การปรับปรุงการผลิตไมโคอีพ็อกซีไดอีนโดยราเอนโดไฟต์ *Phomopsis* sp. Hant25 ที่แยกจากกระเบาใหญ่

นางสาวนฤจพร ธรรมจาริก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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IMPROVEMENT OF MYCOEPOXYDIENE PRODUCTION BY ENDOPHYTIC FUNGUS *Phomopsis* sp. Hant25 ISOLATED FROM *Hydnocarpus anthelminthicus*

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Thesis Title	IMPROVEMENT OF MYCOEPOXYDIENE PRODUCTION BY ENDOPHYTIC FUNGUS *Phomopsis* sp. Hant25 ISOLATED FROM *Hydnocarpus anthelminthicus*
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นฤจพร ธรรมจารึก: การปรับปรุงการผลิตไมโคอีพ็อกซีไดอีนโดยราเอนโดไฟต์ *Phomopsis* sp. Hant25 ที่แยกจากกระเบาใหญ่ (IMPROVEMENT OF MYCOEPOXYDIENE PRODUCTION BY ENDOPHYTIC FUNGUS *Phomopsis* sp. Hant25 ISOLATED FROM *Hydnocarpus anthelminthicus*) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. นงลักษณ์ ศรีอุบลมาศ, อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. สุเทพ ไวยครุฑธา, 115 หน้า.

สารไมโคอีพ็อกซีไดอีนเป็นสารที่มีฤทธิ์ในการต้านเซลล์มะเร็งของมนุษย์ได้หลายชนิด จึงทำให้เกิดความสนใจที่จะศึกษากลไกการออกฤทธิ์ของสารดังกล่าวและศึกษาในด้านอื่น ๆ ที่มีความเกี่ยวข้องต่อไป การศึกษาเหล่านี้ต้องการสารบริสุทธิ์ในปริมาณที่มากเพียงพอต่อการทดลอง ในการวิจัยนี้จึงมีเป้าหมายในการปรับปรุงการผลิตสารไมโคอีพ็อกซีไดอีนจากการหมักเชื้อราเอนโดไฟต์ *Phomopsis* sp. Hant25 ที่แยกได้จากต้นกระเบาใหญ่ในรูปแบบต่าง ๆ เพื่อเพิ่มการผลิตสารชนิดนี้ และมีการพัฒนาวิธีการตรวจสอบปริมาณสารไมโคอีพ็อกซีไดอีนที่ได้จากน้ำหมักเชื้อในเบื้องต้น โดยการใช้วิธี agar diffusion assay ซึ่งพบว่าไมโคอีพ็อกซีไดอีนสามารถยับยั้งการเจริญเติบโตของเชื้อ *Candida albicans* ได้เมื่อทดสอบร่วมกับการใช้คีโตโคนาโซลในความเข้มข้น 12.5 µg/mL ในการวิจัยนี้ได้มีการปรับเปลี่ยนวิธีการหมักเชื้อราในหลายรูปแบบ และจากการวิจัยพบว่าอาหารเลี้ยงเชื้อมีผลต่อการสร้างสารไมโคอีพ็อกซีไดอีนของเชื้อราและการหมักเชื้อราแบบดั้งเดิมโดยการเขย่าและวางนิ่งในอาหารเหลวนั้นได้ปริมาณสารที่ต่ำ นอกจากนี้ได้ศึกษาการตรวจสอบปริมาณสารไมโคอีพ็อกซีไดอีนในน้ำหมักเชื้อโดยการใช้โครมาโทกราฟีแบบของเหลวสมรรถนะสูงซึ่งพบว่าในการวิจัยนี้ ปริมาณสูงสุดในการผลิตสารไมโคอีพ็อกซีไดอีนเพิ่มขึ้นจากเดิม 13.56 เท่า ซึ่งได้ปริมาณสารเท่ากับ 333 mg/L โดยหมักเชื้อในอาหารเลี้ยงเชื้อ MM1D ที่มีการใช้กระดาษกรองเป็นตัวยึดเกาะที่อุณหภูมิ 25 องศาเซลเซียส ในสภาวะที่มีการวางนิ่งก่อนเป็นเวลา 6 วัน ก่อนนำไปเขย่าต่อที่ 200 rpm เป็นเวลาอีก 12 วัน และยังพบว่ารูปสัณฐานของเชื้อราในระหว่างการหมักมีผลต่อการผลิตสารไมโคอีพ็อกซีไดอีน

ภาควิชา....ชีวเคมีและจุลชีววิทยา.... ลายมือชื่อนิสิต.....

สาขาวิชา.....จุลชีววิทยา..... ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

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NARUKJAPORN THAMMAJARUK: IMPROVEMENT OF MYCOEPOXY-DIENE PRODUCTION BY ENDOPHYTIC FUNGUS *Phomopsis* sp. Hant25 ISOLATED FROM *Hydnocarpus anthelminthicus*. THESIS ADVISOR: ASSOCIATE PROFESSOR NONGLUKSNA SRIUBOLMAS, Ph.D., THESIS CO-ADVISOR: ASSISTANT PROFESSOR SUTHEP WIYAKRUTTA, Ph.D., 115 pp.

Mycoepoxydiene exhibited broad spectrum of cytotoxicity against human tumor cell lines. According to the interesting cytotoxic activities of this compound, its mechanism of action and related studies should be pursued, and these experiments require a large amount of mycoepoxydiene. This study aims to improve mycoepoxydiene yield by varying fermentation conditions of *Phomopsis* sp. Hant25, an endophytic fungus isolated from *Hydnocarpus anthelminthicus*. Agar diffusion assay to determine relative amounts of mycoepoxydiene produced by different fermentation conditions was developed. Mycoepoxydiene was found to be clearly active against *Candida albicans* when tested in medium containing 12.5 µg/mL of ketoconazole. Several fermentation conditions were conducted. Culture media affected mycoepoxydiene production by the fungus. Mycoepoxydiene obtained from conventional shaken and stationary liquid fermentations were low. Analysis by HPLC revealed that mycoepoxydiene amount was increased approximately 13.56 times yielding 333 mg/L when cultured in MM1D medium with filter support at 25°C under static condition for 6 days and then agitation at 120 rpm for 12 days. Fungal morphology in culture medium during fermentation was found to be related to mycoepoxydiene yield.

Department: Biochemistry and Microbiology Student's Signature……..……..…..

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

AIDS	=	Acquired immune deficiency syndrome
^0C	=	Degree Celsius
CH_2Cl_2	=	Dichloromethane
CzYB	=	Czapek Yeast autolysate Broth
d	=	Day
DMSO	=	Dimethyl sulfoxide
e.g.	=	For example
et al.	=	And other
EtOAc	=	Ethyl acetate
g	=	Gram
µg	=	Microgram
h	=	Hour
HIV	=	Human immunodeficiency virus
HPLC	=	High performance liquid chromatography
IAA	=	Indole-3-acetic acid
IC_{50}	=	Inhibitory concentration required for 50% inhibition of growth
IPYA	=	Indole-3-pyruvic acid
L	=	Liter
µL	=	Microliter
MCzA	=	Malt Czapek Agar
MCzB	=	Malt Czapek Broth
MeOH	=	Methanol
mg	=	Milligram
mL	=	Milliliter
mm	=	Millimeter
MM1D	=	Modified M1D medium

MS	=	Mass spectroscopy
MW	=	Molecular weight
PDA	=	Potato Dextrose Agar
rpm	=	Round per minute
RT	=	Retention time
SD	=	Standard deviation
SDA	=	Sabouraud's Dextrose Agar
sp.	=	Species
TLC	=	Thin layer chromatography
USA	=	United States of America
UV	=	Ultraviolet
v	=	Volume
w	=	Weight

CHAPTER I

INTRODUCTION

1.1 Introduction

An increase in public health problem of people caused by various cancers, respiratory infections, HIV/AIDS, diarrhoeal diseases, tuberculosis and malaria are the leading killers among diseases. The first-line drugs of these diseases have been observed to be ineffective. Hence, many resistance cases were changed the treatment to second or alternative drugs which more expensive and less effective. Increased efforts are required to search and develop for new drugs from natural products. Nature has been a source of several medicines for curing the diseases in human. Evidently, natural products are naturally derived metabolites or byproducts from plants, animals and microorganisms. Bioactive compounds from microbes, especially fungi have been found to be a major source of potential pharmaceuticals such as penicillin from *Penicillium chrysogenum*, cephalosporin from *Cephalosporium acremonium*, griseofulvin from *Penicillium griseofulvum*, and lovastatin from *Aspergillus terreus* (Guo *et al*., 2008). Fungi are abundance and beneficial sources for the discovery of new valuable and potential products. Considering that 6 out of 20 of the most commonly prescribed medications are of fungus origin (Gloer, 2007) and approximately 5% of the fungi have been described (Hawksworth, 1991; Hawksworth, 2001), fungi offer a huge potential for novel products.

Endophytic fungi are fungi that live for an imperceptibly short period to a lifetime in the tissue of living plants with unapparent and asymptomatic infections (Saikkonen *et al*., 2004). At first the endophyte research began with their demonstrable role in mediation interactions of herbivores with some grass host plants (Petrini, 1991) and then, most of them have been carried out using plants from temperate and tropical regions. Currently, endophytic fungi are focused as an outstanding resource of bioactive

products because there are enormous species of them occupying in the vascular plants and growing in the unusual conditions. Natural products from endophytic fungi have been observed to inhibit and kill a wide variety of pathogens such as bacteria, fungi, yeasts, viruses, and protozoans (Strobel *et al*., 2004). For example, the fungal endophytes *Taxomyces andreanae* and *Pestalotiopsis micorspora*, and several other fungi which are isolated from the barks of the pacific yew trees, are potential new sources of the anticancer drug, paclitaxel (Taxol®) (Zhang *et al*., 2006). Furthermore, camptothecin could be produced by a fungal endophyte isolated from the inner bark of the plant *Nothapodytes foetida* from the western coast of India (Guo *et al*., 2008). Originally, it was isolated from the wood of *Camptotheca acuminate* Decne (Nyssaceae), which is a plant native to mainland China. Camptothecin and its derivatives show strong antineoplastic activity. The drug is already used in China for the treatment of skin diseases. Recently, comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown. This compares with only 38% of novel substances from soil microflora (Strobel and Daisy, 2003). Some of these endophytic fungi produce bioactive substances that may involve in a host-endophyte relationship. As a direct role, these secondary metabolites may play the importance role for their host or themselves in nature; they may ultimately have application in medicine. A scientific effort to isolate endophytic fungi and study their natural products is now on process.

In Thailand, research on endophytic fungi for their bioactive compounds has been in progress. For examples, studies by Wiyakrutta *et al*. (2004) have reported that endophytic fungi were isolated from 81 Thai medicinal plant species collected from forests in four geographical regions of Thailand, and crude extracts of these fungi were evaluated for biological activities. Prachya *et al*. (2007) determined endophytic fungi from *Hydnocarpus anthelminthicus* Pierre ex Laness., a Thai medicinal plant, because the stem, leaf, root, fruit, and seed of this plant have been used traditionally as a folk remedy for cancer, inflammatory, tuberculosis, leprosy, scabies, impetigo and some other dermatitis (Panthong *et al*., 1986; Panthong *et al*., 1991). They isolated

mycoepoxydiene from *Phomopsis* sp. Hant25 culture broth extract. Compound mycoepoxydiene was found to be highly active against several cancer cell lines.

According to the interesting cytotoxic activities of mycoepoxydiene, its mechanism of action and related studies should be conducted further. These experiments require high amount of mycoepoxydiene. However, the conventional shaken or stationary liquid fermentation could produce mycoepoxydiene in low yield. It is interesting in alternative fermentation methods to improve the production of this bioactive metabolite by endophytic fungus. *Phomopsis* sp. Hant25 fermentation should be improved to obtain high yield of mycoepoxydiene.

1.2 Objectives

The objective of this study was to develop practical fermentation methods for efficient production of mycoepoxydiene from *Phomopsis* sp. Hant25. Specifically:

1. To cultivate the endophytic fungus, *Phomopsis* sp. Hant25, in different fermentation methods and to evaluate the conditions producing the high yield of mycoepoxydiene
2. To validate the feasible analysis of mycoepoxydiene by using anti-*Candida albicans* activity
3. To validate the feasible analysis of mycoepoxydiene by using high performance liquid chromatography (HPLC).

CHAPTER II

REVIEW OF LITERATURE

2.1 Association of the endophytic fungi and plants

Endophytes are fungal or bacterial microorganisms which live in the healthy plant tissues, as shown in Figure 1, for an imperceptibly short period or all lifetime without causing symptoms of disease (Saikkonen *et al.*, 2004). An estimate of 1.5 million endophytes is reasonable on the rates of vascular plants to fungal species at 1:6. To date, fungal species have been described approximately 80,000-100,000 species, with about 900,000 fungi still unknown (Hawksworth, 1991). Endophytic fungi represent an important and quantifiable component of fungal diversity, with an estimate of at least 1 million species living in plants (Dreyfuss and Chapela, 1994).

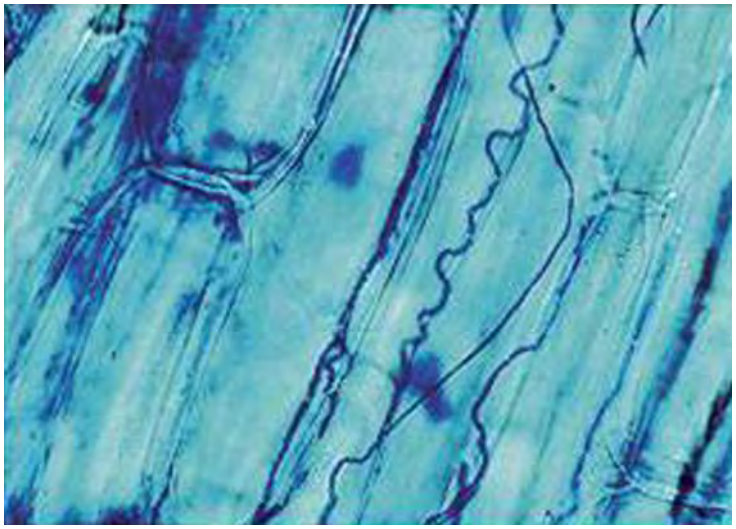


Figure 1 Microscopic picture of tall fescue leaf sheath cell with endophyte mycelium (From: http://www.noble.org/ag/research/Articles/TallFescueToxicity/Index.htm)

A variety of relationship exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic (Strobel *et*

al., 2004). Endophytes can confer an important beneficial role in the physiology of their host plants. Plants infected with endophytes often grow faster than endophyte-free ones. This effect perhaps comes from the endophytes' production of phytohormones e.g. indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPYA), cytokines, and other plant growth-promoting substances like vitamins and partly owing to the fact that endophytes can enhance the hosts' uptake of nutritional elements such as nitrogen fixation and phosphorus assimilation, that they regulate nutritional qualities such as the carbon-nitrogen ratio (Tan and Zou, 2001). Endophytes promote their host plants to grow in dry environmental conditions. The drought tolerance mechanism may be from the condition that endophytes improve the osmotic adjustment which maintain high turgor in growing zone, stunt desiccation and allow rapid resumption of leaf growth upon relief of stress. Moreover, endophytes assist in the secretion and storage of sugars and alcohols which protect enzymes and membranes of host plants from desiccation damage and modify the structure of leaf, that decreases transpiration losses (Zhang *et al*., 2006). Furthermore, host plants may benefit from enhanced competitive abilities and raised ability to resisting invasion of herbivores, insects, pathogen, and various abiotic stresses by attaining the metabolic substances of endophytes (Tan and Zou, 2001; Zhang *et al*., 2006).

Endophytic fungi have been investigated to be an immense potential source of novel active secondary metabolites which possess unique structures and great bioactivities, representing an enormous reservoir which offers a huge potential for exploitation for pharmaceutical, agricultural and industrial uses (Pietra, 1997; Tan and Zou, 2001; Zhang *et al*., 2006). Considering the increasing demand of pharmaceuticals for novel medicine and intermediate to combat dreaded diseases like HIV, severe acute respiratory syndrome (SARS), cancer and dominant infections, endophytic fungus have been recognized to produce novel metabolites of pharmaceutical importance (Strobel *et al*., 2004; Zhang *et al*., 2006).

2.2 History of mycoepoxydiene discovery

In 1999, Ping Cai *et al.* isolated a novel epoxycyclooctadiene, mycoepoxydiene (Figure 2), from solid-state fermentation of a fungus designated as OS-F66617 which collected from twig litter of the deadwood forest near Curitiba, in the state of Parana, Brazil. This organism was cultured on agar-based medium containing 1.8% agar, 0.5% yeast nitrogen base (Sigma), and 1% dextrin (type 1, Sigma) in deionized water at 22°C for 11 days, lyophilized and extracted with methanol. The combined organic solvent was concentrated under vacuum to produce a crude extract and partition between methanol and hexane, followed by chloroform. This compound was acquired as colorless needle-shaped crystals from methanol (Cai *et al*., 1999).

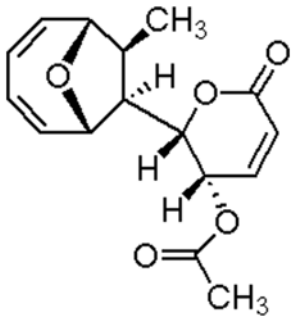


Figure 2 Chemical structure of mycoepoxydiene (Prachya *et al*., 2007)

Xin Lin *et al*. (2005) could extract this compound from the culture of *Diaporthe* sp., a marine fungus which was isolated from the submerged leaves of *Kandelia candel* collected in the mangrove natural conservation areas of Fugong, China. The fungus was cultured in potato-dextrose (PD) liquid medium which compose of 50% (v/v) sea water for 7 days at 25°C in a rotary shaker (120 rpm). The culture was filtered and extracted twice with an equal volume of ethyl acetate. The combined organic solvent was concentrated to produce a crude extract in vacuo. The extract was separated by column chromatography over silica gel, eluting with a gradient of cyclohexane-ethyl acetate and methanol, yielding twenty fractions. Fraction 8 was subjected to further

column chromatography over silica gel, eluting with a gradient of cyclohexane-chloroform and methanol, yielding eighteen fractions. Fraction 7 contained mycoepoxydiene as colorless needles in methanol. In this experiment the compound was produced in the amount of 0.8 mg/L in PD fermentation broth (Lin *et al*., 2005).

In Thailand, mycoepoxydiene has been found from an endophytic fungus, *Phomopsis* sp. Hant25, isolated from the twig of *Hydnocarpus anthelminthicus*, a Thai medicinal plant for the treatment of leprosy and some kinds of dermatitis, cancer, and tuberculosis. This endophytic fungus was cultured in malt Czapek (MCz) medium and incubated at 25°C for 21 days. The fungal culture (5 L) was filtered through cheesecloth to remove mycelia and extracted three times with an equal volume of ethyl acetate to obtain a crude broth extract. The broth extract was subjected to column chromatography on Sephadex LH-20, eluted with 100% methanol, to yield forty fractions. Fraction 20-22 were combined and recrystallized from methanol to acquire mycoepoxydiene 65.6 mg. Later, the mother liquid was chromatographed again on Sephadex LH-20 column, eluted with 100% methanol, to yield twenty fractions. Fraction 7 and 8 were combined and subjected to further column chromatography over silica gel, eluting with 1:9 mixture of ethyl acetate/dichloromethane, to yield ten fractions. Fraction 1 gave mycoepoxydiene 61.2 mg, so this experiment can produce mycoepoxydiene totally 25.36 mg/L (Prachya *et al*., 2007). Moreover, mycoepoxydiene was also purified from the crude extract of Diaporthaceous fungus PSU-H2, an endophytic fungus isolated from *Garcinia hombroniana*, collected in Songkhla province, Thailand (Sommart *et al*., 2009).

