disease development on banana fruits. These indicated that both yeasts had more efficacy to compete for the space and nutrients than other tested yeasts. The components which contained in PDA especially for glucose as carbon source, resulting in a good competition efficiency of yeast *A. pullulans* (Prashanthi and Kulkarani, 2005). Moreover, metabolites from these yeasts also showed the highest potential to control anthracnose disease on bananas. These results indicated that antagonistic yeasts had more than one mechanisms against plant pathogen, and exploiting all modes of action will increase the efficacy of the biological control agents (Alvindia and Natsuaki, 2008). Competition for nutrients and space is the basis of their biological control activity as presented in our previous study and other reports (Ippolito *et al.,* 2000; Castoria *et al.,* 2001; Bencheqroun *et al.,* 2007; Tongsri and Sangchote, 2008).

Other modes of action, yeast *A. pullulans* and *C. uitils* had a potential to secrete some enzymes, especially hydrolytic enzymes including ß-1,3-glucanase and chitinase into liquid media. It was proven that glucose which contained in the media induced the highest amount of glucanase secretion in yeasts (Saligkarias *et al.,* 2002b). It is possible that these enzymes play an important role in degradation of pathogen cell wall as previously shown in other reports (Mathivanan *et al.,* 1998; Zhang and Yuen, 2000; Saligkarias *et al.,* 2002a, 2002b; Chanchaichaovivat *et al.,* 2008). These enzymes might affect membrane permeability of pathogens, thereby inhibiting their growth (Punja *et al.,* 2003). Chitinolytic enzymes from bacteria also caused swelling and vacuolation of *Bipolaris sorokiniana* conidia, discoloration, malformation and degradation of germ tubes (Zhang and Yuen, 2000). Chitinase also produced by the strains of *Trichoderma* species exhibited increased antagonist activity against *Rhizoctonia solani* (Prabavathy *et al.,* 2006).

Antagonistic yeasts also had efficacy to secrete some antifungal substances into liquid medium and environment (Castoria *et al.,* 2001; Wang *et al.,* 2009). This hypothesis was confirmed by the appearance of extracellular hydrolytic enzymes, ß-1,3-glucanase and chitinase in culture filtrates of yeasts as well as the occurrence of clear inhibition zones of these metabolites to *C. musae* and *Cladosporium cladosporioides* on TLC plates. Furthermore, these substances which contained in culture filtrates also had a direct effect on the *in vitro* mycelial growth, spore germination and germ tube elongation of *C. musae*. This phenomenon has been presented in other reports that cell wall degradation enzymes secretion by various antagonists against a number of pathogens (Guetsky *et al.,* 2002; Alvindia and Natsuaki, 2008; Leelasuphakul *et al.,* 2008). Additionally, the report by Yan *et al.* (2008) have found that crude chitinase obtained from recombinant rice chitinase significantly inhibited the spread of gray mold in loquat fruit caused by *Botrytis cinerea.*

It is possible that the production of degrading enzymes and antifungal substances in liquid medium may cause synergistic effect of metabolites on the suppression of pathogen growth as reported by Leelasuphakul *et al.* (2006). However, antifungal properties in culture filtrate of yeast *A. pullulans* and *C. utilis* can be decreased by heat, according to the results of heated metabolites at 90°C for 20 min showed higher disease severity on banana fruits compared with the fruit treated with non-heated metabolites from both yeasts. Furthermore, heated metabolites still decreased anthracnose disease on banana fruits compared with no metabolites treated fruits. This indicated that some antifungal substances in culture filtrates, especially defensive enzymes like ß-1,3-glucanase and chitinase, which are protein molecules, may be denatured or destroyed at high temperature condition (Leelasuphakul *et al.,* 2006; Zhang *et al.,* 2006). In contrast, the heat stable components were due to antibiotics (Leelasuphakul *et al.,* 2006).

Volatile compounds, other secondary metabolites, produced by both yeasts that had the potential to suppress the pathogen growth in this study (Tongsri *et al.,* 2010) might be corresponded to many compounds such as alcohols, acids, acetates or

ethyl esters as shown in the report by Molina *et al.* (2009). Those volatile compounds which generated from the strains of yeasts could be divided into groups according to their probable origins as amino acid catabolism, lipid oxidation, microbial esterification and carbohydrate fermentation products (Andrade *et al.,* 2009). Volatile compounds from other microorganisms had been demonstrated to control plant pathogenic fungi by several researchers such as bacterium *Bacillus subtilis* had been shown the potential to inhibit the growth of *Penicillium digitatum* up to 70% (Leelasuphakul *et al.,* 2008). *Bacillus mycoide* volatile metabolites inhibited the growth of *Botrytis cinerea* and also activated the defense system on strawberry (Guetsky *et al.,* 2002). Additionally, the report by Wan *et al.* (2008) had found that volatile substances from *Streptomyces platensis* F-1 had the potential to control plant pathogenic fungi; *Rhizoctonia solani, Sclerotinia sclerotioum* and *Botrytis cinerea*. However, volatile metabolites from the strains of yeasts *Debaryomyces melissophilus, Rhodotorula glutinis, Cryptococcus laurentii* and some species of *Candida* had no reports on the inhibition of the fungal growth (Ragaert *et al.,* 2006; Andrade *et al.,* 2009).

Antifungal compounds which investigated from the crude extracts had failed to give all of inhibition zones at various R_f values on TLC plates. This might explain that the presence of other substances in crude extracts probably caused poor separation for antifungal compounds while running in mobile phase as the faint inhibition zones had been shown on TLC plates. These mixed substances in crude extracts might also cause the shifted R_f values of inhibition areas (Leelasuphakul *et al.,* 2006). Fractionation by column chromatography using organic solvent mixtures to modify the polarity gave the best separation of individual antifungal compound. Antifungal compounds which contained in crude extracts of both yeast metabolites could be mainly divided into four groups depended on the position of inhibition zones where derived from each fraction. From the results, crude extracts eluted with four solvent mixture series including hexane : ethyl acetate (50:50), hexane : ethyl acetate (25:75), absolute ethyl acetate and ethyl acetate : methanol (95:5) revealed the best inhibition zones according to the compounds were separated with suitable ratio of solvent mixtures (Houghton and Raman, 1998). Antifungal compounds which located

in the position of these inhibition zones were separated by the solvent strength or the range of solvent polarity (Houghton and Raman, 1998).

In the case of fractionation of *A. pullulans* metabolite extract, the position of inhibition zone at R_f 0.65 which was probably major component of active antifungal compounds showed the biggest area of inhibited zone but showed no colour when sprayed with Anisaldehyde reagent. Antifungal compound produced by *A. pullulans* R106 which reported by Ikai *et al.* (1991) is Aureobasidin A. This compound belonged to a cyclic depsipeptide consisting of eight amino acid unit and play a significant inhibitory role with various pathogenic fungi and yeasts such as *Candida* species, *Cryptococcus neoformans,* and some *Aspergillus* species (Zhong *et al.,* 2000). Mode of action of this compound corresponded to inhibition of inositol phosphorylceramide synthase that is essential for fungal growth (Zhong *et al.,* 2000). However, from this study, preliminary test on the inhibition growth of *Cladosporium cladosporioides* by Aureobasidin A on TLC plate showed the position of inhibition zone at R_f value around 0.37 (data not shown). This might conclude that the pure compound, Aureobasidin A did not related to the antifungal compounds which obtained from R_f 0.65.

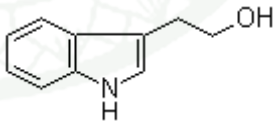
On the other hand, the inhibition zones derived from fractions of *C. utilis* metabolite extract showed the largest area at R_f 0.35 where nearly closed to the position of inhibition zone of Aureobasidin A at R_f 0.37. When TLC plates were sprayed with Anisaldehyde reagent, the former clear zone at R_f 0.35 showed violet colour but the latter showed no colour. This completely indicated that the major component of antifungal compound which contained in *C. utilis* metabolite extract was not Aureobasidin A. However, Aureobasidin A also affected on the growth inhibition of *C. musae* at MIC value of 6.25 μg/mL (data not shown).

The inhibition zones at R_f 0.72 and 0.76 where obtained from *C. utilis* and *A. pullulans* metabolite extracts, respectively, ran too high in ratio of dichloromethane : methanol 98 : 2 which used as running solvent. These clear zones also could not be separated to good quality of antifungal compounds. Therefore, solvent without

polarity like hexane or lower polarity should be added until satisfactory R_f values are obtained (Houghton and Raman, 1998).

The inhibition zones at R_f 0.37 and 0.35 where obtained from hexane-ethyl acetate fractions of yeast metabolite extracts produced orange colour which indicated the alkaloids. At the same R_f values of inhibition zones, these areas also showed violet colour which belonged to the terpenoids. These have been indicated that both antagonistic yeasts, *A. pullulans* and *C. utilis* had capability on the production of both types of compound groups. This enabled to investigate biosynthetic pathway in microorganisms (Guzman-Lopez *et al.,* 2007). However, from the results both alkaloids and terpenoids located at the same position of the inhibition zones on TLC plates which used dichloromethane : methanol 98 : 2 as mobile phase. This indicated that this solution mixture could not separate the group of compounds from each other. Different ratios of solvents or other solvent mixtures should be tested (Houghton and Raman, 1998) and also the fractionation of crude extract of yeast metabolites by using different solvent mixture series should be investigated.

From the NMR measurement, the one type of the antifungal compounds which contained in both yeast metabolite extracts was identified as tryptophol. The properties of this compound were shown as below.

Molecular structure	
Molecular formula	$C_{10}H_{11}NO$
Molecular weight	161.20
Melting point	57-60 ºC
Water solubility	10 g/L (20 ºC)

Source: http://www.tryptophol, beta-3-Indolylethanol, 3-(2-Hydroxyethyl)indole, 2-(1H-Indol-3-yl)ethan-1-ol, 526-55-6.htm (2009)

The presence of tryptophol production had been reported in several strains of fungi and yeasts such as *Candida* spp., *Zygosaccharomyces* spp., *Saccharomyces* spp.,

Schizosaccharomyces spp., *Ceratocystis* spp., *Pythium* spp. and *Aspergillus niger* (Rosazza *et al.,* 1973; Bycroft, 1988; Rey *et al.,* 2001; Guzman-Lopez *et al.,* 2007). Tryptophol production had also been reported in bacteria *Agrobacterium tumefaciens* and *Rhizobium* sp. (Bycroft, 1988). Tryptophol is produced from tryptophan where there are reaction of deamination and decarboxylation (Guzman-Lopez *et al.,* 2007). The biosynthetic pathway for producing tryptophol and indole-3-acetic acid (IAA) which one of the indolic compounds by yeast *Zygosaccharomyces priorianus* had been presented in Figure 38. This yeast converted tryptophan to tryptophol and small quantities of IAA via the intermediated indole-3-pyruvic acid and inole-3-acetaldehyde (Figure 38). The addition of tryptophan or indole precursors in liquid culture leading microorganisms released larger amounts of both tryptophol and IAA (Rosazza *et al.,* 1973; Rey *et al.,* 2001). Of a large number of microorganism tested, the yeasts possessed the highest capacity for degrading tryptophan to tryptophol (Rosazza *et al.,* 1973). Furthermore, tryptophol had been reported to have antibacterial activity against gram-positive bacteria and *Candida albicans* (Erdogan *et al.,* 2000), and it also had been reported to have phytotoxic activity (Guzman-Lopez *et al.,* 2007).

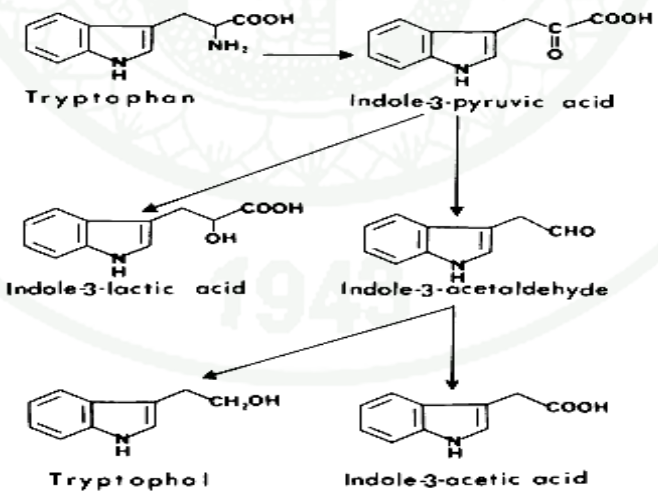


Figure 38 General pathway for the production of tryptophol and indole-3-acetic acid (IAA) from tryptophan by yeasts.