Mycoepoxydiene was chemically synthesized by Takao *et al.* in 24 steps with a 1.6% yield from the Diels-Alder adduct of furan and maleic anhydride (Takao *et al*., 2002). It is recognized that organic chemical synthesis of compounds will produce some pollutants to the environment. Moreover, chemical synthesis provided low yield of mycoepoxydiene. Therefore, endophytic fungus has been the interesting source of mycoepoxydiene. Fermentation condition could be adjusted to improve the production yield of this bioactive metabolite by endophytic fungus.

2.3 Bioactivity of mycoepoxydiene

Mycoepoxydiene exhibited cytotoxicity toward cell lines as shown in Table 1.

Table 1 Cytotoxicity of mycoepoxydiene

Cell lines	IC_{50}, µg/ml	References
Human chronic myelogenous leukemia cell line (K562)	2.30	(Takao *et al*., 2004)
Human hepatocellular carcinoma cell line (HepG2)	3.10 2.25	(Takao *et al*., 2004) (Prachya *et al*., 2007)
Human lung carcinoma cell line (A549)	1.95	(Prachya *et al*., 2007)
Hepatocellular carcinoma cell line (HCC-S102)	2.80	(Prachya *et al*., 2007)
Human cholangiocarcinoma cancer cells (HuCCA-1)	0.27	(Prachya *et al*., 2007)
Human epidermoid carcinoma of the mouth (KB)	2.33	(Prachya *et al*., 2007)
Cervical adenocarcinoma cell line (HeLa)	1.93	(Prachya *et al*., 2007)
Human breast cell line (MDA-MB231)	1.30	(Prachya *et al*., 2007)
Human mammary adenocarcinoma cell line (T47D)	2.57	(Prachya *et al*., 2007)
Human promyelocytic leukemia cell line (HL-60)	0.79	(Prachya *et al*., 2007)
Murine leukemia cell line (P388)	0.73	(Prachya *et al*., 2007)
Human breast cancer (MCF-7)	2.24	(Sommart *et al*., 2009)

2.4 Fermentation methods

In the pharmaceutical and biotechnology industries, fermentation is a large-scale cultivation of microbes or other single cells. In the research, fermentation is often performed in a test tube, flask, or bottle in volumes from a few milliliters to two liters. At the production and manufacturing level, large vessels called fermentors or bioreactors are used. Today, applied fermentations are performed to improve production of secondary metabolites such as solid state fermentation method, static/shaking fermentation method, solid supports fermentation method, fed-batch fermentation method and solid-liquid two phase partitioning fermentation method.

2.4.1 Submerged fermentation method

Fungal fermentations in liquid media, aside from alcoholic beverages such as wine, beer and fermented milk, came about in the pre-penicillin days. As far as can be studied, Kluyver and Perquin (1933) were the first researchers that published and illustrated a method of using liquid medium with agitation to produce a fungal metabolite. They exhibited their paper with a figure of a flask of liquid medium containing *Aspergillus flaws* mycelium balls in a kojic acid fermentation. To produce mycelium that was homogeneous, was the objective of Kluyver and Perquin's research. This cannot be done if fungus colonies grow on the surface of liquid or solid medium (Bulder *et al*., 1989).

Agitation of liquid substrates in flasks was adopted for most mold fermentations and was used initially in the development of penicillin. The determination of nutrients, fermentation time, improved strains, agitation rate, and product recovery were made in shaken flasks on a variety of shaker. The first commercial penicillin was produced by growing *Penicillium* on the surface of static liquid medium. This technique was rapidly substituted by fermentation in which amount of gallons with liquid media were vigorously agitated in tanks. This was the practice for the fungus-like *Streptomyces* antibiotic fermentations (Hesseltine, 1987).

Submerged liquid culturing is usually preferred for large-scale fermentations and has been used extensively for industrial production of antibiotics, amino acids, ethanol, organic acids, baker's and distiller's yeasts. This technological experience provides a critical knowledge base as well as industry acceptability for production of secondary metabolites. Submerged fermentation is considered more readily available, economical, and practical than other methods for mass production of secondary metabolites (Yu *et al*., 1997). It is generally believed that liquid fermentation is preferred or required to produce low-cost metabolite products (Jackson, 1997). One-stage submerged cultivation can produce spores faster than other methods (Rombach, 1989). Equipments (flasks or bioreactors) of this process can be utilized without modification. Liquid cultures are homogenous, which makes them simpler to control,

maintain, and monitor (Jackson, 1997). Parameters affecting microbial growth and secondary metabolite production, such as heat, pH, nutrient conditions, and oxygen, can also be controlled in liquid fermentation. The metabolites produced in this fermentation are usually relatively easy to recover using centrifugation or filtration methods, which are normally more efficient than most harvest techniques used in solid-state fermentation. Liquid media are generally easier to be sterilized compared to solid media and may be more readily applied with scale-up (Robinson *et al*., 2001). These benefits may account for low cost of production (Jackson, 1997). However, submerged fermentation can also have a detrimental effect on product formation, because its yield is proportional to the amount of biomass. The high cell density causes the increase of viscous liquid media and the difficulty in stirring and oxygen mass transfer. Therefore, solid state fermentation technology can be an alternative strategy, allowing better oxygen circulation (Wei *et al*., 2007).

2.4.2 Solid state fermentation method

Solid state fermentations are those processes in which all or parts of the substrate are in a solid state (Hesseltine, 1972). They are more natural than liquid fermentations because the growth conditions mimic natural growth habitat of microorganism. While some groups of microorganisms, such as some bacteria and yeasts, grow in liquid conditions, a large number of microorganisms grow on solid substrates. The major groups of microorganisms used in solid state fermentation are bacteria, actinomycetes, yeasts, and fungi.

Frequently, bacteria grow on a solid object in the soil, on the roots of plants, inside plants, and on leaf surfaces. The human and animal pathogens also grow on surfaces. For example, *Streptococcus*, which brings about illness in human, grows on the throat cell surface. A pile of bacteria is a good example of a multiple culture solid state fermentation. Bacterial cell walls are composed of exopolysaccharides, which attach the cell to solid matrix surfaces. Most bacteria in nature grow in/on a solid state matrix but some of them are free of attachment as they grow in water, blood, and milk.

Although many species and genera of actinomycetes inhabit in soil, some actinomycetes grow and multiply in water.

Fungi grow mostly on or in solid material e.g. *Aspergillus* species typically grow on and in cereals, soil matrix and plant material. Yeasts certainly grow and reproduce freely in fruit juices and other liquids containing carbohydrates but also may grow on solid substrates.

Therefore, it is not surprising that solid state fermentations have been used and are still used widely. Because the growth conditions are similar to those fermentations which many organisms have grown for a long time (Hesseltine, 1987).

Traditionally, solid substrate fermentation has been widely used in the east to manufacture fermented foods such as soy sauce, sake etc. In the western zone briefly practiced solid substrate fermentation for the production of antibiotics and enzymes was substituted by submerged fermentation which provided the higher benefits in terms of automation and large scale fermentation (Mazumdar-Shaw and Suryanarayan, 2003). Due to the great success of large-scale submerged fermentation processes, solid state fermentation was almost completely neglected (Larroche and Gros, 1997). However, over the past 15–20 years, solid state fermentation has been the interesting process because it exhibits certain advantages over submerged fermentation (Barrios-Gonzalez and Mejia, 1996). A brief summary of historical evolution of solid state fermentation is shown in Table 2.

Solid state fermentation systems have a successful application for the production of microbial enzymes (Palma *et al*., 2000), fine chemicals (Shojaosadati and Babaeipour, 2002), antibiotics (Lotfy, 2007), and immunosuppressants (Sekar *et al*., 1997). In antibiotics and other secondary metabolites production, solid state fermentation systems are associated with higher yields in shorter time periods compared to the alternative submerged fermentation approach (Barrios-González and Mejýa, 2008). Furthermore, some antibiotics are only produced in solid state fermentation system (Bigelis *et al*., 2006). The reason for this different physiology in solid state fermentation is not completely understood, but it is often called “physiology of

solid medium". Table 3 presents several advantages of solid state fermentation over the traditionally employed submerged fermentation and disadvantages of solid state fermentation.

Table 2 History of development of solid state fermentation (Pandey, 1992)

Period	Development
2,600 BC*	Bread making by Egyptians
BC in Asia (recorded history 1,000 BP**)	Cheese making by *Penicillium roqueforti*
2,500 BP	Fish fermentation/preservation with sugar, starch, salts, etc. Koji process
7th Century	Koji process from China to Japan by Buddhist priests
18th Century	Vinegar from pomace, Gallic acid used in tanning, printing, etc
1860-1900	Sewage treatment
1900-1920	Fungal enzymes (mainly amylases), kojic acid
1920-1940	Fungal enzymes, gluconic acid, rotary drum fermentor, citric acid
1940-1950	Fantastic development in fermentation industry. Penicillin production by solid state fermentation and submerged fermentation
1950-1960	Steroid transformation by fungal cultures
1960-1980	Production of mycotoxins, protein enriched feed
1980-present	Various other products like alcohol, gibberellic acid

*BC, before Christ

**BP, before present

Table 3 Advantages and disadvantages of solid state fermentation over submerged fermentation (Pérez-Guerra *et al*., 2003)

Advantages	Disadvantages
Similar or higher yields than those obtained in the corresponding submerged cultures	Only microorganisms that can grow at low moisture levels can be used
The low availability of water reduces the possibilities of contamination by bacteria and yeast. This allows working in aseptic conditions in some cases	Usually the substrates require pre-treatment (size reduction by grinding, rasping or chopping, homogenization, physical, chemical or enzymatic hydrolysis, cooking or vapor treatment)
Similar environment conditions to those of the natural habitats for fungi which constitute the main group of microorganisms used in solid state fermentation	Biomass determination is very difficult
Higher levels of aeration, especially adequate in those processes demanding an intensive oxidative metabolism	The solid nature of the substrate causes problems in the monitoring of the process parameters (pH, moisture content, and substrate, oxygen and biomass concentration)
The inoculation with spores (in fungal processes) facilitates their uniform dispersion through the medium	Agitation may be very difficult. For this reason static conditions are preferred
Culture media are often quite simple. The substrate usually provides all the nutrients necessary for growth	Frequent need of high inoculum volumes
Simple design reactors with few spatial requirements can be used due to the concentrated nature of the substrates	Many important basic scientific and engineering aspects are yet poor characterized. Information about the design and operation of reactors on a large scale is scarce
Low energetic requirements (in some cases autoclaving or vapor treatment, mechanical agitation and aeration are not necessary)	Possibility of contamination by undesirable fungi
Small volumes of polluting effluents. Fewer requirements of dissolvents are necessary for product extraction due to its high concentration	The removal of metabolic heat generated during growth may be very difficult
The low moisture availability may favor the production of specific compounds that may not be produced or may be poorly produced in submerged fermentation	Extracts containing products obtained by leaching of fermented solids are often of viscous nature
In some cases, the products obtained have slightly different properties (e.g. more thermotolerance) when produced in solid state fermentation in comparison to submerged fermentation	Mass transfer limited to diffusion
Due to the concentrated nature of the substrate, smaller reactors in SSF with respect to submerged fermentation can be used to hold the same amounts of substrate	In some solid state fermentation, aeration can be difficult due to the high solid concentration
	Spores have longer lag times due to the need for germination
	Cultivation times are longer than in submerged fermentation

Two types of solid state fermentation systems can be distinguished depending on the nature of the solid phase used. The first and most commonly used system is based on cultivation on natural solid substrates. The second system is based on cultivation on impregnated inert supports with a liquid medium (Barrios-González *et al.*, 2008). The cultivation on natural substrates system uses natural materials as a support and a nutrient source. These materials are typically agricultural products or agro-industrial sources such as grains, cassava, potato, beans and sugar beet. The substrate costs cheap and is widely available (Wei *et al.*, 2007). However, natural substrates have a major problem that the carbon source is a part of their structure. During the microorganism fermentation, the substrates are degraded and affected the geometric and physical characteristics of the medium (Oriol *et al.*, 1988). Consequently, heat and mass transfer can be decreased. This disadvantage can be overcome by the use of an inert support with a more or less constant physical structure throughout the process, enabling improved control of heat and mass transfer. The solid support of the second system can be natural origin or synthetic materials such as hemp, perlite, polyurethane foam (PUF), sugarcane bagasse and vermiculite. The advantage of inert supports is less complicated product recovery. The products can be simply extracted from the inert support with fewer impurities compared to the natural substrates. Other advantages of using inert support are the ability to design the proper production media and control process with easily because all components of the production medium are known and can be analyzed. The use of supports impregnated with liquid media offers the medium creation for the optimal production of metabolites. Moreover, the effects of medium components on the growth and physiology of the microorganism, and on the production of metabolites can be determined when using the defined media. In addition, process consistency is increased because the defined medium enhances more reproducible process. Conversely, the heterogeneity or batch-to-batch variation of the natural media results in variation of process performance (Ooijkaas *et al.*, 2000).

Lovastatin is a fungal secondary metabolite with capable of lowering the cholesterol level in blood. It is the best selling pharmaceuticals in the USA. Barrios-

González *et al*. (2008) developed the lovastatin production process by solid state fermentation on polyurethane foam. This system could induce a large productivity, as a 30 times higher lovastatin production was obtained, compared to submerged fermentation conditions. The difference in lovastatin production between both culture systems correlates with difference in expression of genes involved in lovastatin production. The higher lovastatin production in solid state fermentation relates with higher transcription of its biosynthesis genes (Barrios-González *et al*., 2008).

Application of submerged fermentation and solid state fermentation

Using microorganisms to carry out processes and fermentations goes back to primitive age. Table 4 lists products that produced by using solid substrate and liquid media. In ancient fermentation products, a large number of useful microbial processes were employed on solid substrate. Many of these solid substrate fermentations are still used at present (Costerton, 1984). Frequently, more traditional fermentation products are still made by solid state fermentation than by liquid fermentation. However, many new fermentation products are produced by liquid fermentation, as shown in Table 5 (Hesseltine, 1987).

Table 4 Ancient fermentation products

Solid state	Liquid
Sauerkraut	Wine
Koji (for rice wine, miso, and shoyu)	Acid milks
Cheese	Beer
Bread	Vinegar
Mushrooms	Yogurt
Shoyu	
Miso	
Natto	
Tempeh	
Ragi and related starters	
Fermented fish	
Fermented vegetables	
Sausages	
Silage	
Tea, coffee, vanilla, and cocoa	
Compost	
Kaffir beer	
Decomposition of plant and animal material	

Table 5 Modern fermentation products (Hesseltine, 1987)

Solid state	Liquid
Enzymes	Penicillin
Mycotoxins	Other antibiotics
Clean water from trickling filters	Citric acid
	Gluconic acid
	Torula and bakers yeast
	Enzymes
	Steroid transformations
	Amino acids
	Xanthan gum
	Vitamin B_{12}
	Riboflavin
	Lactic acid

2.4.3 Static/shaking fermentation method

Static/shaking fermentation method is a process which performs preincubation without agitation period allowing a mycelial mat to form on medium surface, followed by agitation for weeklong period. Bigelis *et al.* (2006) used static/shaking fermentation method to produced anthraquinones and flavomannin by *Penicillum* sp. *LL*-WF159 in liquid fermentation and compared between static/shaking and conventional fermentation method. In the first procedure, the organism was preincubated with static for 1 week, and then shaken for 1 week at 200 rpm. In the second procedure, it was agitated for 2 weeks. The relative yields of rugulosin, skyrin, flavomannin and compound WF 159-A were determined. It was found that the production of four bioactive compounds was increased in static/shaking fermentation method, as shown in Table 6. Furthermore, the levels of the four bioactive compounds

were further increased by the addition of a polymeric disc and forming of an artificial mycelial mat.

Table 6 Relative yields (mg/L) of four metabolites in shaken and stationary/shaken fermentations with *Penicillium* sp. *LL*-WF159 (Bigelis *et al*., 2006)

Disc added to PDB	Flavomannin		Compound WF159-A		Rugulosin		Skyrin	
	Shake[a]	Stat/shake[b]	Shake[a]	Stat/shake[b]	Shake[a]	Stat/shake[b]	Shake[a]	Stat/shake[b]
PDB only	418	4,205	184	460	1,955	4,586	245	1,068
Polyurethane	3,784	9,384	779	758	8,784	6,778	984	1,246
Polypropylene 1	140	9,049	1,167	1,048	5,918	7,578	942	1,759
Polypropylene 2	89	9,683	606	889	6,287	7,563	1,448	1,352
Polypropylene cellulose	316	6,197	1,003	758	5,407	7,852	829	1,573
Polyester–cellulose	157	4,585	778	533	4,020	6,329	397	1,037

All productive cultures were dark yellow to yellow-orange revealing colors characteristic of anthraquinones. Static 2-week cultures contained pale white, nonpigmented mycelia

[a]Shaken 14 days at 200 rpm

[b]Static 7 days, then shaken 7 days at 200 rpm

2.4.4 Solid supports fermentation method

The solid supports fermentation method is a process that uses an inert solid support bearing liquid medium. This condition increases the surface area for fungal growth on the solid supports during fermentation and the tendency of microorganisms to adhere to solid surfaces, possibly mimicking their natural growth habits (Bigelis *et al*., 2006). Solid supports may be natural materials or a variety of synthetic materials that possess properties as the followings: inert and non-toxic to fungus, resistance to degradation and metabolize from culture and ability to be autoclaved.

Bigelis *et al.* (2006) investigated the growth of *Penicillium* sp. *LL*-WF159 in shake-flasks containing a polymeric disc. The organism was grown in PDB with and

without discs composing of polyurethane, polypropylene (two types), polypropylene cellulose, or polyester–cellulose. Two different fermentation protocols were employed, and the relative yields of the four antibiotics were determined. All shake-flasks were either agitated for 2 weeks, or they were maintained static for 1 week followed by a period of shaking for 1 week. The support matrices permitted mycelial attachment and growth and elevated relative metabolite yields as determined by HPLC analysis (Table 6).

The levels of the four antibiotics could be further elevated by the addition of a polymeric disc, as shown in Table 6. The physical nature of the support system influenced the relative yields of rugulosin, skyrin and flavomannin to varying degrees (Table 6). In general, the relative yields were increased, though in a few cases, the polymeric disc lowered metabolite production owing to possible interference with mixing and aeration (Bigelis *et al*., 2006).