Source: Rosazza *et al.* (1973)

However, the method for detecting indole componds and indole derivatives had been investigated with *p*-dimethylaminobenzaldehyde (PDAB-$NaNO_2$) spray reagent and they gave blue to purple spots on the TLC plates. They also were visualized in gray when TLC plates were sprayed with $FeCl_2$ (Rosazza *et al.,* 1973).

Both yeasts *A. pullulans* and *C. utilis* had the capability of terpenoid production which had been shown violet colour at the clear zones on the TLC plates. Terpenoid compounds had been produced by several microorganisms such as *Penicillium digitatum, Mucor plumbeus* and *Ceratocystis moniliformis* (Lanza and Palmer, 1977; Demyttenaere and De Kimpe, 2001; Fraga *et al.,* 2001). Non-volatile and volatile terpene compounds produced by several strains of yeasts such as *S. cerevisiae*, *Metschnikowia pulcherrima, Debaryomyces hansenii, Kluyveromyces thermotolerans, Hanseniaspora uvarum, Pichia kluyveri* and *Candida molischiana* also had been reported (Fernandez-Gonzalez *et al.,* 2003; Jackson *et al.,* 2003; Carrau *et al.,* 2005) but no report on antimicrobial activity. Terpenoids were produced via different biosynthetic pathways. Mevalonate pathway is the main route for these compounds as shown in *Trichoderma* species (Figure 39). Another biosynthetic pathway for terpenoids is deoxyxylulose phosphate pathway which had been discovered only recently in plants and certain bacteria (Eisenreich *et al.,* 2001; Rohdich *et al.,* 2002).

In this study, although the type of terpene compounds could not be elucidated by NMR measurement, the presence of inhibition zones at the same position of violet in colour on TLC plates clearly indicated these compounds had enabled to show antifungal activity. This might indicate that the efficiency of *C. musae* growth inhibition might affect by the synergistic effects among terpenes, tryptophol and/or other secondary metabolites. This result correlated with the study of Cardoza *et al.* (2007) reported that terpene compounds produced by *Trichoderma harzianum* had the antifungal activity against *R. solani* and *F. oxysporum.* Liu *et al.* (2009) also reported that terpene essential oils produced by *Ganoderma japonicum* had inhibitory activity against several strains of bacteria, fungi and yeasts. The antifungal effects of terpenes

on *Trichophyton mentagrophytes* caused distorted and collapsed hypha, and cell membrane and organelles were irreversibly damaged (Park *et al.*, 2009).

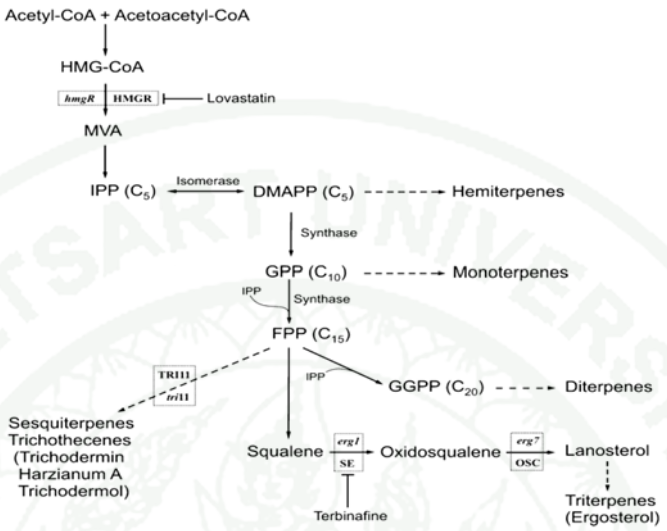


Figure 39 Pathway for the production of terpenes by *Trichoderma* species. HMG-CoA = 30-hydroxy-30-methylglutayl-CoA, MVA = mevalonate, IPP = isopentenyl diphosphate, DMAPP = dimethylallyl diphosphate, GPP = geranyl diphosphate, FPP = farnesyl diphosphate, GGPP = geranyl geranyl diphosphate, HMGR = hydroxy-methylglutaryl-CoA reductase, SE = squalene epoxidase, OSC = oxidosqualene cyclase

Source: Cardoza *et al.* (2007)

By the way, from the studies of Ippolito *et al.* (2000) reported that yeast cells of *A. pullulans* had the efficacy of induction of resistance to postharvest diseases. In this study, metabolites from both yeast *A. pullulans* TISTR 3389 and *C. utilis* also stimulated host defense mechanisms in banana fruit. From this result revealed that these yeast metabolites acted as PAL, ß-1,3-glucanase and chitinase inducers. PAL is a key enzyme in phenylpropanoid pathway and was stimulated starting from the beginning of storage time at day 0, this indicated that elicitors caused a rapid stimulation of PAL synthesis as an early in the defense response (Edwards *et al.*,1985) for involving rapid accumulation of phenolic compounds to slow or stop invasion of pathogen (Abayasekara *et al.*, 1998). PAL was also involved in

biosynthesis of phytoalexin like phenylphenalenones against *C. musae* and lignification in banana fruit peels (Abayasekara *et al.,* 1998; Kamo *et al.,* 1998, 2001).

From the results, ß-1,3-glucanase activity was stimulated within 48 h and remained elevated throughout storage to day 4, while chitinase activity became elevated later at 72 h similar to the infection of *Alternaria alternata* on tomato fruit also induced higher levels of chitinase enzyme, after 2 and 8 days of storage (Cota *et al.* 2007). Therefore, banana fruit treated with metabolites and followed by inoculation with *C. musae* and storage for 48 h, did not show increasing levels of chitinase in all treatments. Inoculation of *C. musae* on non treated fruit and incubation for 24 h had no significant effects on the stimulation of PAL, ß-1,3-glucanase or chitinase activity. This indicated that the pathogen penetrated under the cuticle and remained quiescent there (Abayasekara *et al.*, 1998). Increases in defensive enzyme activity were not expressed and plant tissues had no enzymatic defense responses during a short period during which quiescent infection established (Troncoso-Rojas and Tiznado-Hernández, 2007). However, enhancement of PAL and hydrolytic enzymes in plant tissues were induced by physical and environmental factors (Wang *et al.*, 2007; Charles *et al.*, 2009). Furthermore, from the results, YMB medium also slightly activated the activities of PAL and hydrolytic enzymes in both conditions of banana storage within 24 h and longer time period. This indicated that yeast extract which derived from the cell wall of yeast *Saccharomyces cerevisiae* and contained in liquid culture medium had the efficiency to induce defense mechanisms in the plant cells (Pauw *et al.,* 2004).

The inducer, salicylic acid at the concentration of 0.5 mM which used in this study was not harmful to *C. musae* growth and had the potential to promote the activity of defensive enzymes. Similar to the report of Wen *et al.* (2005) found that 0.15 mM salicylic acid induced the accumulation of *PAL* mRNA and the synthesis of new PAL protein, and lead to increase the activity of PAL enzyme in grape berry tissues. Salicylic acid at higher concentration (2 mM) suppressed the growth and spore germination of *C. musae*, which was contradictory to the report of Xu and Tian

(2008) that 2 mM salicylic acid did not inhibit *Penicillium* growth and also activated antioxidant defense responses in cherry fruit against this pathogen.

Antifungal or other substances in yeast culture filtrates might act as elicitors which had the potential to induce defense mechanisms against pathogen via several biosynthetic pathway. From this results, tryptophol which belonged to indoles may acted as signal molecule to stimulate the level of defensive enzymes. Lambrecht *et al.* (2000) and Yu *et al.* (2008) also found that indole-3-acetic acid (IAA) which was produced by yeast and bacterium via the same pathway of tryptophol production had capability of the induction of defense mechanisms in host plants. Additionally, Pauw *et al.* (2004) reported that fungal elicitors from yeast extract activated the generation of ROS like hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) which might function in plant cells as signal molecules in the induction of plant defense response. H_2O_2 was also activate the accumulation of mRNAs encoding glutathione S-transferase, ascorbate peroxidase or glutathione peroxidase which are ROS scavenging enzymes leading to setup the balance of biochemical changes in the plant cells (Garcia-Brugger *et al.,* 2006). Pathogenesis-related protein against pathogen in the plant cells also had been induced by H_2O_2 (Levine *et al.,* 1994; Chamnongpol *et al.,* 1998).

From the gene expression analysis using RT-PCR approach, PAL and ß-1,3-glucanase were highly sensitive to the signal from *C. utilis* metabolites treatments. Especially for PAL gene which could be regulated at transcriptional and/or translational levels since the PAL mRNA was expressed in bands as well as the increasing PAL enzyme accumulation was significantly found at 0 h after treatment (Sanchez-Ballesta *et al.,* 2000; Chen *et al.,* 2009). Whereas the response of ß-1,3-glucanase gene was likely be regulated at the translational level but not at the transcriptional level because ß-1,3-glucanase mRNA was clearly visible in bands at initial time at 0 h until 24 h after treatment which contradictory to ß-1,3-glucanase activity (protein level) was highly activated at 48 h after treatment.

From the results, it was possible there were signal molecules in yeast metabolites that triggered the induction of the PAL and ß-1,3-glucanase gene expression similar to the induction of these genes by salicylic acid. Whereas the expression of chitinase gene was not able to detect because chitinase gene family has a high level of genetic diversity (Van Loon *et al.,* 2006), each member colud be very different in terms of nucleotide sequences. In this experiment, chitinase gene sequence in genus *Musa acuminata* was obtained from NCBI and was used to design primers. Three pairs of primers were designed and used for chitinase gene expression analysis but none was succeeded. The most likely reason for the unsuccessful attempts was the variation in nucleotide sequences of this gene. The chitinase sequence obtained from NCBI was generated from banana AAA genome "Grand Nain" group but banana used in this study was from AAA genome "Gros Michel" group.

From this experiment, salicylic acid and *C. utilis* metabolite treatments were able to activate the induction of PAL and ß-1,3-glucanase genes after the application of the treatments. In order to investigate the effect of salicylic acid and *C. utilis* metabolite treatments in more details, a shorter time period of tissue collection for gene expression analysis were required, for example at 3, 6, 12 and 24 h after treatment (Chen *et al.,* 2009). This shorter time period will also have a better chance to obtain good quality of RNA. During the experimental period, banana tissue will turn from green to yellow colour and finally to brown colour, this phenomenon correlates with the quality of extracted RNA from these tissues.

In conclusion, metabolites from yeasts have a potential as control agents in controlling the growth of *C. musae* and anthracnose disease on banana fruit. They are capable of inducing the accumulation of PAL, ß-1,3-glucanase and chitinase against pathogen in banana fruits. They also can be used to activate the signal transduction of banana fruit similar to salicylic acid treatment.

CONCLUSION

3-(2-hydroxyethyl)-indole (common name as tryptophol) or other antifungal compounds which contained in metabolites from both yeasts *A. pullulans* and *C. utilis* had an efficiency of inhibition of *C. musae* growth and suppressed anthracnose disease severity. Both hydrolytic enzymes; ß-1,3-glucanase and chitinase which secreted by yeasts significantly inhibited the spore germination of pathogen as well as volatile compounds produced by these yeasts also suppressed the growth of *C. musae*. 3-(2-hydroxyethyl)-indole or other secondary metabolites in yeast culture filtrates probably acted as signal molecules for activation of defensive enzymes; PAL, ß-1,3-glucanase and chitinase against pathogen in tissues of banana fruit peels. The expression of PAL gene induced by yeast metabolites could be regulated at levels of transcription (RNA level) and/or translation (protein level), whereas the ß-1,3-glucanase gene expression was found at the translational level. The expression of chitinase gene was not able to detect.