2.4.5 Fed-batch fermentation method

Fermentations can be operated in batch, fed-batch or continuous process. In batch process all components are included in the beginning of the fermentation. During process there are neither input nor output flows until complete process. In fed-batch process, no culture is removed during the fermentation, but supplementary growth medium is added in system due to increasing metabolite products in fermentation process. There are both input and output flows in a continuous process, but the volume of fermentation is kept stable. Fed-batch process is widely used in industrial applications because they combine the advantages of both batch and continuous processes. Initial process is started as a batch process and the new substrate will be added when the microbial growth reaches the steady state. The fermentation is continued at a certain growth rate until some limitation inhibits the cell growth (Irving *et al*., 2005). The use of fed-batch culture takes advantage of the fact that it enhanced high cell densities due to extension of working time, the concentration of specific substrates were controlled during fermentation, the production of byproduct

and catabolite repression was limited owing to addition of substrates solely required for product formation (Sengupta and Modak, 2001), and the replacement of water lost during fermentation process.

Penicillin G production is an example for the use of fed-batch in the production of a secondary metabolite. The fermentation is separated in two phases: the rapid-growth phase and the slow-growth phase in which penicillin is produced. Phosphorus plays the important role in both phases. During the rapid-growth phase, phosphorus is associated with the increase of biomass due to a cellular component of nucleic acids, lipids, membrane, and cell wall. Insufficient phosphorus supply in this phase leads to nutrient imbalance and inhibition of mycelium growth. During the production phase, phosphorus is commonly involved in regulation of secondary metabolite biosynthesis through gene transcriptions. Therefore, feeding a suitable concentration of phosphorus is an efficient and feasible way to improve the productivity of penicillin G (Li *et al*., 2005). The similar repeated fed-batch fermentation processes were applied in production of many products, such as cephalosporin C (Silva *et al*., 2001), lovastatin (Kumar *et al*., 2000), dihydroxyacetone (Hekmat *et al*., 2003), and lysine (Pfefferle *et al*., 2003).

2.4.6 Solid-liquid two phase partitioning fermentation method

Two phase partitioning system consists of aqueous phase and immiscible liquid organic phase or solid adsorbents in system that absolutely separates each other (Collins and Daugulis, 1996). The later phase acts as a sponge that effectively absorbs/adsorbs and partitions concentrations of toxic compounds from the aqueous phase (Prpich and Daugulis, 2005). Immiscible organic solvents have been prosperously used in two phase partitioning system for the removal of toxic compounds from solution. A limitation of these systems is the toxicity of the organic solvent to biocatalyst. This has been exhibited in the lengthy lag phases and premature termination of the bioprocess owing to the cumulative toxic effect of both product and solvent. Solid adsorbents, such as polymeric resins (Amberlite™ XAD), and

thermoplastic polymers, represent an alternative to organic solvents in two phase partitioning system. These materials have been revealed to successfully remove toxic factors resulting in the higher overall yields and productivities. A limitation of solid adsorbents may be the non-specific adsorption of organic compounds from solution.

XAD-7, synthetic neutral resins, is a polymeric adsorbent and is moderate polar. It is a non-ionic aliphatic acrylic polymer which derives its adsorptive properties from its structure. This property makes Amberlite XAD-7 to have an excellent physical and thermal stability. Due to its aliphatic nature, it can adsorb non-polar compounds from aqueous systems, and can also adsorb relatively polar compounds from non-aqueous solvents. Applications of Amberlite XAD-7 (Rohm and Haas Singapore Pte. Ltd. 2002) from the product data sheet claimed the following properties:

1. Removal of relatively polar compounds from non-aqueous solvents
2. Removal of non-aromatic compounds from polar solvents
3. Recovery of plant extracts
4. Recovery of antibiotics, enzymes and proteins
5. Removal of organic pollutants from aqueous wastes, ground water and vapor streams
6. Enzyme immobilization

Based on the structure of Amberlite XAD-7, it can be used to adsorb compounds which compose of ester or/and ketones groups including aliphatic structure molecules from an aqueous solution due to the principle “like attracts like”. Hence, some reports was applied to recover and separate stevioside that have ester group in molecule from crude extract which corresponded with the second case of applications (Hafizuddin *et al.*, 2003).

Thermoplastic polymers in solid phase have been implemented for the enhanced bioproduction of 3-methylcatechol from toluene via *Pseudomonas putida* MC2 by integration of fermentation and recovery process. 3-Methylcatechol is a precursor in the production of fine chemicals such as polycyclic pyrimidinone. 3-Methylcatechol has been shown to impact toxic effects on the biocatalyst,

Pseudomonas putida MC2, and leading to inhibition of growth followed by biocatalytic inactivity. To solve this solution, HYTREL™, a thermoplastic polymer, was used to recover 3-methylcatechol from the culture broth. HYTREL™ polymer was studied in three designs: a 10% (w/w) solid (polymer beads), a polymer sheet and a polymer beads packed in external extraction column. A batch biotransformation containing a 10% (w/w) polymer beads resulted in an overall 3-methylcatechol productivity of 353 mg/L-h, which compares to the single phase productivity of 128 mg/L-h. HYTREL™ polymer sheet around the circumference of the bioreactor could remove 3-methylcatechol from solution resulting in 3-methylcatechol productivity of 344 mg/L-h. Lastly, a continuous biotransformation with external extract column yielded the overall 3-methylcatechol productivity of 270 mg/L-h (Prpich and Daugulis, 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Culture media and chemicals

3.1.1 Culture media

Culture media used for cultivation of endophytic fungi, *Phomopsis* sp. Hant25, were potato dextrose agar (PDA) (Merck), malt extract (Merck), yeast extract (Merck), peptone from soymeal (Merck), agar base (agar-agar ultrapure granulated, Merck), malt Czapek medium (agar and broth) (MCzA, MCzB) (Paterson and Bridge, 1994), Czapek yeast autolysate broth (CzYB) (Paterson and Bridge, 1994), and Modified M1D medium (MM1D) (Pinkerton and Strobel, 1976). MCzB, CzYB and MM1D were prepared according to a formula shown in Appendix A. Culture medium for *C. albicans* in the antifungal assay was Sabouraud's dextrose agar (SDA) (Merck).

3.1.2 Chemicals

Chemicals used in this study are as the followings: boric acid (H_3BO_3) (Merck, GR), ammonium tartrate (Merck, GR), sodium nitrate ($NaNO_3$) (BHD, AR), sodium chloride (NaCl) (Merck, GR), sodium dihydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (Merck, GR), calcium dinitrate tetrahydrate ($Ca(No_3)_2).4H_2O$ (Merck, GR), potassium nitrate (KNO_3) (Merck, GR), ferric chloride ($FeCl_3$) (Merck, GR), manganese sulphate ($MnSO_4$) (Merck, GR), potassium iodide (KI) (Merck, GR), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) (Merck, GR), potassium chloride (KCl) (RiedeldeHaen, AR), dipotassium hydrogen phosphate (K_2HPO_4) (Merck, GR), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (Merck, GR), copper sulphate heptahydrate ($CuSO_4.7H_2O$) (Merck, GR), ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Merck, GR),

sucrose (commercial grade), 95 % ethanol (industrial grade), methanol (CH_3OH) (Labscan, AR), dichloromethane (CH_2Cl_2) (Labscan, AR), ethyl acetate (EtOAc) (Labscan, AR), dimethylsulfoxide (DMSO) (Fisher Scientific, AR), acetonitrile (Fisher Scientific, HPLC grade) and acetic acid (Merck, AR). For antifungal drug, ketoconazole (USP24; Karingo, Italy), was kindly provided by Siam Pharmaceutical Co., Ltd.

3.2 *Candida albicans* strain

Candida albicans ATCC 90028 was used for testing throughout this study. *C. albicans* was maintained on Sabouraud 2% dextrose agar (SDA).

3.3 Sources of endophytic fungus and inoculum preparation

Endophytic fungus *Phomopsis* sp. Hant25 was isolated from the twigs of Thai medicinal plant, *Hydnocarpus anthelminthicus* Pierre ex Laness (family Flacourtiaceae) (Prachya *et al.*, 2007). The fungus was stored in sterile distilled water at 2-8° C. Inoculum was prepared by growing the fungus on PDA at room temperature for 7 days. After that, the fungus was cut by cork borer no. 4 in the same radius, as shown in Figure 3 and taken as the inoculum.

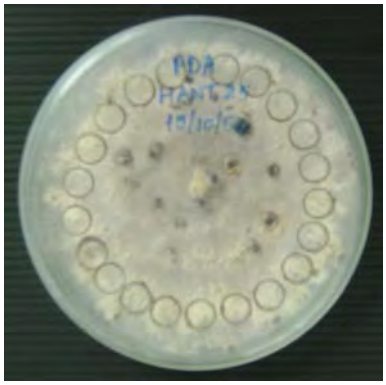


Figure 3 *Phomopsis* sp. Hant25 inoculum on PDA

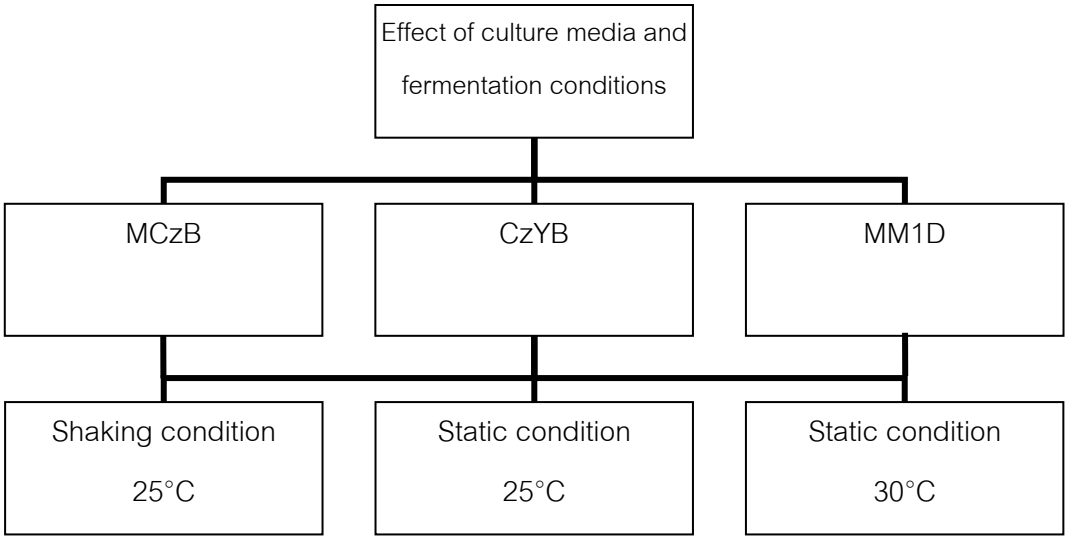
3.4 Preliminary submerged fermentation for mycoepoxydiene production

Inoculum culture of *Phomopsis* sp. Hant25 was prepared as described above in Section 3.3. Two pieces of inoculum were grown in 250 mL Erlenmeyer flasks (Pyrex No. 4442) containing 50 mL of MCzB and further incubated under stationary condition at 25°C for 21 days. After incubation, the whole of sample in each flask was taken periodically every 3 days. Fungal mycelia were then separated from culture broth by using vacuum pump system. Mycoepoxydiene concentration in the culture broth was measured by HPLC. The fungus mycelia were dried at 70°C until the weight was stable. The dried mycelia were weighed to get cell dry weight.

3.5 Alternative fermentation and processing of cultures

3.5.1 Determination of effects of culture media and fermentation conditions on mycoepoxydiene production

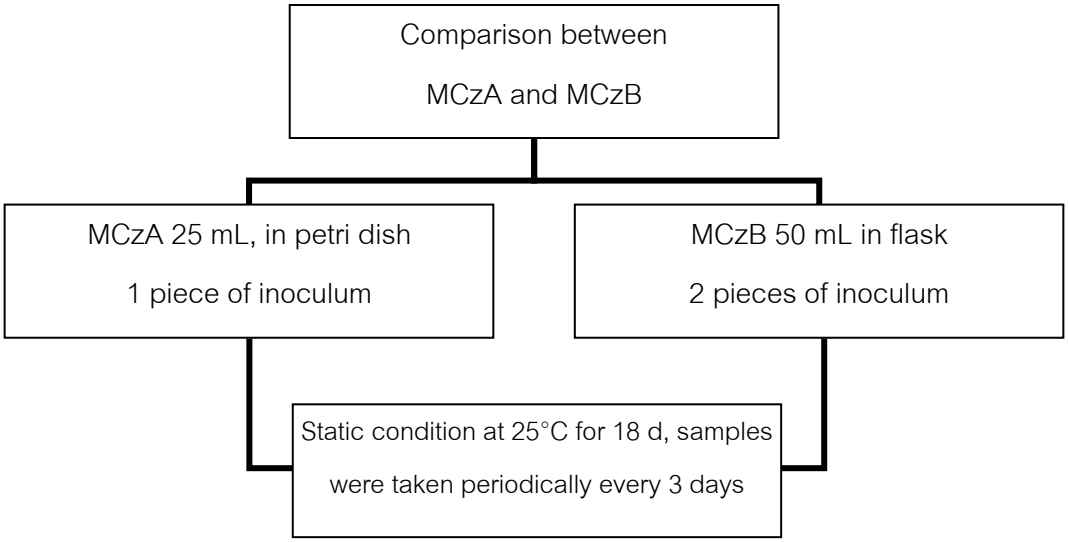
Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of CzYB, MCzB and MM1D. The experiment was performed in triplicate. Each inoculated medium was incubated for 36 days in 3 conditions: stationary conditions at 25° C and 30°C, and shaking condition at 120 rpm 25°C, as shown in Scheme 1. During cultivation, culture broth in a volume of 1.5 mL from each flask was taken periodically every 3 days. Relative quantity of mycoepoxydiene in collected culture broth was detected by agar diffusion assay as described in Section 3.8.



Scheme 1 Experimental design for determination of effects of culture media and fermentation conditions on mycoepoxydiene production

3.5.2 Comparison between submerged fermentation and solid state fermentation in malt Czapek medium on mycoepoxydiene production

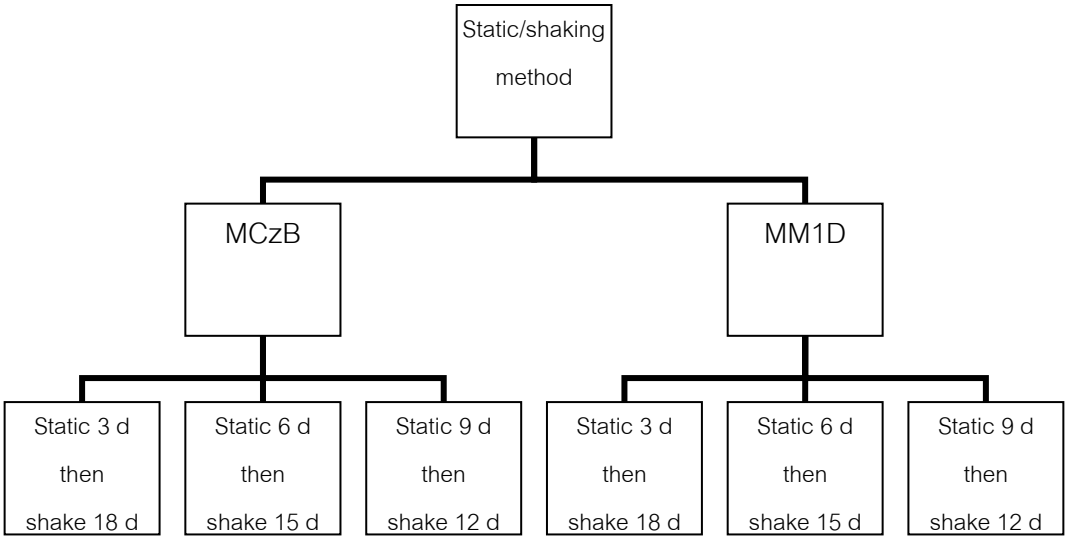
Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MCzB and one piece of inoculum was cultured in 20 X 90 mm petri dish containing 25 mL of MCzA. The experiment was performed in triplicate. The inoculated media were incubated in stationary condition at 25° C for 18 days. Scheme 2 presents experimental design of this experiment. During cultivation, 3 flasks and 3 plates of samples were taken periodically every 3 days. The whole of culture broth from each flask and the whole of culture agar from each plate were extracted with ethyl acetate as described in Sections 3.6.1 and 3.6.2, respectively. Relative quantity of mycoepoxydiene in crude extract was detected by TLC bioautography analysis as described in Section 3.9.



Scheme 2 Experimental design for comparison between submerged fermentation and solid state fermentation in malt Czapek medium on mycoepoxydiene production

3.5.3 Static/shaking fermentation method on mycoepoxydiene production

This method is performed by preincubation in stationary condition that allowed a mycelial mat to form on medium surface, followed by agitation for weeklong period. Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MCzB and MM1D. The experiment was performed in triplicate. Both media were divided into three groups and incubated at static condition for 3, 6 and 9 days at 25°C. In the next step, each group was shaken for 18, 15 and 12 days, respectively, at 120 rpm 25°C. Scheme 3 presents experimental design of this experiment. During cultivation, culture broth in a volume of 1.5 mL from each flask was taken periodically every 3 days. Relative amount of mycoepoxydiene and mycoepoxydiene in culture broth were determined by agar diffusion assay and HLPC as described in Sections 3.8 and 3.10, respectively.



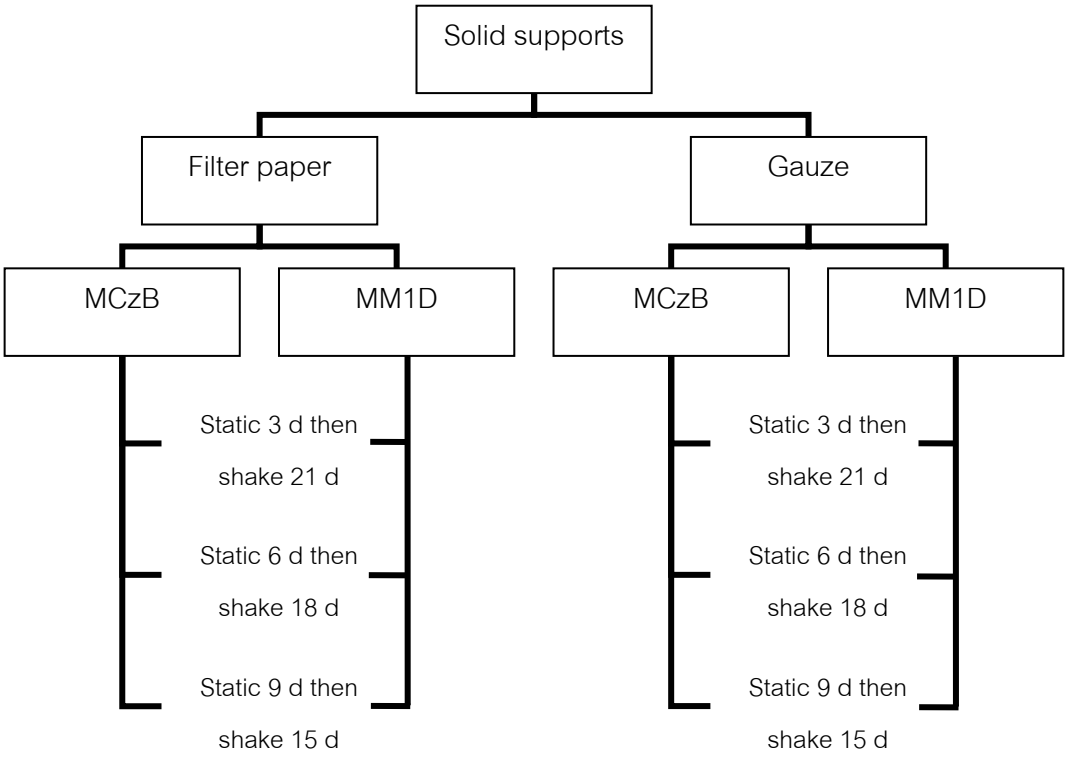
Scheme 3 Experimental design of static/shaking fermentation method on mycoepoxydiene production

3.5.4 Solid support fermentation method on mycoepoxydiene production

The solid support fermentation is a method that uses an inert solid support bearing liquid medium which increases surface area for fungal growth on the solid supports during fermentation. In this experiment, filter paper (Whatman chromatography paper, Cat no. 3001690) and four layers of gauze (Medigauz® absorbent cotton gauze, Thailand) were employed and put in MCzB and MM1D media before sterilization. They were 7 cm in diameter. These two materials were selected according to the following properties: (i) the ability to withstand sterilization by autoclave; (ii) the flexibility to insert into flask; (iii) being inert and non-toxic to fungus; (iv) fungus attachment on solid supports, and (v) being not utilized as substrate for fungus.