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APPENDICES

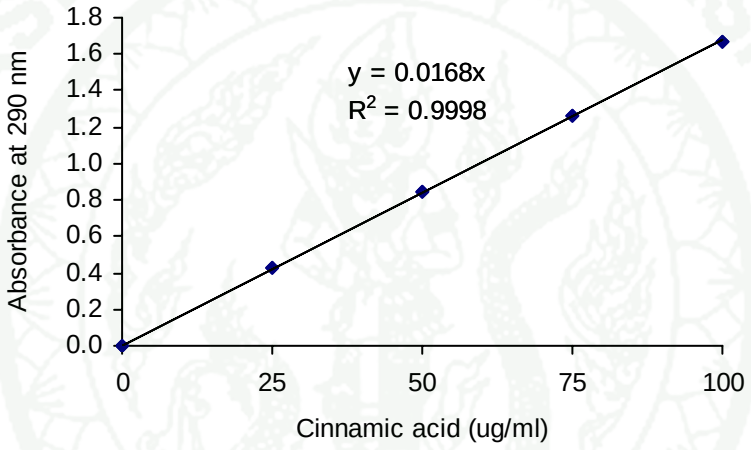
Appendix A

Chemicals and standard curve for enzyme activity analysis

1. Analysis of PAL

1.1 Standard curve of cinnamic acid

Dilution of cinnamic acid at various concentrations of 0, 25, 50, 75 and 100 µg/ml were prepared. The reaction was analyzed by the same method as described in 6.3.1. The absorbance at OD 290 nm of each reaction was plotted and used as standard curve (Appendix Figure A1).



Appendix Figure A1 Standard curve of cinnamic acid.

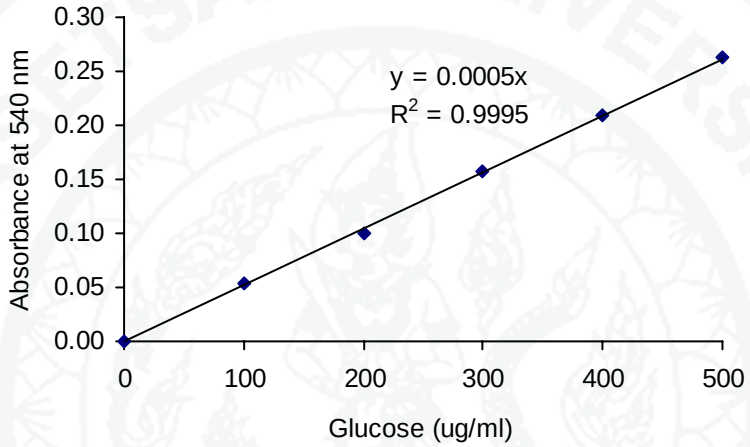
2. Analysis of ß-1,3-glucanase

2.1 Chemicals

3,5-dinitrosalicylic acid (DNS) solution: 1 g of DNS was dissolved in 20 ml of 2M NaOH at 80˚C, then mixed with 30 g of sodium potassium tartrate in 50 ml of ddH_2O and stirred. Distilled water was added to total volume of 100 ml and kept at room temperature.

2.2 Standard curve of glucose

Dilution of glucose at various concentrations of 0, 100, 200, 300, 400 and 500 µg/ml were prepared. The reaction was analyzed by the same method as described in 6.3.2. The absorbance at OD 540 nm of each reaction was plotted and used as standard curve (Appendix Figure A2).



Appendix Figure A2 Standard curve of glucose.

3. Analysis of Chitinase

3.1 Chemicals

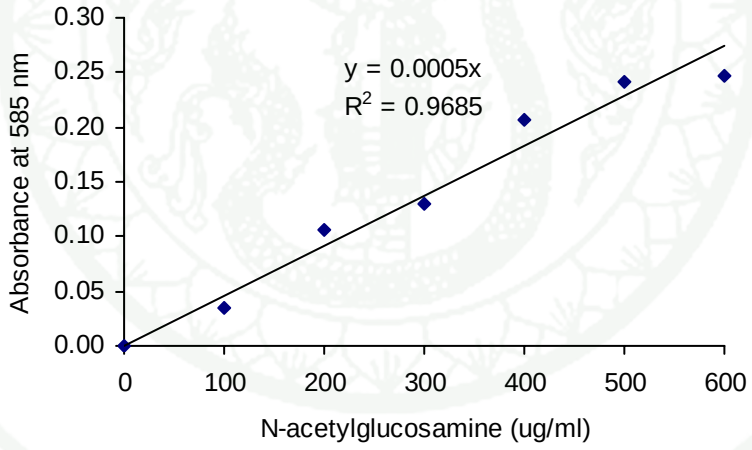
Swollen chitin (modified from the method of Monreal and Reese, 1969): 5 g of chitin was mixed with 50 ml of phosphoric acid (H_3PO_4) and kept at 4˚C for 24 h. The suspension was rinsed and washed with distilled water until pH 5.0, then adjusted with 1N NaOH to pH 7.0. The water was drained off and resuspended with distilled water again, then centrifuged at 8000 rpm for 10 min. Swollen chitin was collected and stored at 4˚C until used.

0.05 M citrate-phosphate buffer, pH 6.6: 13.6 ml of 0.05 M citric acid mixed with 36.4 ml of 0.05 M Na_2HPO_4 (modified from the method of Vivckananthan *et al.*, 2004).

Dimethylaminobenzaldehyde (DMAB) reagent: 1 g of DMAB dissolved in 100 ml of glacial acetic acid with 1.25% (v/v) of 10 N HCl.

3.2 Standard curve of N- acetylglucosamine (NAG)

Dilution of NAG at various concentrations of 0, 100, 200, 300, 400, 500 and 600 µg/ml were prepared. The reaction was analyzed by the same method as described in 6.3.3. The absorbance at OD 585 nm of each reaction was plotted and used as standard curve (Appendix Figure A3).



Appendix Figure A3 Standard curve of N- acetylglucosamine.