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of media with solid supports. Cultures were first maintained in stationary condition for 3, 6 and 9 days, and then shaken for 21, 18 and 15 days, respectively at 120 rpm 25°C. During cultivation, culture broth in a volume of 1.5 mL from each flask was taken periodically every 3 days. Relative amount of mycoepoxydiene and mycoepoxydiene in culture broth were determined by agar

diffusion assay and HLPC as described in Sections 3.8 and 3.10, respectively. The experiment was performed in triplicate. Scheme 4 summarizes experimental design of this experiment.



Scheme 4 Experimental design of solid support fermentation method on mycoepoxydiene production

3.5.5 Floating solid support fermentation method on mycoepoxydiene production

The floating solid supports fermentation is performed by using a floating inert solid support bearing liquid medium to increase surface area for fungal growth during fermentation. In this experiment, polypropylene (PP, non-woven polypropylene spunbound) (7 and 8.5 cm dia. for flask and Petri dish, respectively) was selected owing to floating property which separated between fungus and medium, containing of pores or holes on material that allowed mycelia to absorb nutrient from media, and other

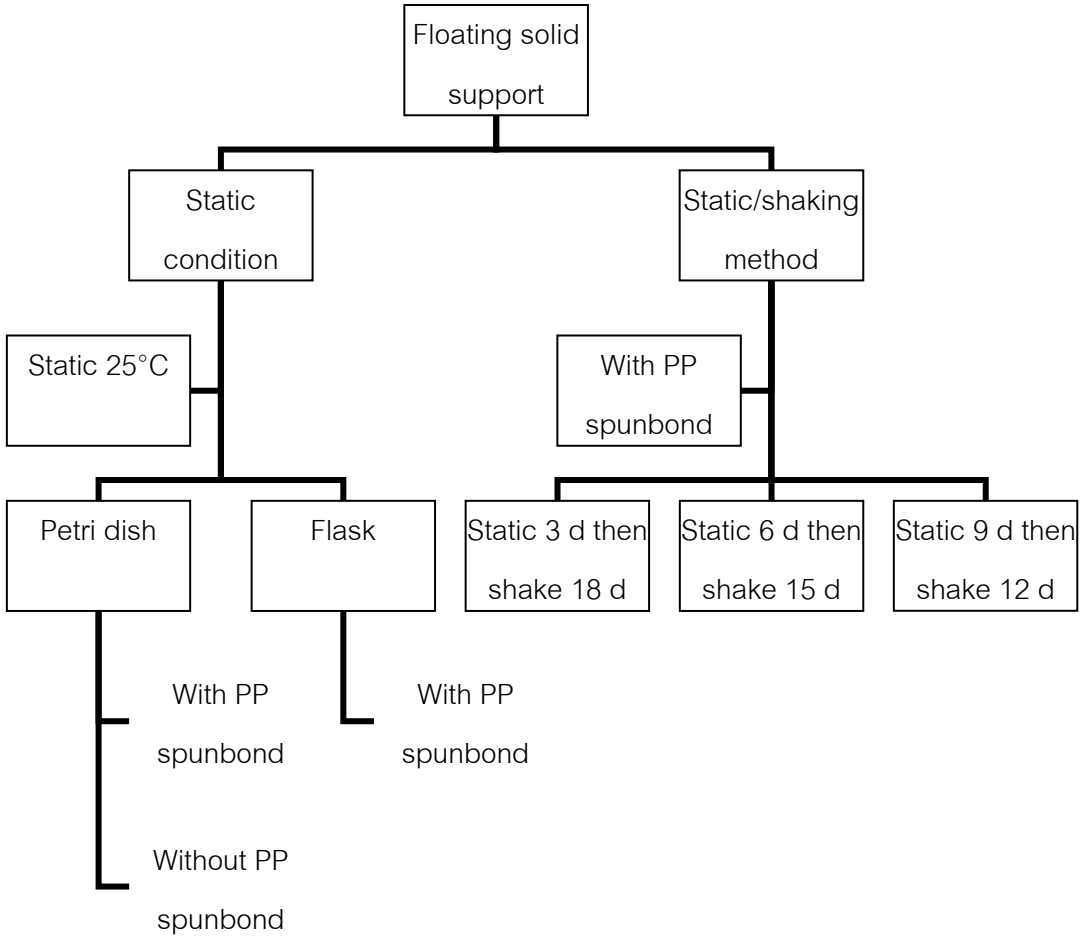
suitable properties as described previously for solid support in Section 3.5.4. This method consists of two experiments with different fermentation conditions.

3.5.5.1 Static condition

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MCzB and MM1D. One piece of inoculum was cultured in 20 X 90 mm Petri dish containing 25 mL of MCzB and MM1D. Both containers were divided into 2 groups: with and without PP support. These were done in triplicate. The inoculated media was cultured in stationary condition at 25° C for 21 days. During cultivation, 3 samples from each group were taken periodically every 3 days and processed as follows: (i) culture broth in a volume of 1.5 mL from each container was taken to determine the relative amounts of mycoepoxydiene by agar diffusion assay and mycoepoxydiene by HPLC as described in Sections 3.8 and 3.10, respectively; (ii) the whole of sample remained from each container was filtered using vacuum pump. The culture broth filtrate was extracted with ethyl acetate yielding crude extract as described in Section 3.6.1. Relative quantity of mycoepoxydiene in crude extract was detected by TLC bioautography analysis as described in Section 3.9.

3.5.5.2 Static/shaking condition

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MCzB and MM1D with PP support (7 cm dia.). The experiment was performed in triplicate. Cultures were first maintained in stationary condition for 3, 6 and 9 days, and then shaken for 18, 15 and 12 days, respectively at 120 rpm 25°C. During cultivation, culture broth in a volume of 1.5 mL from each container was taken periodically every 3 days. Relative amounts of mycoepoxydiene and mycoepoxydiene in culture broth were determined by agar diffusion assay (as described in 3.8) and HPLC (as described in 3.10), respectively. Scheme 5 summarizes the experimental design of this experiment.

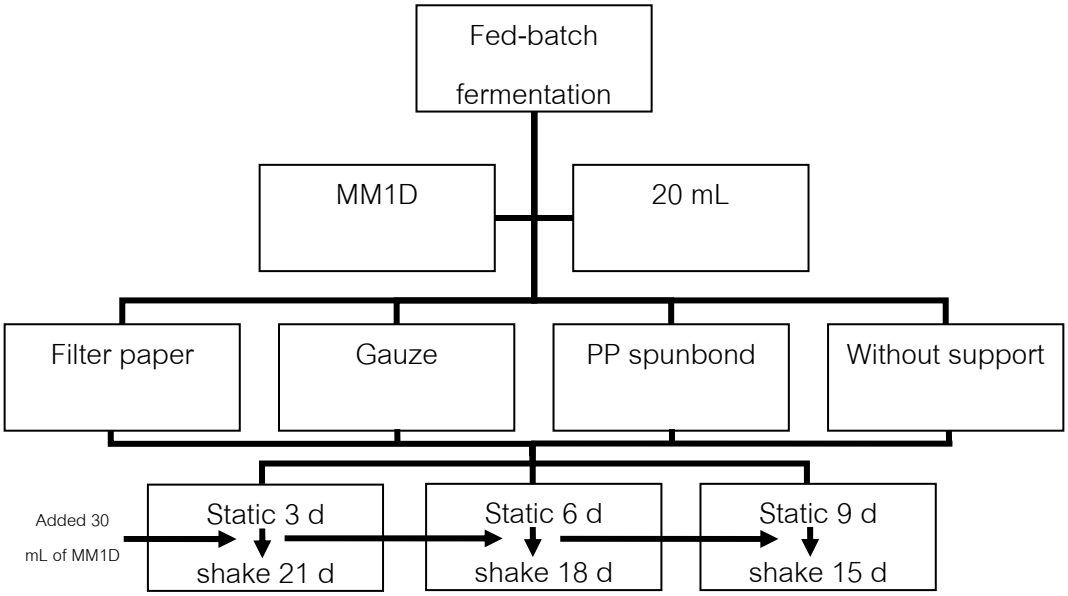


Scheme 5 Experimental design of floating solid support fermentation method on mycoepoxydiene production

3.5.6 Fed-batch fermentation method on mycoepoxydiene production

Fed-batch fermentation is a production technique which supplementary growth medium is added during the fermentation, but no culture is removed until the end of the batch. In this experiment, filter paper, four layers of gauze and PP were employed to determine the effect of solid supports on mycoepoxydiene production in the fed-batch fermentation. These materials were 7 cm in diameter. Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 20 mL of MM1D. Cultures were maintained in stationary condition for 3, 6 and 9 days, and then 30 mL of MM1D was added into each flask. Consequently, cultures were shaken for 21, 18 and

15 days, respectively at 120 rpm, 25°C. During cultivation, culture broth in a volume of 1.5 mL from each flask was taken periodically every 3 days. Relative amounts of mycoepoxydiene and mycoepoxydiene in culture broth were determined by agar diffusion assay (as described in Section 3.8) and HPLC (as described in Section 3.10), respectively. The experiment was performed in triplicate. Scheme 6 summarizes the experimental design of this experiment.



Scheme 6 Experimental design of fed-batch fermentation method on mycoepoxydiene production

3.5.7 Solid-liquid two phase partitioning fermentation method on mycoepoxydiene production

3.5.7.1 Pretreatment of Amberlite XAD-7 beads

Amberlite XAD-7 (Fluka Chemie AG, Switzerland) in bulk quantity was supplied as a water-wet product imbibed with sodium chloride and sodium carbonate salts to retard bacterial growth. Before use, the resin was washed with deionized water to remove the salts and then dried at 50°C.

3.5.7.2 Determination of adsorption of mycoepoxydiene on Amberlite XAD-7 beads and elution in small scale

Culture MM1D broth collected in Section 3.5.4 was used in this study. Mycoepoxydiene concentration in the MM1D broth was analyzed by HPLC. Then, washed Amberlite XAD-7 was added into 1 mL of this culture broth in a final concentration of 1, 2, 3, and 4 % (w/v). Each group was performed in duplicates. All of them were mixed by Fisher Roto-Rack machine for 1 h, then the culture broth and beads were separated. Mycoepoxydiene remained in the culture broth and that adsorbed in the beads were determined by HPLC. Before analysis, mycoepoxydiene in the bead residue from one set of each sample was eluted by ethyl acetate for 3 times and methanol for 3 times. In each step of elution, beads were mixed with the solvent for 1 h. Mycoepoxydiene in the bead residue from another set of sample was eluted sequentially with methanol and ethyl acetate.

3.5.7.3 Determination of adsorption of mycoepoxydiene on Amberlite XAD-7 beads and elution in actual scale

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MM1D. Cultures were first maintained in stationary condition for 3 days, and then shaken for 15 days at 120 rpm, 25°C. The experiment was performed in triplicate. After that, washed Amberlite XAD-7 was added in a final concentration of 1% (w/v). On day 21 of cultivation, samples were taken and the culture media were separated from beads by using vacuum pump system. Mycoepoxydiene left in culture broth and that adsorbed in beads were determined quantitatively by HPLC as described in Section 3.10. Before analysis, mycoepoxydiene in the beads was eluted with 5 mL of methanol for 4 times. At each step of elution the beads were mixed with solvent by shaking at 200 rpm for 1 h.

3.5.7.4 Solid-liquid two phase partitioning fermentation method

3.5.7.4.1 Static condition with 1% Amberlite XAD-7 beads

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MM1D and 1% of Amberlite XAD-7 beads prepared as described in Section 3.5.7.1. The inoculated medium was cultured in stationary condition at 25° C for 21 days. Samples were taken periodically every 3 days. Each sample was performed in triplicate. The whole of culture broth from each sample was separated from beads by using vacuum pump system. Mycoepoxydiene left in culture broth and that adsorbed in beads were determined quantitatively by HPLC as described in Section 3.5.7.3.

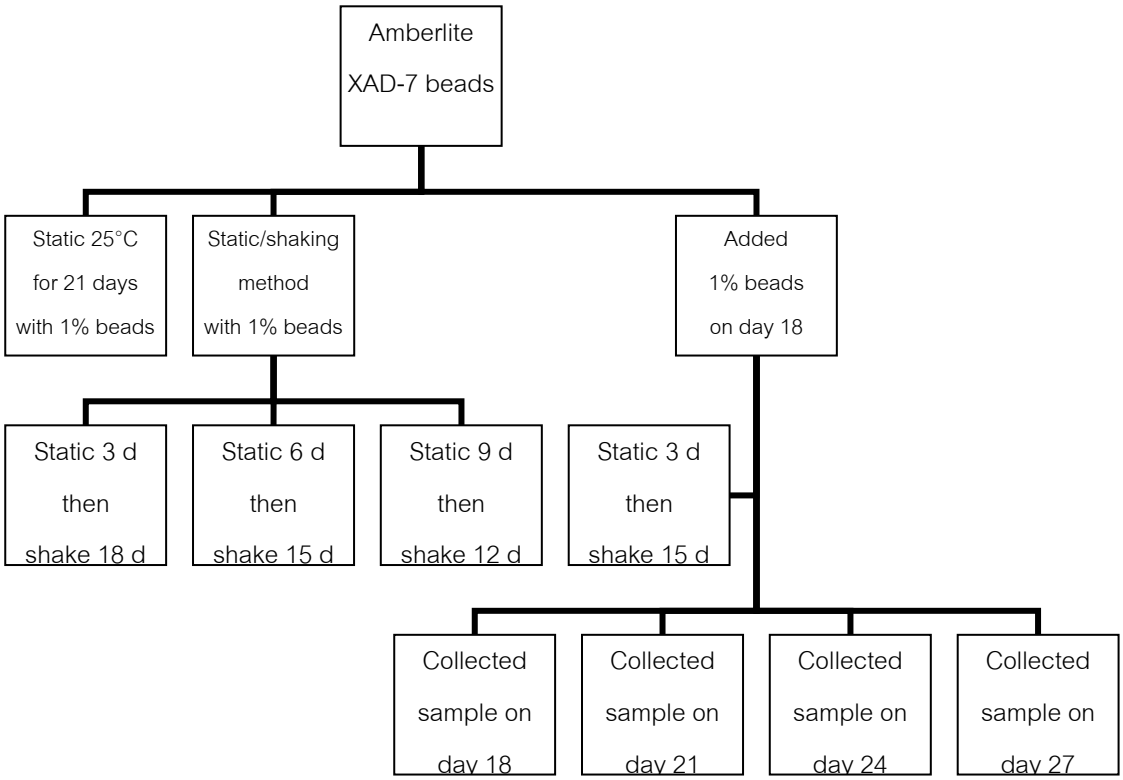
3.5.7.4.2 Static/shaking condition with 1% Amberlite XAD-7 beads

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MM1D and 1% of Amberlite XAD-7 beads. Cultures were first maintained in stationary condition for 3, 6 and 9 days, and then shaken for 18, 15 and 12 days, respectively at 120 rpm, 25°C. Samples were taken periodically every 3 days. Each sample was performed in triplicate. The whole of culture broth from each sample was separated from beads by using vacuum pump system. Mycoepoxydiene left in culture and that adsorbed in beads were determined quantitatively by HPLC as described in Section 3.5.7.3.

3.5.7.4.3 Static/shaking condition with 1% Amberlite XAD-7 bead addition on day 18

Two pieces of *Phomopsis* sp. Hant25 inoculum were grown in 250 mL Erlenmeyer flasks containing 50 mL of MM1D. Cultures were first maintained in stationary condition for 3 days, and then shaken for 15 days at 120 rpm,

25°C. Culture broth in a volume of 1.5 mL was taken on day 18 to detect the amount of mycoepoxydiene by HPLC, as described in Section 3.10, before adding Amberlite XAD-7 beads. This quantity of compound was used as the control to compare after eluting from beads. After that, Amberlite XAD-7 beads was added into the culture in a final concentration of 1% (w/v) and sample was further incubated for 1 h. Then sample was taken and the whole of culture broth from each sample was separated from beads by using vacuum pump system. On days 21, 24 and 27 of cultivation, samples were taken and processed as sample on day 18 after addition of beads. Each group of them was performed in triplicate. Mycoepoxydiene left in culture and that adsorbed in beads were determined by HPLC as described in Section 3.5.7.3. Scheme 7 summarizes the experimental design of solid-liquid two phase partitioning fermentation method on mycoepoxydiene production.



Scheme 7 Experimental design of solid-liquid two phase partitioning fermentation method on mycoepoxydiene production

3.6 Extraction of mycoepoxydiene from culture medium

3.6.1 Extraction from liquid medium

Mycelia were separated by filtration and the filtrate was extracted three times with an equal volume ethyl acetate. The combined organic solvent was concentrated under vacuum at 50°C to yield a crude extract. The crude extract was kept at -20°C until use.

3.6.2 Extraction from agar medium

Mycelia were separated from agar medium. Then the agar was cut by a sterile sharp blade as block of 5 x 5 mm dimension and extracted three times with an equal volume of ethyl acetate by using magnetic stirrer to mix between agar and solvent. The combined organic solvent was concentrated under vacuum at 50°C to yield a crude extract. The crude extract was kept at -20°C until use.

3.7 Development of mycoepoxydiene analysis by agar diffusion assay

Simple method to determine the relative amount of mycoepoxydiene in culture broth was developed based on agar diffusion assay modified from “plate” method of antibiotic assay described in United States Pharmacopeia 32–National Formulary 27 (2009).

3.7.1 Preparation of test sample

Phomopsis sp. Hant25 inoculated MCzB in a volume of 3 mL was extracted with ethyl acetate as described in Section 3.6.1. The dried crude extract was dissolved in 100 µL of dimethyl sulfoxide (DMSO).

3.7.2 Preparation of *C. albicans* inoculum

The suspension of *C. albicans* was prepared by picking colonies from overnight culture plate and suspending in the normal saline solution (0.85% NaCl). The turbidity of yeast suspension was adjusted to match that of 50%T at 580 nm using a spectrophotometer (BioSpec-1601, Shimadzu).

3.7.3 Preparation of assay plate and determination of anti-*C. albicans* activity

The yeast inoculum was added in a final concentration of 1% into melted SDA containing 2.5, 6.25 or 12.5 µg/mL of ketoconazole. The inoculated SDA (9 mL) was poured into 20x90 mm Petri dish and allowed to harden. Filter paper disk impregnated with 5 or 10 µL of crude extract in DMSO was placed onto *C. albicans* inoculated SDA-plates. All plates were placed in room temperature for 1 h and incubated at 37°C. After 24 h of incubation, the clear zones of inhibition of *C. albicans* growth were observed.

3.8 Determination of relative amount of mycoepoxydiene in culture broth by agar diffusion assay

The yeast inoculum was prepared as described in Section 3.7.2 and added in a final concentration of 1% into melted SDA containing 12.5 µg/mL of ketoconazole. The inoculated SDA (9 mL) was poured into 20x90 mm Petri dish and allowed to harden. Stainless steel cylinder cups (6 x 10 mm) were placed onto the surface of SDA and 300 µL of *Phomopsis* sp. Hant25 culture broth were filled into the cup. All plates were placed in room temperature for 1 h and incubated at 37°C. After 24 h of incubation, the clear zones of inhibition of *C. albicans* growth were measured.

3.9 TLC bioautography analysis

TLC bioautographic method is a simple and effective method which combines thin-layer chromatographic isolation and in situ detection of anti-microbial activity. This technique facilitates the localization and target-directed isolation of active components in a crude extract. Bioautographic technique depends on inhibition of the microbial growth to detect active components of extracts chromatographed on a TLC plate (Shahverdi *et al.*, 2007). After loading of crude extract on a silica gel 60 F_{254} plate (Merck, Darmstadt, Germany), thin layer chromatography (TLC) was developed to a distance of 7 cm in an eluent system of dichloromethane-ethyl acetate (9:1) (Kinghorn, 1997) and solvent was removed from TLC plate by air drying. The standard mycoepoxydiene 10 µg and crude extract 300 µg were spotted on TLC plate to identify the location of mycoepoxydiene on TLC plate. The plate was visualized under ultraviolet light at 254 nm and the separated spots were marked to show their locations. The developed TLC plate was placed inversely over the surface of the SDA plate seeded with 1% *C. albicans* and contained 12.5 µg/mL of ketoconazole. All plates were placed in room temperature for 1 h and then incubated at 37°C for 24 h. The presence of the clear inhibition zones around mycoepoxydiene spots were compared one another to show the relative level of mycoepoxydiene in each crude extract.

Analytical thin-layer chromatography condition

Technique	: one dimension ascending
Adsorbent	: silica gel F_{254} coated on aluminium sheet (E. Merck)
Layer thickness	: 250 µm
Distance	: 7 cm
Temperature	: laboratory temperature 25 ^{o}C
Detection	: 1. Visual detection under daylight
	2. Visual detection under ultraviolet light at wavelengths of 254 and 356 nm

3.10 HPLC quantitative analysis

Mycoepoxydiene yields in culture broth were analyzed by high performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC) condition

Column	: Hewlett-Packard, ODS Hypersil, 5 µm, 125x4 mm with LiChrospere 100 RP-18, 5 mm, 4 x 4 mm guard column
Injection volume	: 20 µL
Sample loading	: The culture medium sample was dissolved in H_2O then injected into the loop of the column.
Flow rate	: 1.5 mL/min, with a run time of 12 min
Detection	: UV-photodiode array detector monitored at 260 nm
Mobile phase	: 0.1% acetic acid in H_2O: acetonitrile (75:25)

Samples were delivered for a run time of 12 min with the mycoepoxydiene peak eluted at approximately 9.5 min, as shown in Figure 4. Mycoepoxydiene UV detection was achieved at 260 nm, and its concentration was determined using a standard calibration curve prepared with mycoepoxydiene standard. The standard calibration curve was created by running 8 different concentrations of standard (0.0098, 0.0195, 0.0391, 0.0781, 0.1563, 0.3125, 0.625, 1.25 mg/mL). Each concentration gave a peak area of 77, 196, 424, 897, 1891, 3949, 7754 and 15588, respectively. Peak areas were then plotted against the concentrations (Figure 5). Hence, the peak area of mycoepoxydiene in culture broth could convert to concentration by equation of this graph.

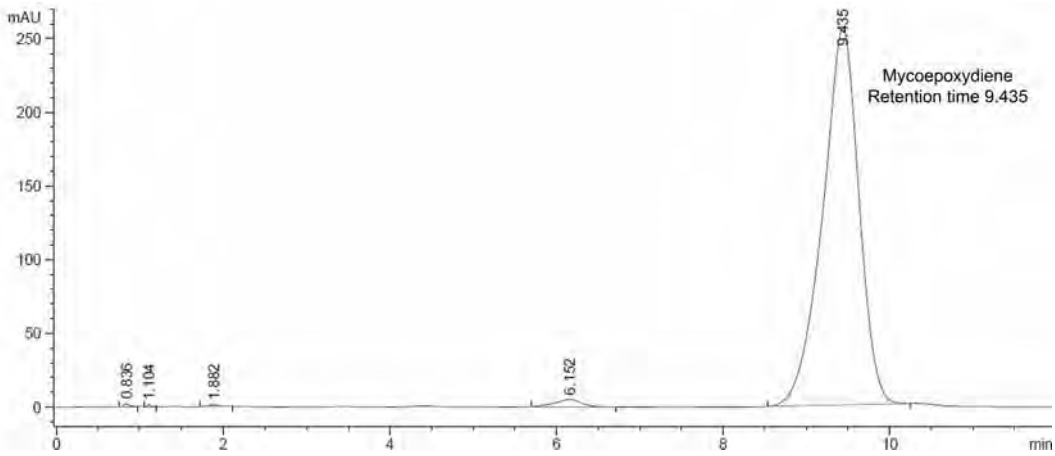


Figure 4 HPLC chromatogram of standard mycoepoxydiene. Mobile phase was acetonitrile/0.1% acetic acid (25:75 v/v). Flow rate was 1.5 mL/min. Retention time of standard mycoepoxydiene was 9.43 min.

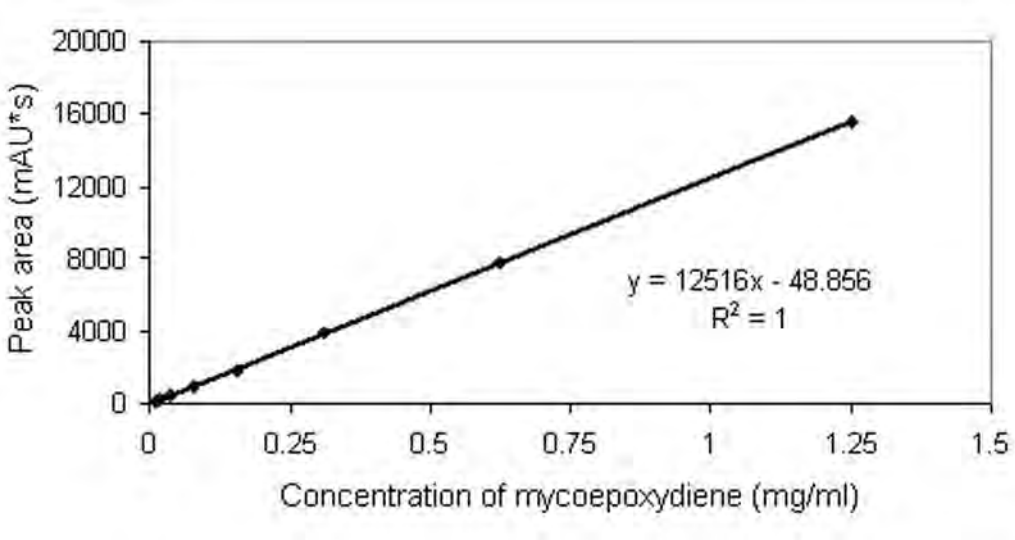


Figure 5 The standard calibration curve of mycoepoxydiene

3.11 Preservation of endophytic fungus, *Phomopsis* sp. Hant25

The endophytic fungus stock cultures were prepared as agar blocks in sterile distilled water and stored at 4°C until use.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Development of mycoepoxydiene analysis by agar diffusion assay

Simple method which can determine the relative amount of interesting metabolite in fermentation broth is required in the study to improve production yields of microbial secondary metabolites by fermentation methods. Determination of the antimicrobial activity of fermentation broth is recognized to be one simple method which can be conducted in a general microbiology laboratory. In this study agar diffusion assay was developed to determine relative amounts of mycoepoxydiene in fermentation broths. Several nonantifungals, for examples FK506 (Sun *et al*., 2008) and berberine (Iwazaki *et al*., 2009) were found to be synergistic with azole drugs against *C. albicans*. Mycoepoxydiene was reported to have potent cytotoxic activity against several human tumor cell lines (Prachya *et al*., 2007; Sommart *et al*., 2009). It has weak activity against *C. albicans*. Therefore, mycoepoxydiene was assayed by using *C. albicans* inoculated SDA-plates containing subinhibitory concentrations of ketoconazole (2.5, 6.25 or 12.5 µg/mL), as described in section 3.7.

As shown in Figure 6, mycoepoxydiene in 5 µL of crude extract could not completely inhibit *C. albicans* in plates containing 2.5 and 6.25 µg/mL of ketoconazole but showed the clear inhibition zone in plates containing 12.5 µg/mL of ketoconazole. For this reason, if the quantity of mycoepoxydiene in crude extract was low, the clear zone of inhibition might not be shown in test plate containing low concentration of ketoconazole and the presence of mycoepoxydiene could not be detected. Therefore, ketoconazole in a final concentration of 12.5 µg/mL was used in all experiment to determine relative amount of mycoepoxydiene by agar diffusion assay and TLC bioautography analysis.

Although the disk diffusion assay had many advantages, the biggest problem of this assay was extraction of crude extract from the culture broth, especially in detection of a large number of samples. As a result of this limitation, application of culture broth onto the surface of test plate was employed. It was found that culture broth in a volume of 300 µL, which contained equivalent amount of mycoepoxydiene to 10 µL of crude extract, could exhibit clear zone of inhibition, as shown in Figure 7. Therefore, a 300 µL volume of culture broth was directly tested for relative amount of mycoepoxydiene.

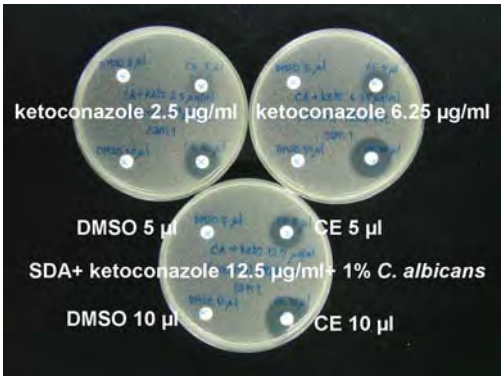


Figure 6 The disk diffusion assay exhibiting activity of mycoepoxydiene in crude extract against *C. albicans* in SDA containing 2.5, 6.25 or 12.5 µg/mL of ketoconazole

Figure 7 Agar diffusion assay exhibiting activity of mycoepoxydiene in culture broth against *C. albicans* in SDA containing 12.5 µg/mL of ketoconazole. Culture broth (300 µL) was filled in stainless steel cylinder cup which placed on the surface of inoculated SDA.

4.2 Preliminary submerged fermentation for mycoepoxydiene production

Fermentation of *Phomopsis* sp. Hant25 in MCzB at 25°C under standstill condition was performed to provide the baseline concentration of mycoepoxydiene to compare with other methods. It was found that mycoepoxydiene was produced on day 12 at a concentration of 24.56 mg/L. As shown in Figure 8, mycoepoxydiene was produced in the stationary phase of fungal growth. This corresponded to the production period of other microbial secondary metabolites (Blanch and Clark, 1997). Unexpectedly, mycoepoxydiene produced was later degraded.

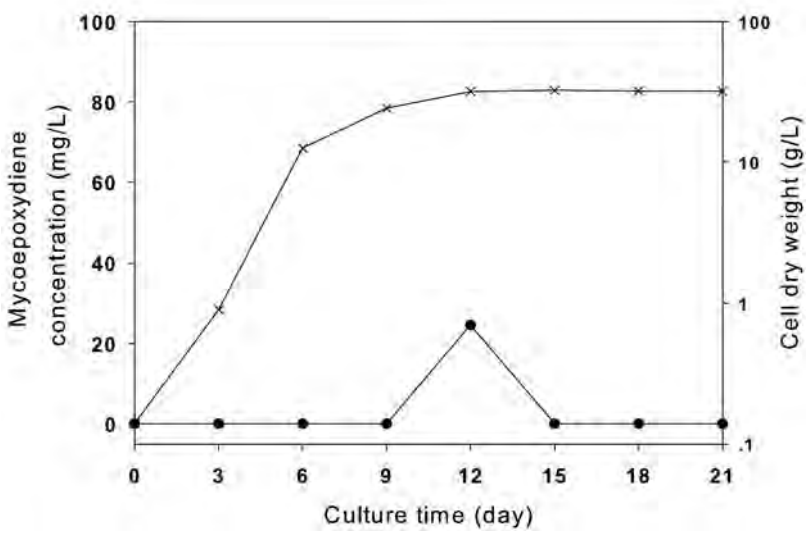


Figure 8 Mycoepoxydiene concentrations (mg/L) in culture broth produced by *Phomopsis* sp. Hant25 cultivated in MCzB at 25°C under standstill condition. (●: mycoepoxydiene concentration; x: cell dry weight)

4.3 Alternative fermentation for mycoepoxydiene production

4.3.1 Effects of culture media and fermentation conditions on mycoepoxydiene production

Relative amounts of mycoepoxydiene produced by *Phomopsis* sp. Hant25 cultivated in MCzB, MM1D and CzYB at 25°C and 30°C under standstill

condition and at 25°C under shaking condition were determined by agar diffusion assay based on antifungal activity against *C. albicans*, as described in Section 3.8. As shown in Figure 9, *Phomopsis* sp. Hant25 could produce higher levels of mycoepoxydiene when grown in MCzB and MM1D than that grown in CzYB. It was found that *Phomopsis* sp. Hant25 could not produce detectable level of mycoepoxydiene when grown at 30°C in CzYB and MM1D. Therefore, MCzB and MM1D were selected as the media and 25°C was selected as incubation temperature for cultivation of *Phomopsis* sp. Hant25 in the experiments to improve production yield of mycoepoxydiene. In this study, aeration was found to affect mycoepoxydiene production of the fungus.

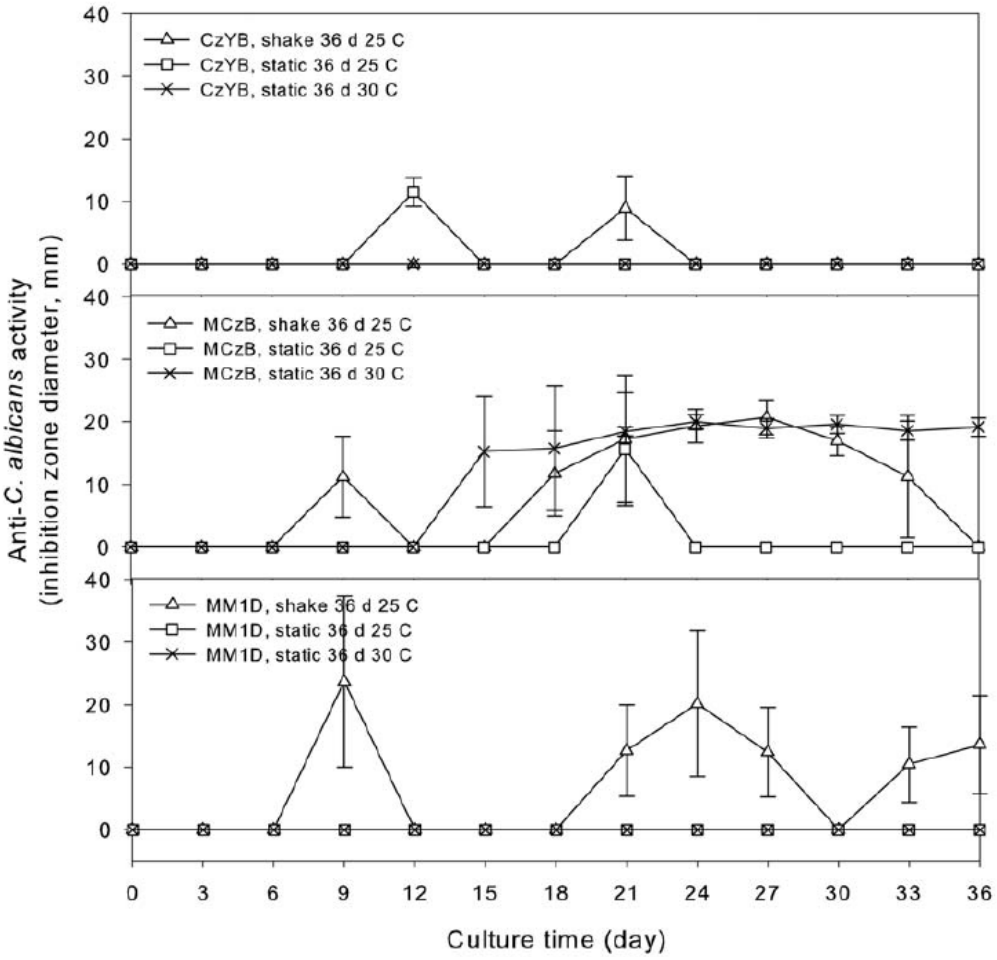


Figure 9 Anti-*C. albicans* activity of fermented broths obtained from static and shaken fermentations of *Phomopsis* sp. Hant25

4.3.2 Comparison between submerged fermentation and solid state fermentation on mycoepoxydiene production

Solid state fermentation has been applied successfully for production of microbial enzymes and secondary metabolites (Krishna, 2005). In the case of antibiotics and other secondary metabolites, production under such conditions is associated with higher yields in short time periods compared to the alternative submerged fermentation (Robinson *et al.*, 2001). In this study, mycoepoxydiene productions by submerged fermentation and solid state fermentation were compared by cultivating two pieces of inoculum and one piece of inoculum in 50 mL of MCzB and on 25 mL of MCzA, respectively, as described in Section 3.5.2. TLC bioautography analysis was used to confirm the presence and anti-*C. albicans* activity of mycoepoxydiene in crude extracts obtained from fermentation media. It was found that both fermentation methods could produce mycoepoxydiene, as shown in Figures 10-11. As expected, not only mycoepoxydiene but also other compounds in crude extracts exhibited activity against *C. albicans* in test plate containing 12.5 µg/mL of ketoconazole, as shown in Figures 12. However, anti *C. albicans* activity was mainly from mycoepoxydiene. Therefore, agar diffusion assay developed in this study could be used to determine relative amounts of mycoepoxydiene in culture broths from different fermentation conditions.