Appendix B

Protocols and base sequence of genes in RNA expression analysis of banana (*Musa acuminata* AAA Group)

PAL gene

>gi|195931990|gb|EU856394.1| Musa acuminata cultivar Calcutta 4
phenylalanine ammonia lyase (PAL) gene, complete cds = **3,915 bp**

```
   1 tcaaagattg atgatttaaa aatttatcgg ataaattcaa tgcataaaac actggtctga
  61 taaagttgtg ttagctcgac ttaggtttga aattctacca aactaaggta taaaatcttg
 121 aaaatcttac acgacagatt tttctaaact attagttata attctaactc ttaatatcga
 181 tattttagaa ccaaatccta tcttattatt taccttataa aaaggtcaaa ataaagtcgc
 241 ttttcaatcc tccccgcatt gctattggaa attgtcatga catttagaca acagtaaatg
 301 aaaaagtaag ttgaggcgag aaataaatca ccactggcct atgtttacta aaattataat
 361 gtgatcagct catcagaaga ataatgccta cgtgatcaga tggggctacc acaagaggat
 421 ggtgaggtct cacgtatatg cttctcaata tgatgataaa cagtgtaata atctgtcact
 481 atcctatttg tttagagaaa atatatatat tcatgacgaa tatagttagg tgcaggaaca
 541 caagtctatg atttgactcc caatgtggag gttgggttag gagagtggga tgcatacaca
 601 tctcatcaca ccaacccctc ccttggcgtt gtggcttcct gacdtccatg ccctgcgata
 661 tggagaatcc caccactctt aatgcatggt ttggtgagac ccaccagaat cgcagaaccg
 721 gatttgttat ttttcgtggt ttgcgattga aatgctctac tgaatccgct ccgctctctt
 781 tgacgagata gaaagaaggt agaaggggag ctgcctccga agcccaccaa ccccctctcc
 841 cgccccaacg caaaccagcg gcctccaagt agcagtcaag catgccatgg gatgatgata
 901 cgagtttgcg cggtcagcaa tgctccgcga gcagcaacta ctcctttggc actcccaaca
 961 accactctgt gatctctcta tttaaacccc taccctcttc taatcttact caagagatcc
1021 agttctacct cagccttctc tcctcgccta tctctaatcc ttgacctatt cacgtattga
1081 aatggagttc gcaccgaaag ctcaagtcgt tgagaacggc gaggcgttct gccttaaggc
1141 ggacccttg aactggatca aggcggcgga gtcgctgacg gggagccacc tcgacgaggt
1201 gaagcgcatg gtggaggagt tccggaagcc gctggtgcgg ctcgagggcg cgaccctgac
1261 gatctcccag gtggcggccg tggccgctgc ccggtcgccc gtgagggtgg agctgtcgga
1321 ggaggcgagg gatggcgtgc gggccagcag cgagtgggtg atggagagca tgaacaaggg
1381 gaccgacagc tacggcgtca ccaccggctt cggcgcgacc tcgcacagga ggaccaagca
1441 aggaggtgct cttcagaagg agctcattag ttgatagaca tgtagatgat gttttctttt
1501 agatccttta cttacgccat ggatctgcca caccaagtaa agttggccta ccattttgtc
1561 ataggatcta ttgcgatctg caataggagg atggatcctg gcatggctgg aaagagaaat
1621 agctgtgcgt tagttggcat gatgctttct tgctggtgta ggtttggcct ttttatttat
1681 ggggatgaga tcagatccaa atgtcgaaga gaacaaaagt cacatctact tgcagttcac
1741 cgacacttct caaggaagaa gtcatgcaca gcccaccaac tccaatcatg aatcctttgt
1801 ccaactctgg gtggatccat gttcttgtgt tctctgacac gatccttgat ggatcctgtc
1861 aagtgtttgg tctggcaatg gctgctaatc attgattgtc tcgtcgcgct gccgttgctg
1921 tcgtgagctg tgagatctct aaccctgaga ttccaattgc tgcagattcc ttaatgccga
1981 ttccttaatg ccggaatatt cggctccggg acggagtccg cccacacgct gccgacaccg
2041 gcggccaggg cggcgatgct cgtacgcgtc aacaccctcc tccaaggcta ctctggcatc
2101 cggttcgaga tcctcgaggc catggccagc ctcctcaact ccggcatcac cccttgcctc
2161 ccgctccgcg gcaccatcac cgcctcgggc gacctcgtcc ccttgtccta catcgctggc
2221 gtgctcaccg gccgctctaa tgccaaagcc gttcgccccg gcggcgaggc ggtcgacgct
2281 gcggaggcct tccgccggtc cggcatcccc catgggttct tcgagctgca gcccaaggag
2341 gggctcgctc tcgtcaacgg caccgccgtc ggctcgggcc tcgcctcggt cgttctgtac
2401 gaagccaacg tcctcgctgt cctcgccgag gtgctgtcgg cggtcttttg cgaggtgatg
2461 caagggaagc cggaattcac cgaccacctc acccacaagc tgaagcacca cccgggccaa
2521 atcgaagccg ccgcgatcat ggagcacgtc ctcgagggca gctcctacat gaagatggcg
2581 aagaagctcc atgagcaaga cccgctccag aagccaaagc aggaccgcta cgccctccgc
2641 acctcgccgc agtggctcgg cccccagatc gaagtcatcc ggtcgtccac gaagtccatc
2701 gaacgtgaga tcaactcggt gaacgacaac cccctcattg acgtctcccg gaacaaggcc
2761 ttgcacggtg gcaacttcca ggggaccccg atcggtgtct ccatggacaa cacccgctta
2821 gccattgctg ccatcggcaa actcatgttc gcacagttct cagagctcgt caacgacttc
2881 tacaacaatg ggctcccctc gaacctttcc ggtggaagaa acccgagctt ggactacggc
2941 ttcaaaggcg cagagatcgc gatggcggct tactgctccg agctccagtt cctggcaaac
3001 ccagtgacca accatgttca gagtgcagag cagcacaacc aggatgtgaa ttccttggga
3061 ctgatctcct ccaggaagac agccgaggca gtagacatcc tgaagctcat gtccacaaca
3121 tacttagtcg cgctctgcca agccatcgac ttgaggcact tggaggagaa tctgaagaac
```