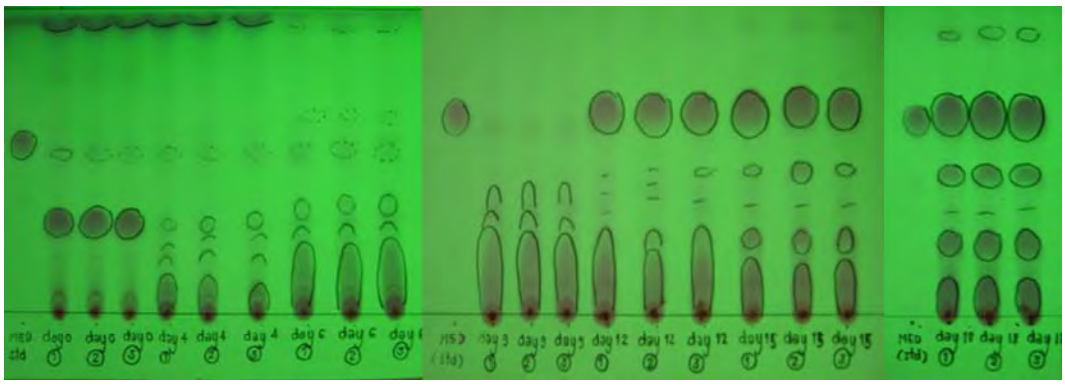


Figure 10 TLC plates exhibiting the presence of mycoepoxydiene in crude extract (300 µg) derived from the fermented MCzB. Leftmost lane is standard mycoepoxydiene.

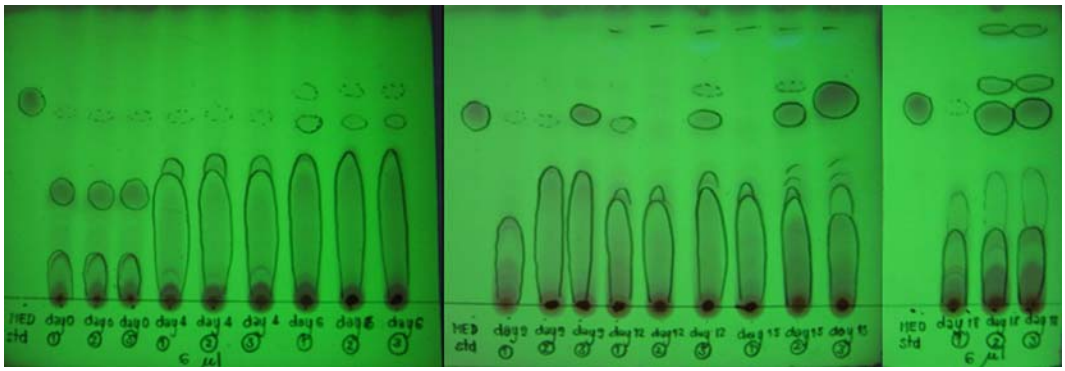


Figure 11 TLC plates exhibiting the presence of mycoepoxydiene in crude extract (600 µg) derived from the fermented MCzA. Leftmost lane is standard mycoepoxydiene.

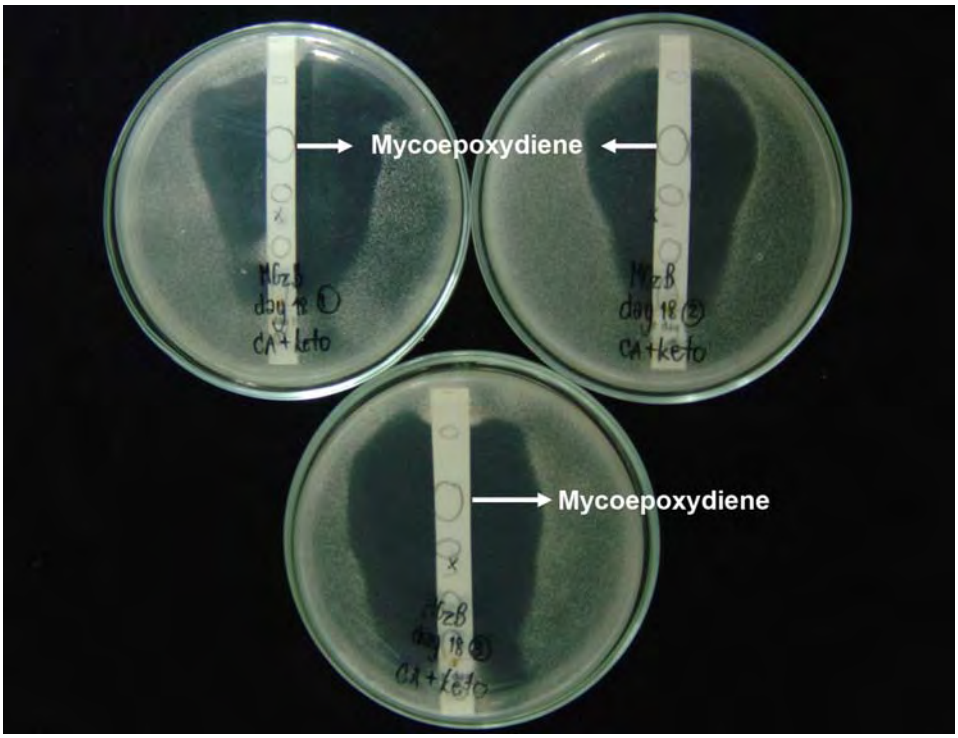


Figure 12 TLC bioautography exhibiting anti-*C. albicans* activity of mycoepoxydiene on test plates containing 12.5 µg/mL of ketoconazole

Relative amounts of mycoepoxydiene in crude extracts, which obtained from each fermentation medium, were determined based on anti-*C. albicans* activity by TLC bioautography, as shown in Figures 10-13. The narrow of SD value of each point in Figure 13, which corresponded with the intensity of mycoepoxydiene spots on TLC plates, suggested that results from submerged fermentation were more reproducible than those of solid state fermentation. These results might occur from the extraction methods. Although the same process was used to extract mycoepoxydiene from fermentation broth and medium, but extraction from solid medium was more complicated than that from liquid medium. Liquid-liquid contact between solvent and fermented broth is better than that between solvent and surface area of solid matrix. Hence, the extraction of solid medium might not completely extract

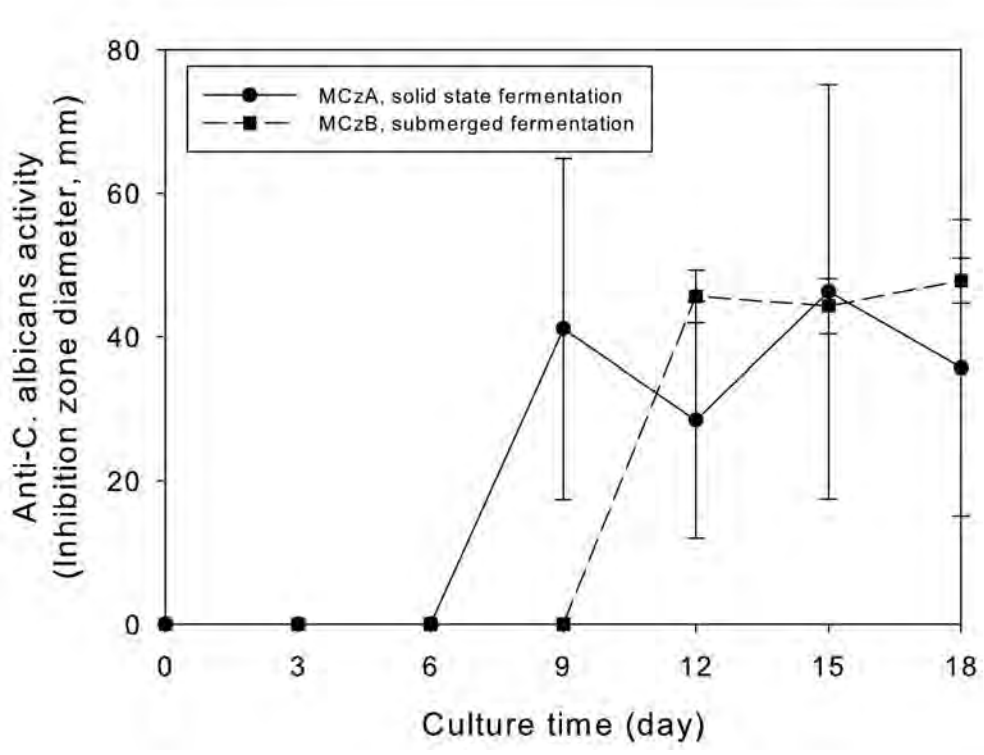


Figure 13 Anti-*C. albicans* activity by TLC bioautography analysis indicating relative amounts of mycoepoxydiene in crude extract (300 µg) from fermented liquid medium and in that (600 µg) from agar medium

mycoepoxydiene. As shown in Figure 13, inhibition zone diameters indicating anti-*C. albicans* activity of mycoepoxydiene in 300 µg of crude extract from fermented MCzB was comparable to that in 600 µg of crude extract from fermented MCzA. This suggested that mycoepoxydiene produced by *Phomopsis* sp. Hant25 cultivated in MCzB was 2 times higher than that produced by solid stage fermentation. In addition, mycoepoxydiene in fermented broth could be directly determined by HPLC without extraction process. According to these factors, submerged fermentation was selected to be a fermentation process used to develop further.

Figure 12 revealed that at least two compounds could inhibit *C. albicans* predominantly. One of them was mycoepoxydiene but another compound was still unknown. Previously, Prachya *et al.* (2007) could isolate three compounds (mycoepoxydiene, Figure 2; deacetylmycoepoxydiene, Figure 14; and 2, 3-dihydromycoepoxydiene, Figure 15) from fermented *Phomopsis* sp.Hant25 in MCzB.

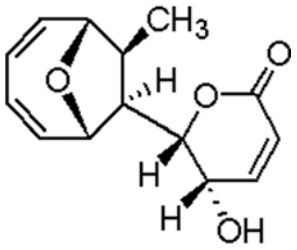


Figure 14 Chemical structure of deacetylmycoepoxydiene

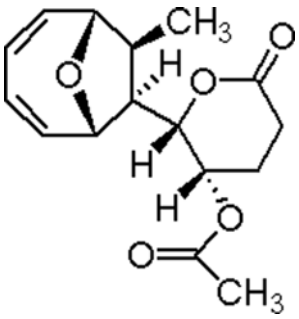


Figure 15 Chemical structure of 2, 3-dihydromycoepoxydiene

To identify compounds in active area on TLC plates, each of three spots was scraped from TLC plate and extracted from silica gel by ethyl acetate. Then, each extracted compound was analyzed by using mass spectrometry. It was found that they were mycoepoxydiene (Figure 2), 2, 3-dihydromycoepoxydiene (Figure 15) and deacetylmycoepoxydiene (Figure 14). Mass spectra of them are shown in Appendix B. In addition, the developed TLC plate was cut into five parts following the spots on TLC plates with clean scissors and was detected for the activity against *C. albicans*. It was found that spot designated 2A, 4A and 5A, as shown in Figure 16, exhibited the activity against *C. albicans*. According to the mass spectrometry data, spots 2A, 3A and 4A were mycoepoxydiene, 2, 3-dihydromycoepoxydiene and deacetylmycoepoxydiene, respectively. However, polar compound at position 5A was still unknown. Activities of mycoepoxydiene, and deacetylmycoepoxydiene obtained in this investigation were in agreement with previous data which revealed that both of them exhibited potent cytotoxic activity but 2, 3-dihydromycoepoxydiene was inactive (Prachya *et al*., 2007; Sommart *et al*., 2009).

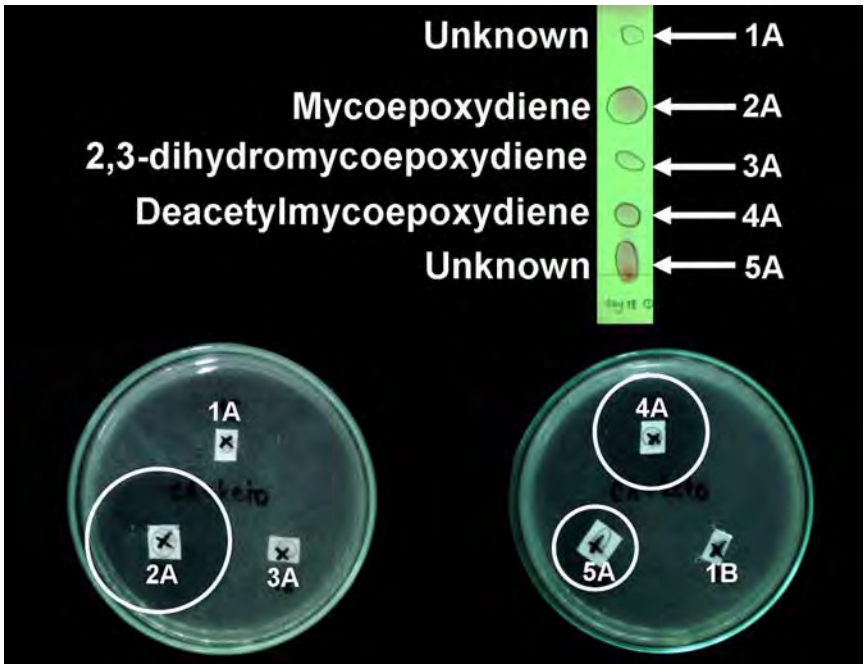


Figure 16 Agar diffusion assay exhibiting anti- *C. albicans* activity of each spot on TLC plate

4.3.3 Static/shaking fermentation method on mycoepoxydiene production

Static/shaking fermentation of *Phomopsis* sp. Hant25 in MM1D resulted in higher anti-*C. albicans* activities of fermented broths than those obtained from fermented MCzB, as shown in Figure 17. Fermented MCzB under static/shaking fermentation of being static for 3 days and then shaken for 18 days exhibited no anti-*C. albicans* activity. Considering fermented MM1D, shorter static periods, i.e. static 3 days then shaken 18 days and static 6 days then shaken 15 days, resulted in fermented broths with higher anti-*C. albicans* activities than activity of fermented broth from static/shaking fermentation process of static 9 days then shaken 12 days. It was found

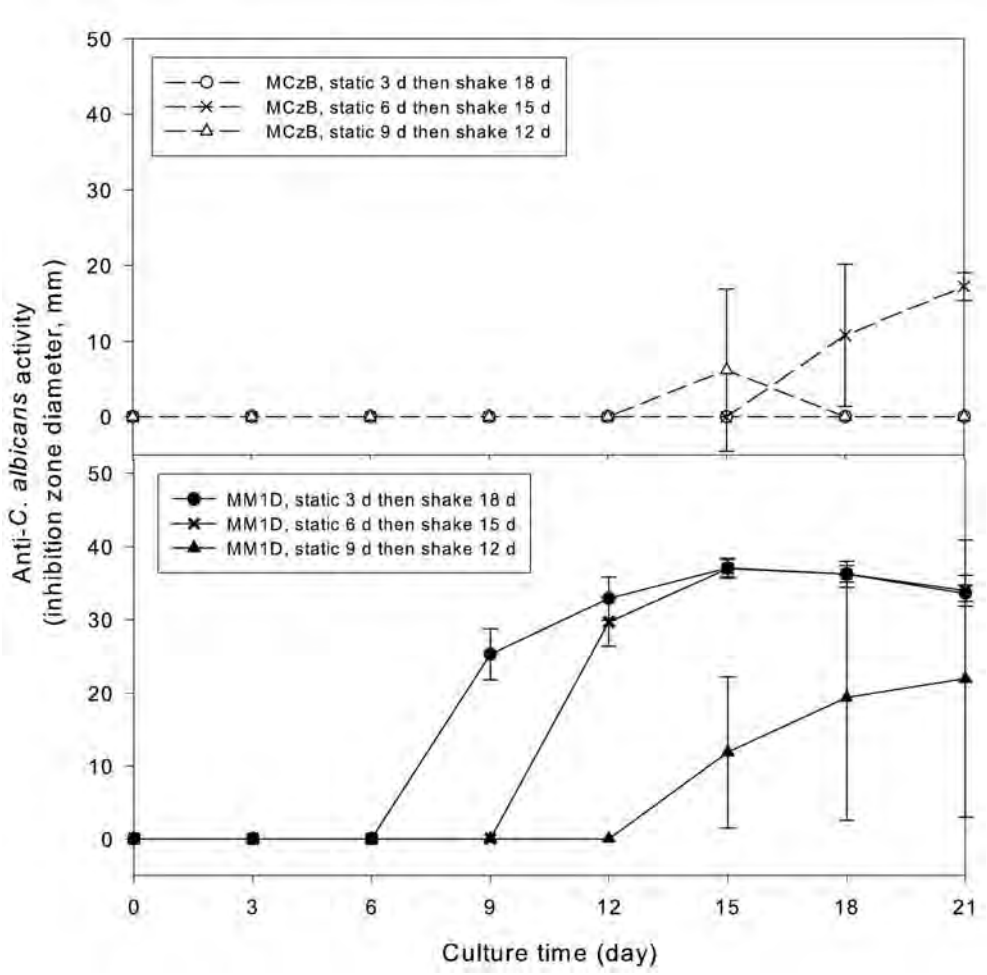


Figure 17 Anti-*C. albicans* activity of fermented broths obtained from static/shaking fermentation conditions

that fermented broths obtained from shaking fermentation and shaking/static fermentation of the fungus resulted in no anti-*C. albicans* activities.

In order to confirm that anti-*C. albicans* activity was the effect of mycoepoxydiene, fermented MCzB and MM1D of each fermentation condition which showed the highest inhibition zone were analyzed by HPLC. Mycoepoxydiene could not be found in any of fermented MCzB samples that showed anti-*C. albicans* activity and in some active fermented MM1D samples (samples on day 9 of static 3 days then shaken 18 days, on day 12 of static 6 days then shaken 15 days and on day 15 of static 9 days then shaken 12 days), as shown in Figure 18. This suggested that anti-*C. albicans* of those samples was from the effect of other compounds. These results implied that anti-

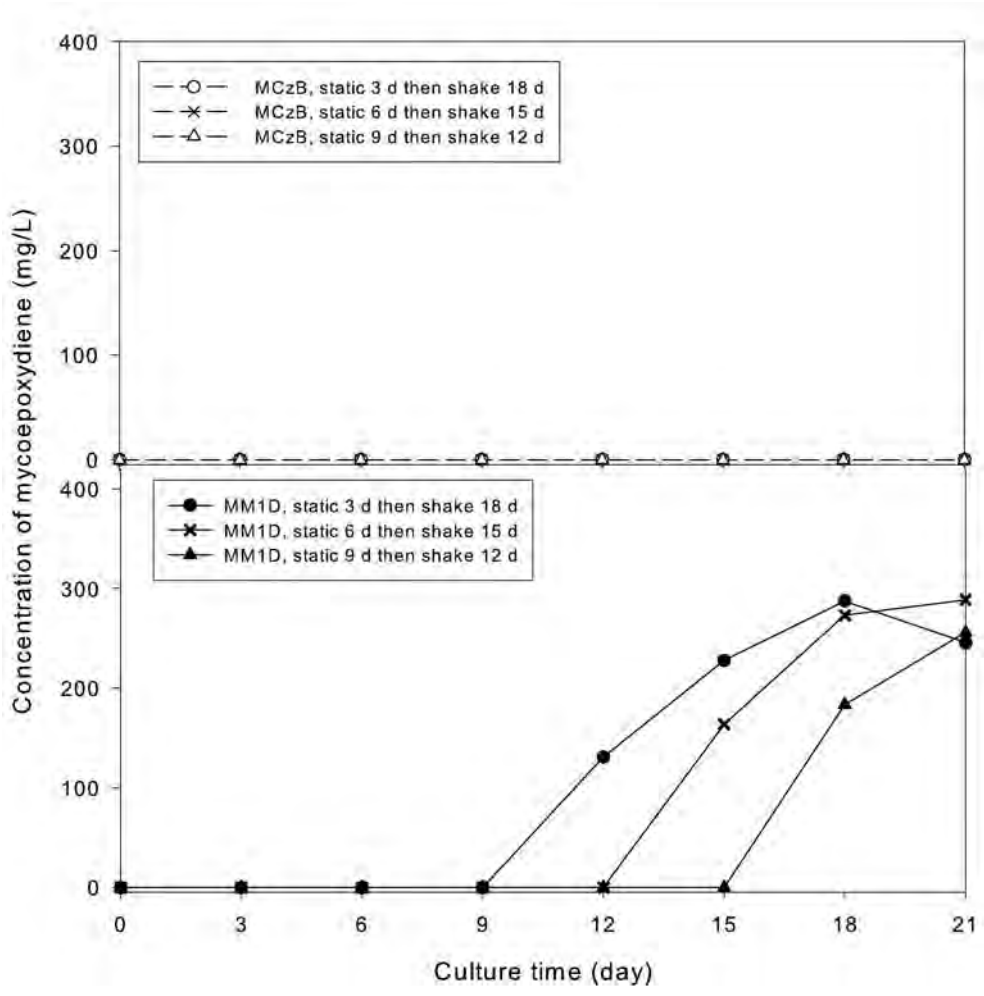


Figure 18 Mycoepoxydiene concentrations in fermented broths obtained from static/shaking fermentation conditions

C. albicans activity of fermented broth only provided preliminary suggestion of possibility to have mycoepoxydiene in sample. Analysis by HPLC should therefore be conducted further to confirm the presence of mycoepoxydiene in sample.

Because of the presence of mycoepoxydiene in MM1D fermented broth rather correlated with anti-*C. albicans* activity, as shown in Figures 17 and 18, all of the MM1D samples showing the clear inhibition zones were further examined for mycoepoxydiene yields by HPLC analysis. It was apparent that maximum production of mycoepoxydiene concentration in static/shaking method on day 18 of cultivation reach 260 mg/L when cultured in MM1D at 25°C with condition of being static for 3 days and then shaken at 120 rpm for 15 days, as shown in Figure 19. In summary, maximum mycoepoxydiene productivity by *Phomopsis* sp. Hant25 grown in MM1D under static/shaking fermentation resulted in 10.59 times increase over that obtained from conventional preliminary submerged fermentation in MCzB. This corresponded to previous report on the increase of anthraquinones and flavomannin productions by *Penicillium* sp. *LL*-WF159 fermented under static/shaking condition compared to those obtained from the fungus grown under conventional fermentation.

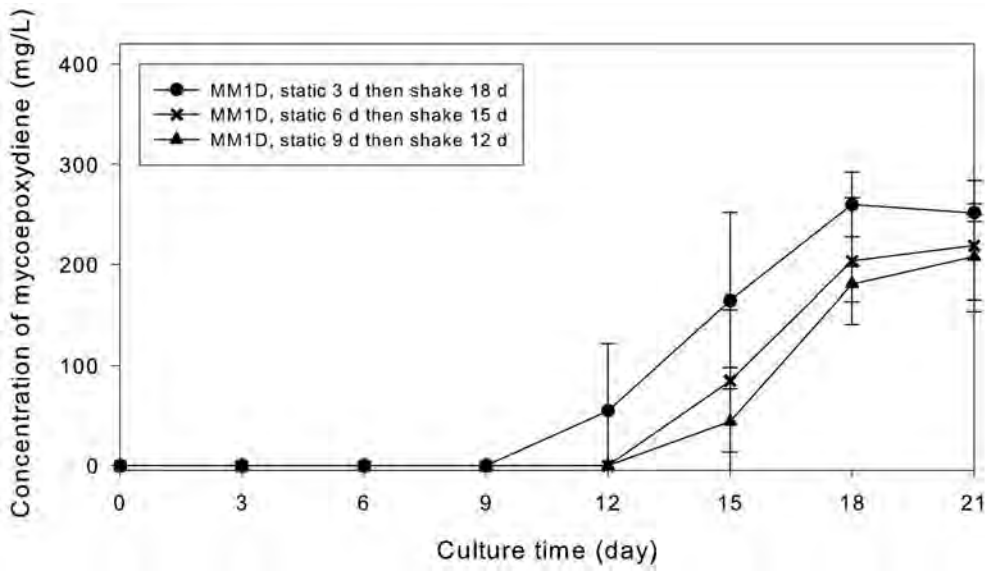


Figure 19 Mycoepoxydiene concentrations in fermented MM1D broths obtained from static/shaking fermentation conditions

4.3.4 Solid support fermentation method on mycoepoxydiene production

4.3.4.1 Filter paper solid support

Filter paper solid support has been applied into fermentation broths to improve mycoepoxydiene yields produced by *Phomopsis* sp. Hant25 incubated under static/shaking fermentation conditions, as described in Section 3.5.4. Patterns of anti-*C. albicans* activity exhibited by broths fermented in the condition of having filter paper solid support (as shown in Figure 20) were in the same manner as shown by the condition without filter paper solid support (as shown in Figure 17).

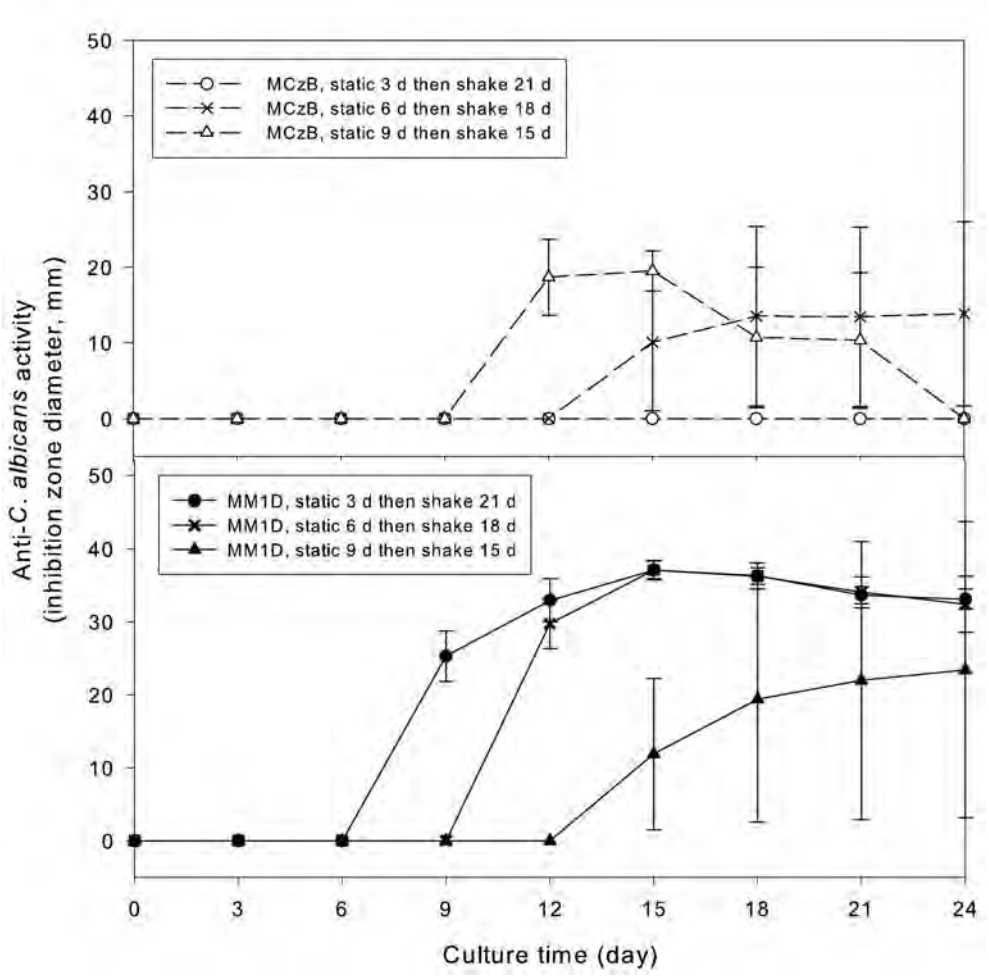


Figure 20 Anti-*C. albicans* activity of fermented broths obtained from static/shaking fermentations containing filter paper solid support

However, activity of active MCzB obtained from the filter paper solid support fermentation was higher than that obtained from fermentation without filter paper solid support.

Fermented broth samples that showed the highest clear inhibition zone from each condition were further analyzed by HPLC. Active fermented MCzB samples were found to contain very low level of mycoepoxydiene. In contrast, fermented MM1D samples exhibited the correlation between the clear inhibition zone and the mycoepoxydiene yield, as shown in Figures 20 and 21. Hence, all of the MM1D samples showing the clear inhibition zone were further examined for mycoepoxydiene yields by HPLC analysis. It was found that static/shaking fermentation containing filter paper solid

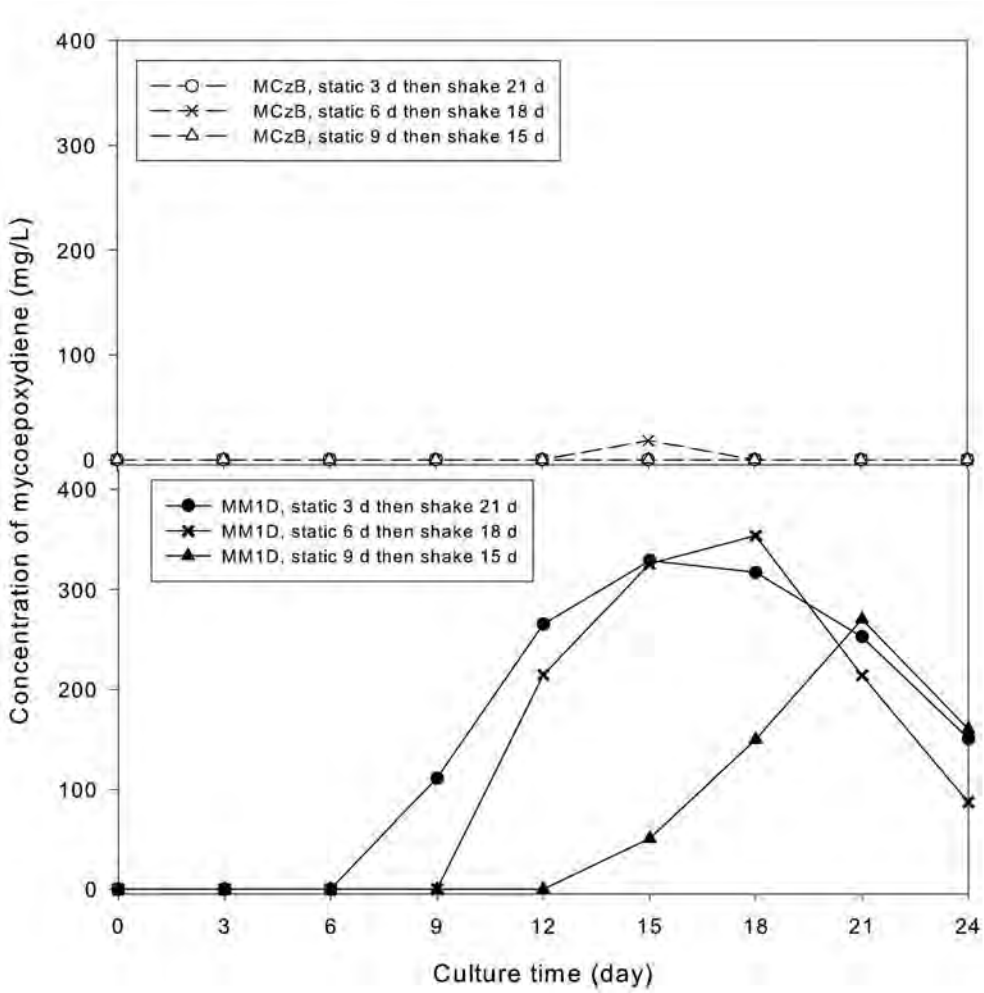


Figure 21 Mycoepoxydiene concentrations in fermented broths obtained from static/shaking fermentations containing filter paper solid support

support promoted mycoepoxydiene production in MM1D medium to reach maximum at 333 mg/L. This high level of mycoepoxydiene was achieved on day 18 of cultivation at 25°C under condition of being static for 6 days and then shaken at 120 rpm for 12 days, as shown in Figure 22. This result indicated that the addition of filter paper solid support in static/shaking fermentation could further increase mycoepoxydiene yield to 13.56 times over that obtained from preliminary submerged fermentation in MCzB. This level of productivity represented a 28.08% increase in performance over the previously performed static/shaking fermentation method without filter paper support. The use of solid supports for fungal attachment in liquid medium is recognized to influence growth, morphology, and production of bioactive secondary metabolites (Bigelis *et al*., 2006).

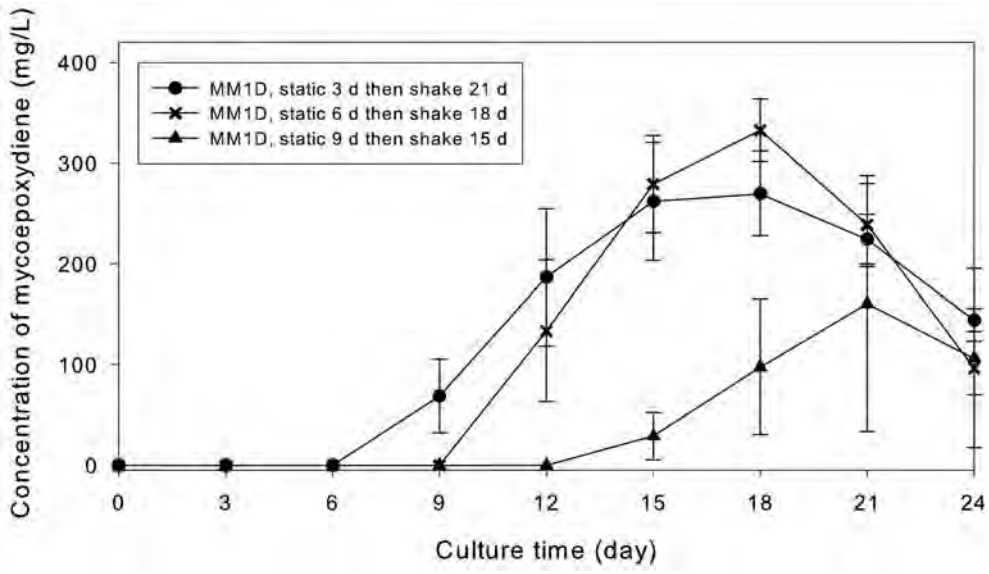


Figure 22 Mycoepoxydiene concentrations in fermented MM1D broths obtained from static/shaking fermentations containing filter paper solid support

4.3.4.2 Gauze solid support

Gauze solid support has been applied into fermentation broths to increase mycoepoxydiene yields produced by *Phomopsis* sp. Hant25 incubated under

static/shaking fermentation conditions, as described in Section 3.5.4. Patterns of anti-*C. albicans* activity exhibited by broths fermented in the condition of having gauze solid support (as shown in Figure 23) were in the same manner as shown by the condition with filter paper solid support (as shown in Figure 20) and without solid support (as shown in Figure 17).

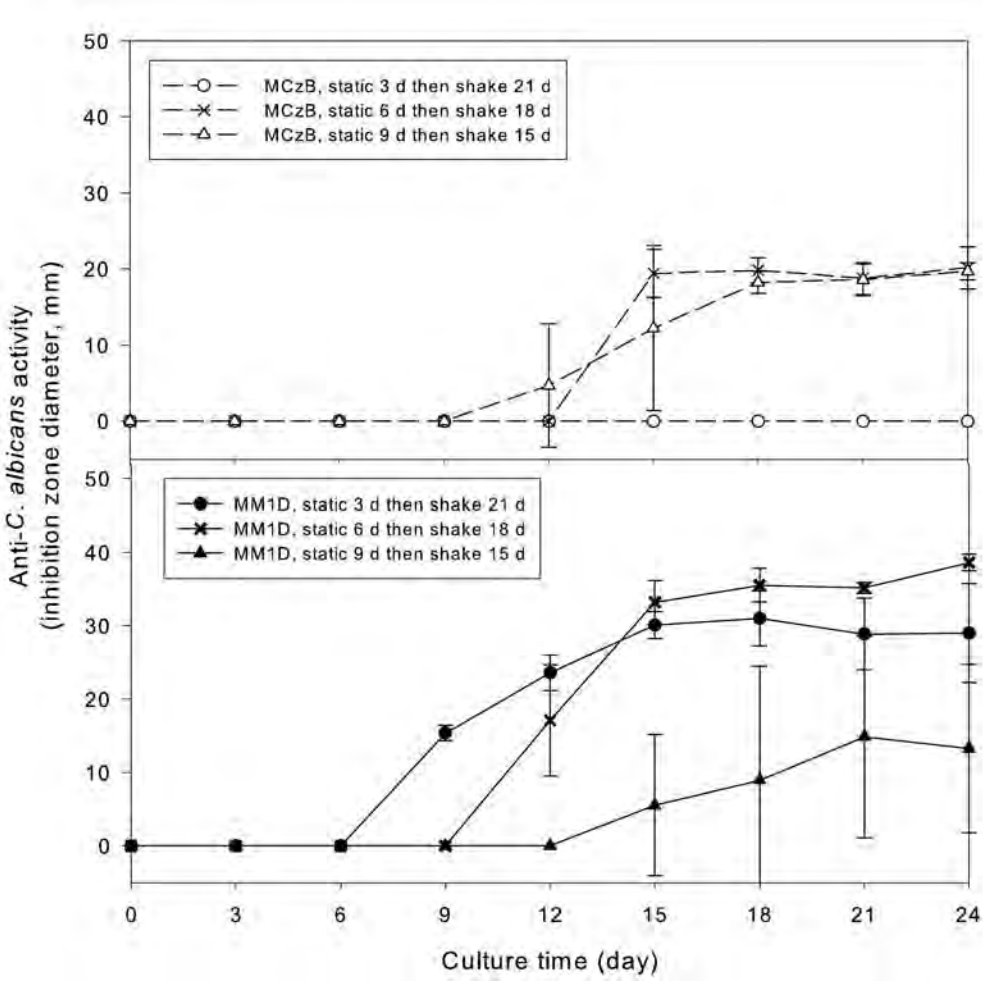


Figure 23 Anti-*C. albicans* activity of fermented broths obtained from static/shaking fermentations containing gauze solid support