Appendix Figure B1 Base sequence of *Musa acuminata* AAA Group cultivar Gran Enano phenylalanine ammonia lyase (PAL) mRNA. Upper rectangle = forward primer, lower rectangle = reverse primer.

```
3181 gccgtaaaga acaccgtgat ccaggtggcg aaaaagaccc tgacgaccgg tgtgaacggg
3241 gagctacacc catccaggtt ctgcgagaag gatctgatca cgatcatcga tcgggaacat
3301 gtctttagct acgtcgacga cccatgcagc tccacgtacc ctctgatgca gaagttaagg
3361 caggtgctcg tggagcatgc actgaacaac ggagagaagg agaaggaccc gaacacctcc
3421 atcttccaaa agatcactgc cttcgaggaa gagctcaagg ctgccctgcc gaaagaagtg
3481 gaggcagcaa gagcggcggt ggagaacggc aacgcggcga ccgcaaaccg gatcgaggaa
3541 tgcagatcgt atcctctgta tagatttgtg agagcagagc tatgcacggg atacctcacg
3601 ggagagaagg tgcggtcgcc gggcgaggat ttcgacaaga tctttgcggc catcaacaaa
3661 gggatggtga tcgatcccct tctcgagtgc ctcaaggaat ggaacggtgc tcccctaccc
3721 atatgctaag atatagatca catggagatg gaggtttata ccttaccaca gtactagttg
3781 gtgatgtagt tgcactagca acagcaggca tgagacgtcc aagttctgct gctgctgctg
3841 ctgctttcgc atgttctttc ttcgtcttcc ccatattatt ccagttgtgt gtgcttatgc
3901 atgaataatt gcaac

OLIGO         start  len    tm      gc%           seq
LEFT PRIMER     564   20   59.96   55.00   TGTGGAGGTTGGGTTAGGAG
RIGHT PRIMER    968   20   60.16   55.00   AGAGTGGTTGTTGGGAGTGC
PRODUCT SIZE: 405,
```

Appendix Figure B1 (Continued)

ß-1,3-glucananse

>gi|6073859|gb|AF001523.2| *Musa acuminata* beta-1, 3-glucananse mRNA, complete cds=1,141 bp

```
   1 atggcaacaa aagcttctct ctccataaaa ggctttgcct tgctggtttc agtccttgta
  61 gcagttccaa caagagtgca atcgattggt gtctgctacg gcatgctcgg caacaatctt
 121 cccccgccca gcgaggtggt cagtctctac aaatccaaca acatcgcgag gatgagactc
 181 tacgatccaa accaagccgc cctgcaagcc ctcaggaact ccaacatcca agtcctgttg
 241 gatgtccccc gatccgacgt gcagtcactg gcctccaatc cttcggccgc cggcgactgg
 301 atccggagga acgtcgtcgc ctactggccc agcgtctcct ttcgatacat agctgtcgga
 361 aacgagctga tccccggatc ggatctggcg cagtacatcc tccccgccat gcgcaacatc
 421 tacaatgctt tgtcctcggc tggcctgcaa aaccagatca aggtctcgac cgcggtcgac
 481 acgggcgtcc tcggcacgtc ctaccctccc tccgccggcg ccttctcctc cgccgcccag
 541 gcgtacctga gccccatcgt gcagttcttg gcgagtaacg gagcgccgct cctggtcaat
 601 gtgtaccctt attttagcta caccggcaac ccgggacaga tctcgctgcc ctacgccctg
 661 ttcacggcct ccggcgtcgt cgtgcaggat gggcgattca gctatcagaa cctgttcgac
 721 gccatcgtcg acgcggtctt cgcggcgctg gagagagtgg gaggggcgaa cgtggcggtg
 781 gtggtgtcgg agagcgggtg gccgtcggcg ggcggaggag ccgaagcgag caccagcaac
 841 gcgcggacgt acaaccagaa cttgatcagg catgttggcg gaggaacgcc gaggagacca
 901 gggaaggaga tcgaggcgta catattcgag atgttcaacg agaaccagaa ggctggaggg
 961 atcgagcaga actttggcct gttttatccc aacaagcagc ccgtctacca aataagcttt
1021 tagaaactaa cttgtaaggt tgatgaatca tctcctacct acctacctac gaataaaaca
1081 tgaaataaag caccagaata aagggagaat tctgatcttg gagaaaaaaa aaaaaaaaaa
1141 a
```

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	329	20	59.84	55.00	CCAGCGTCTCCTTTCGATAC
RIGHT PRIMER	582	20	59.88	50.00	TCCGTTACTCGCCAAGAACT

SEQUENCE SIZE: 630

Appendix Figure B2 Base sequence of *Musa acuminata* beta-1, 3-glucananse mRNA. Upper rectangle = forward primer, lower rectangle = reverse primer.

Chitinase I

>gi|40806505|gb|AY507150.1| *Musa acuminata* basic class I chitinase mRNA, complete cds=**1,419 bp**

..

```
   1 tgtaagcaag agctttctat tatgaaaagt tatatgcatc aatcccacgt gcagtctgtg
  61 aattacttgt tgttctcggt tctgctactc ggtatcgata aggacgggga acagtgcggt
 121 cggcaggcgg gaggagccct ctgcgcttca ggtaaggagt ccttgtgttg tagccaattc
 181 ggatggtgtg ggaatacgga tgactactgt ggttctcagg aggggtgcca gagccaatgt
 241 ccgggtcata agataagcac tttacccagc ttaaagaaag taatagatac cgacacgaac
 301 ttttcagcta ataaactata tgtgaagtac ttcgtcgacc agatgttaaa gcacagaaat
 361 gacaatagtt gcgaagggaa atttctcttt tatagttatt ccgccttcat ttgcgcatcg
 421 gcccgtttta gatactttgg aactacaggc gataccacta ggcttgcccg taagcgagaa
 481 atcgcggctt ttttcgcaca gacctcacac gagaccactg gaggcacgtg ggctactgca
 541 cctgacggcc cttacgcgtg gggttattgt ttcatcaatg agcaagtcag tgcagaacag
 601 tgcggccgac aagcgggagg tgcccctgc gctgcgggaa aaaaatactt tggaagagga
 661 ccaattcaga taagtcataa ctataactat ggaagcggag gccaggctgg aaaagccatt
 721 ggggttgacc tgctaaacaa cccagactta gtagcgaccg atgcgacaat ctccttcaag
 781 tcagcattct gggaatggat gacagcgcaa tctccaaaac cctcctgcca cgacgtaatc
 841 acgggacggt ggagtccatc gtcagctgat tcgtctacag gcaccctgat ccggattggg
 901 tggatacaat tgttcaattt agatcttgag tgtggccatg ggagtgactc acgcgtacaa
 961 gatagaatag gtttttatcg acgctactgc ggaatattag gcgttagtcc gggtgacaat
1021 cttgattgtg gcaatcaaag gagctttggc ttatatacca agtccatggc cctatgttcg
1081 tggaaagaaa tatttagtgg ttttgggact actggtgata caacggcaag gaaagcacaa
1141 tgcccaacat gtggatgggc ctgctgcttc attaccgtac aaaatgtcct gaacgctctc
1201 tatacaccaa atctaatctc atacaatcga ttagtgtacc tatttatata tataggagga
1261 tgggcttcag caccatgcca aacatacgca tggggttact gcttcattac agaacaaaat
1321 gcccagagcg atcactgtac accaagtaat caatggcctt gtgctcctgg aaggaaatat
1381 ttcggacgag gccccatcca aatttcacag taagttacc
```

```
No mispriming library specified
Using 1-based sequence positions
OLIGO        start  len   tm      gc%           seq
LEFT PRIMER   721   20   59.97   50.00    GGGGTTGACCTGCTAAACAA
RIGHT PRIMER  1287  20   60.00   50.00    GTATGTTTGGCATGGTGCTG
```

Appendix Figure B3 Base sequence of *Musa acuminata* basic class I chitinase mRNA. Upper rectangle = forward primer, lower rectangle = reverse primer.

Musa acuminata actin (ACT1)

>gi|151413782|gb|EF672732.1| *Musa acuminata* actin (ACT1) gene, complete cds = **1,440 bp**

```
"
   1 gttcttcctc cttcgatttg gttttgcaga tatccataac atggctgacg gcgaggatat
  61 ccagcctctt gtctgtgaca atggtactgg aatggtcaag gttagacatc cttttcctct
 121 cttatgttat aatttgtccc tctttccctg atcatgttgc aaacaacaaa tataactgtt
 181 ctctttcagg ctggtttcgc tggagatgat gcacctagag ctgtattccc tagcatcgta
 241 ggccgacctc gccacactgg tgtcatggtt gggatgggtc aaaaagatgc ttatgttggt
 301 gacgaggctc aatcaaagag aggtatcctg accttgaagt accctattga acacggcatt
 361 gttagcaact gggatgacat ggagaagatc tggcatcaca ccttctataa tgagcttcgt
 421 gttgcccctg aggagcaccc tgtattgctc accgaagccc ctctcaatcc caaggcaaac
 481 cgagagaaga tgacccaaat tatgtttgaa accttcaatg ttccggctat gtatgtagca
 541 attcaggctg ttctttccct ttatgctagt ggtcgtacaa ccggtatgcc tccttcaact
 601 tgattaaagt ctcaaacatg atgtgtaaat gctgatctgt ggcactcttc ttaataggta
 661 ttgtgcttga ttctggtgat ggtgtgagcc acactgttcc tatatacgaa ggatatgccc
 721 tccctcatgc catccttcgt ttggaccttg ctggtcgtga cctcacagat gctttgatga
 781 agattcttac ggagaggagt tattccttca ctaccactgc tgaacgggaa attgtcaggg
 841 atatcaagga aaagcttgcc tacgttgccc tggattatga acaggagctg gagaatgcca
 901 aaggtagttc ctctgtggaa aagagctatg agttacctga tgggcaggtc atcacgattg
 961 gtgcggagag attcagatgc ccggaggttc tcttccagcc atctctgatt ggcatggagg
1021 ctgctggtat ccatgagacc acttacaatt ccatcatgaa gtgtgatgtg gatatcagaa
1081 aggacctgta tggcaacatc gttctcagtg gtggtacgac tatgtttcct ggaatcgctg
1141 accgcatgag caaggagatc acagcgctcg ccccaagcag catgaagatt aaggtggttg
1201 ccccaccaga gcgcaagtac agtgtctgga ttggaggctc catccttgct tcgctcagca
1261 cttttcagca ggtaatatct tgatccacat ttgcaaatta tcaagattca gaaacgtttc
1321 gctgctattt catgatcata attttttact ctttttttgtt taataatgca ccagatgtgg
1381 atctccaagg gcgaatacga ggaatccggt ccttccattg tccacatgaa gtgcttctga
```

OLIGO	start	len	tm	gc%	seq
LEFT PRIMER	434	20	60.14	55.00	AGCACCCTGTATTGCTCACC
RIGHT PRIMER	864	20	60.01	50.00	CGTAGGCAAGCTTTTCCTTG

Appendix Figure B4 Base sequence of *Musa acuminata* actin (ACT1) gene. Upper rectangle = forward primer, lower rectangle = reverse primer.

CURRICULUM VITAE

NAME : Mrs. Veeranee Tongsri

BIRTH DATE : July 29, 1970

BIRTH PLACE : Surin, Thailand

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE**
1993	Khon Khaen Univ.	B.Sc. (Agriculture)
1999	King Mongkut's Institute of Technology Ladkrabang	M.Sc. (Plant Pest Management Tech.)

POSITION/TITLE : Assistant Researcher

WORK PLACE : Department of Plant Pathology, Faculty of Agriculture, Kasetsart University

SCHOLARSHIP/AWARDS

2007 : The grant from Graduated school, Kasetsart University, Bangkok.

2007 : The grant from Postharvest Technology Innovation Center, Thailand.

2009 : The scholarship from Ministry of Foreign Affairs, Israel for the International Course on Research and Development in Postharvest Practices.

2009 : Bronze Poster Presentation Award in the International Symposium on Go...Organic2009, Bangkok, Thailand.

RESEARCH EXPERIENCE

2008 : Research assistant in a research project for the "Induced resistance of banana fruits against anthracnose by using yeast and its metabolites" at Kasetsart University, Bangkok.

PUBLICATIONS

1. Tongsri, V. and S. Sangchote. 2008. Screening antagonistic yeasts for controlling anthracnose disease of banana cv. Hom Thong, caused by *Colletotrichum musae* (Berk & Curtis). **Agricultural Sci. J.** 39 : 3 (Suppl.) : 35-38 (in Thai). *Poster presentation*

2. Tongsri, V. and S. Sangchote. 2009. Yeast metabolites inhibit banana anthracnose fungus *Colletotrichum musae*. **Asian J. of Food and Agro-Industry** 2: (Special Issue) http://www.ajofai.info

3. Tongsri, V., N. Khewkhom and S. Sangchote. 2010. Mechanisms of metabolites from yeast *Aureobasidium pullulans* TISTR 3389 for controlling anthracnose disease of banana caused by *Colletotrichum musae* (Berk & Curtis). **Agricultural Sci. J.** 41:1 (Suppl.1) : 154 – 157 (in Thai). *Oral presentation*