Fermented broth samples that showed the highest clear inhibition zone from each condition were further analyzed by HPLC. Active fermented MCzB samples were found to contain no mycoepoxydiene. In contrast, fermented MM1D

samples exhibited the correlation between the clear inhibition zone and the mycoepoxydiene yield, as shown in Figures 23 and 24. Therefore, all of the MM1D samples showing the clear inhibition zone were further examined for mycoepoxydiene yields by HPLC analysis. Maximum mycoepoxydiene production in fermented MM1D under static/shaking fermentation with gauze support was reached 279 mg/L on day 18 of cultivation at 25°C in condition of being static for 6 days and then shaken at 120 rpm for 12 days, as shown in Figure 25. Maximum mycoepoxydiene level produced under this fermentation condition was 11.36 times higher than that obtained from the preliminary submerged fermentation method. This level was comparable to

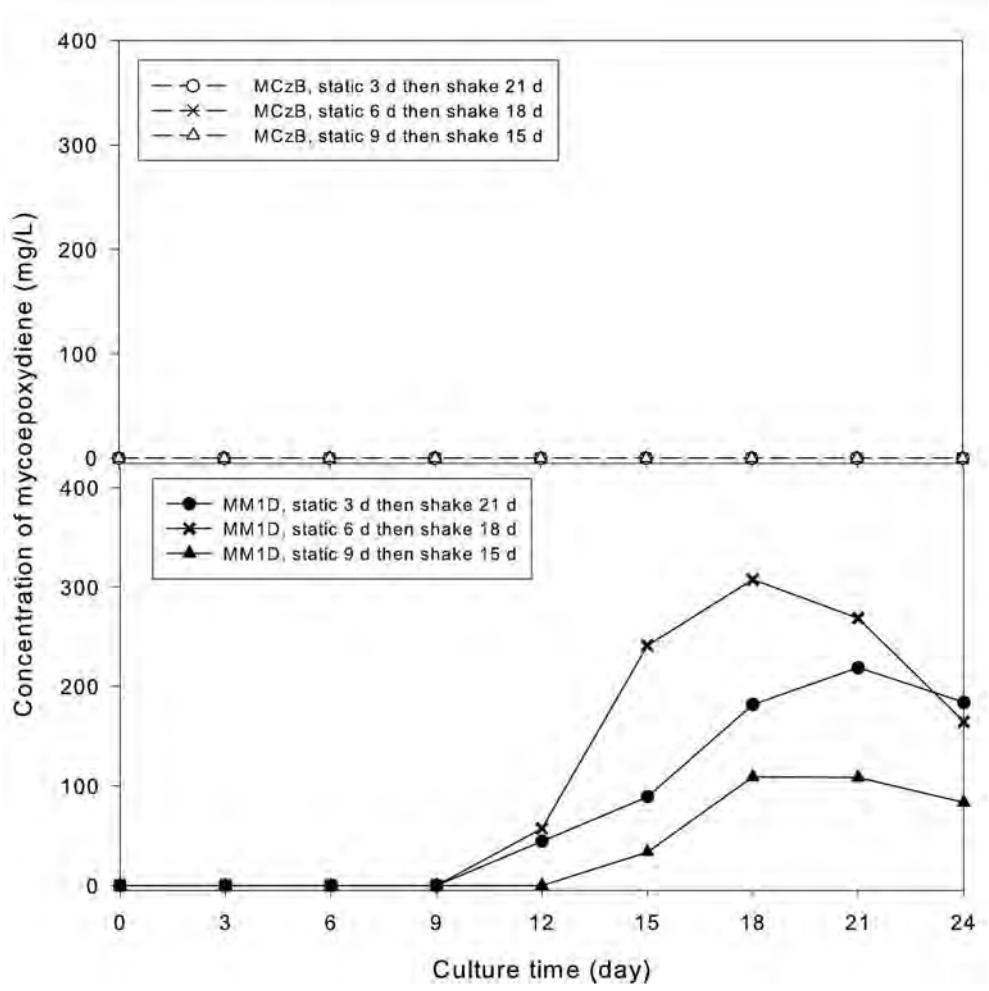


Figure 24 Mycoepoxydiene concentrations in fermented broths obtained from static/shaking fermentations containing gauze solid support

mycoepoxydiene level produced in fermented MM1D under static/shaking fermentation without solid support. This indicated that gauze support was different from filter paper solid support in term of no influence on mycoepoxydiene production under static/shaking fermentation. This might be due to the growth form of fungus. Contrary to attachment of fungal mycelia to gauze support all the time, fungal mycelia were found to attach to filter paper support only during early period of incubation in static condition and then release in the agitation period. For this reason, fungal growth in the gauze support fermentation might obtain the more shear force effect than that grown on the filter paper support. Accordingly, this factor might affect fungal morphology and mycoepoxydiene production.

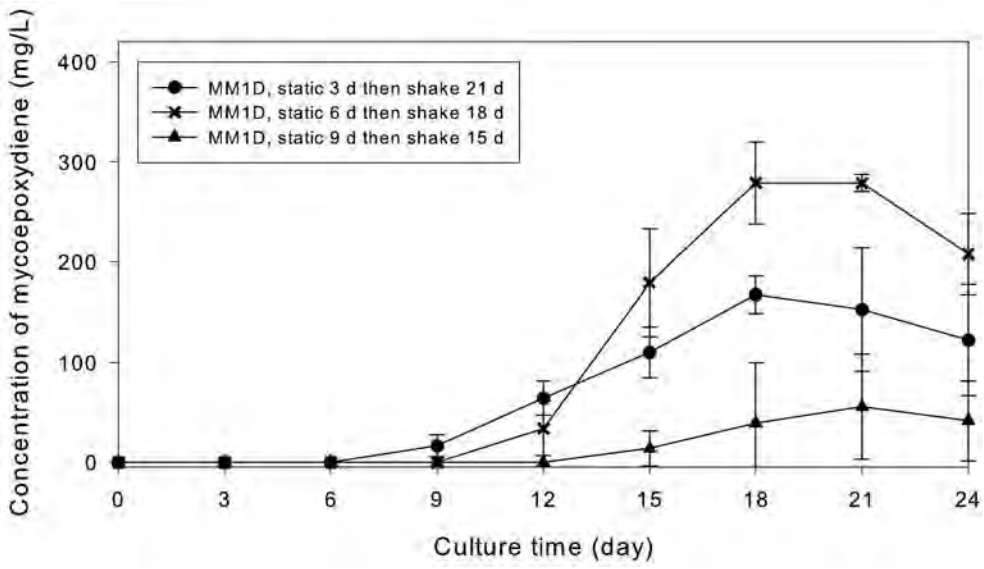


Figure 25 Mycoepoxydiene concentrations in fermented MM1D broths obtained from static/shaking fermentations containing gauze solid support

4.3.5 Floating solid support fermentation method on mycoepoxydiene production

Floating solid support made of polypropylene was used to increase surface area of fungal growth on the solid support during fermentation, as described in 3.5.5.

4.3.5.1 Static condition

In order to evaluate the effect of surface area for fungal growth and dissolved oxygen possibility on mycoepoxydiene production, *Phomopsis* sp. Hant25 was grown in 20 x 90 mm Petri dish containing 25 mL of media which increased surface area of fungal growth and allowed higher possibility of oxygen transfer into the media compared to 250 mL Erlenmeyer flask containing 50 mL of media. In addition, fermentation with PP support was also investigated, as described in section 3.5.5.1. Relative amounts of mycoepoxydiene in fermented broths were determined by agar diffusion assay. The results are shown in Figure 26. The maximum anti-*C. albicans*

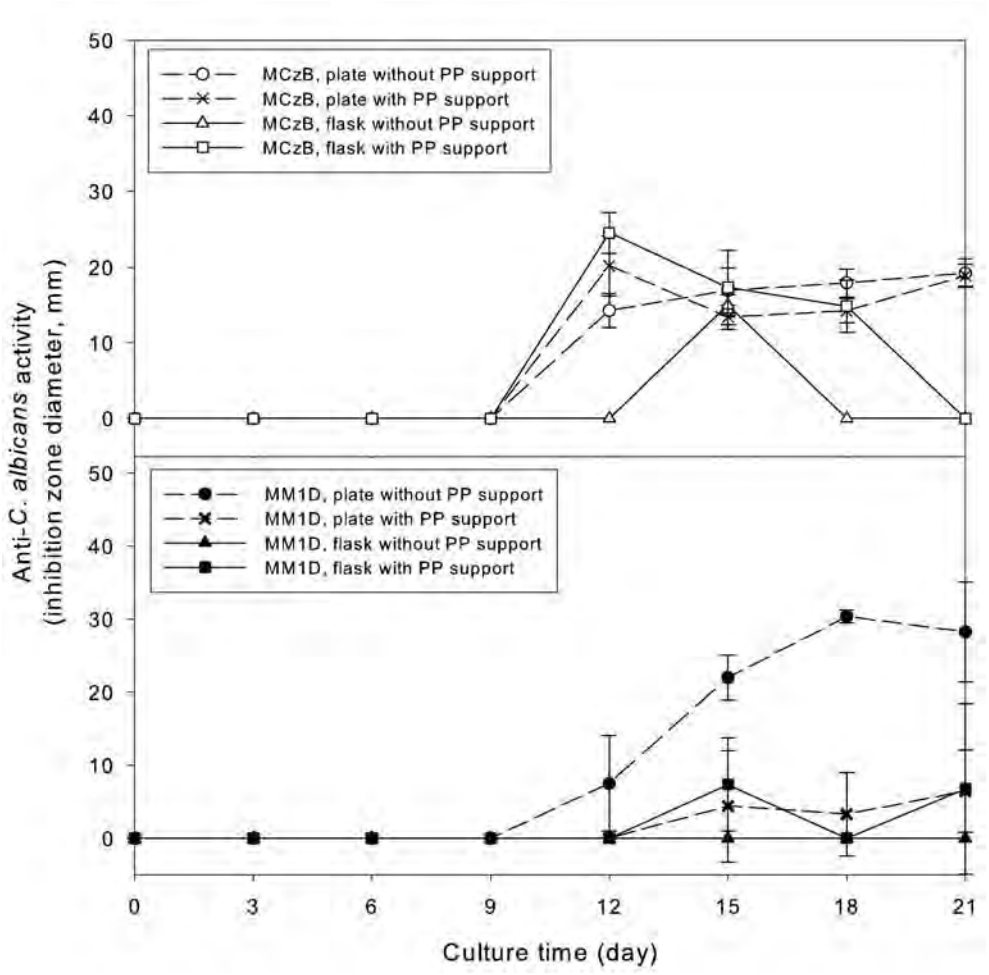


Figure 26 Anti-*C. albicans* activity of fermented broths obtained from floating solid support fermentations with static condition

activity of fermented MCzB was observed in sample taken from MCzB fermented in flask containing PP support under static condition for 12 days. The maximum anti-*C. albicans* activity of fermented MM1D was observed in sample taken from MM1D fermented in plate without PP support under static condition for 18 days. In order to determine whether the anti-*C. albicans* activity of fermented broth was resulted mainly from mycoepoxydiene, crude extracts obtained from fermented broths were examined by TLC bioautography. The results are shown in Figure 27. Patterns of relative amounts of

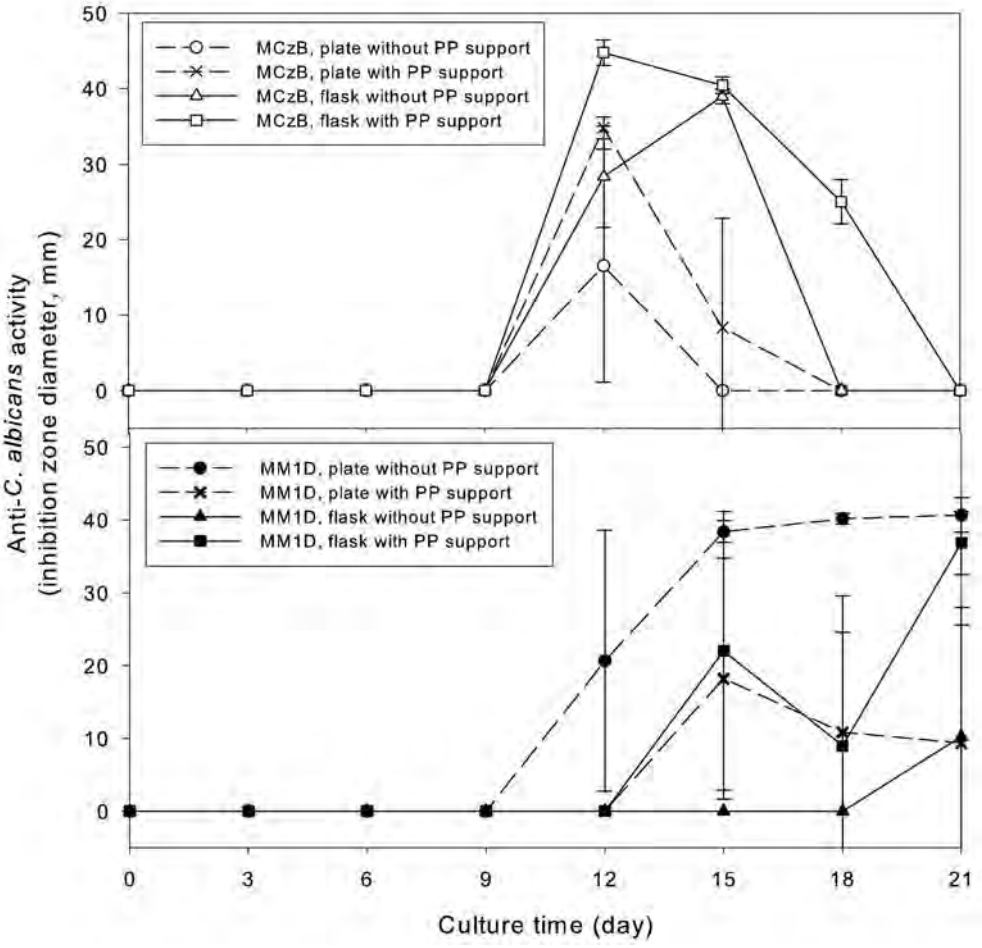


Figure 27 Anti-*C. albicans* activity by TLC bioautography analysis indicating relative amounts of mycoepoxydiene in crude extract (300 µg) obtained from media fermented in flask and in that (600 µg) obtained from media fermented in Petri dish

mycoepoxydiene produced in fermented broths determined by agar diffusion assay and TLC bioautography were resemblance except for fermented MCzB in plate with and without PP support, as shown in Figures 26 and 27.

In order to determine exact amount of mycoepoxydiene in fermented broth, samples exhibiting the highest clear inhibition zone by agar diffusion assay from each condition were selected to be analyzed by HPLC. The results are shown in Figure 28. Very low levels of mycoepoxydiene in fermented MCzB were found only in media fermented in plate and flask containing PP support. They were maximum in media fermented for 12 days. For fermented MM1D, the highest amount of

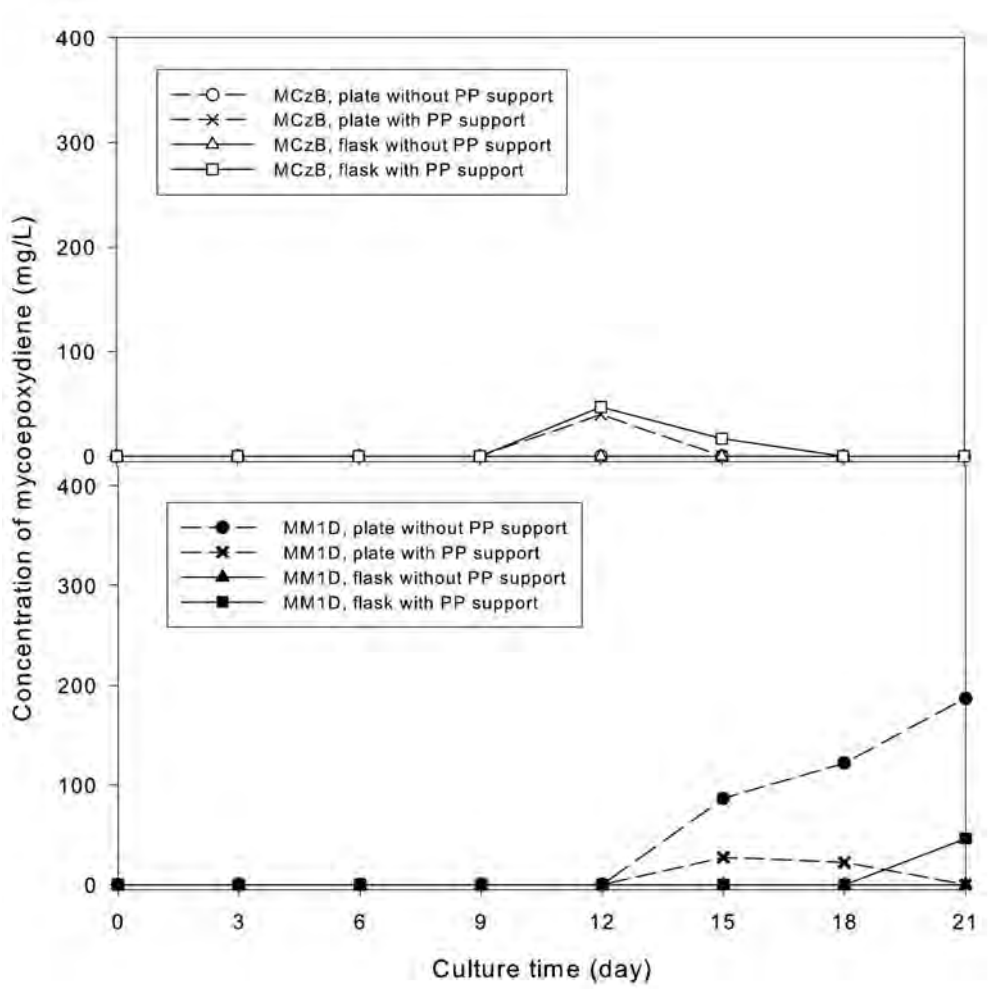


Figure 28 Mycoepoxydiene concentrations in fermented broths obtained from floating solid support fermentations under static condition

mycoepoxydiene was found in media fermented in plate without PP support. This was in agreement with the results from agar diffusion assay (Figure 26) and TLC bioautography (Figure 27).

All of the MM1D samples showing anti-*C. albicans* activity were further analyzed by HPLC. As shown in Figure 29, the highest yield of mycoepoxydiene was increased approximately 4.76 times from 24.56 mg/L (submerged fermentation in MCzB) to 117 mg/L when cultured in MM1D Petri dishes without PP support at 25°C on day 18 of cultivation, with stationary condition. These results suggested that cultivation of fungus in Petri dish, which confers large surface area for fungal growth and relatively high dissolved oxygen compared to culture in Erlenmeyer flask, favored mycoepoxydiene production by *Phomopsis* sp. Hant25 grown in MM1D medium. In addition, growing of fungus on PP floating solid support in Petri dish showed detrimental effect on mycoepoxydiene production.

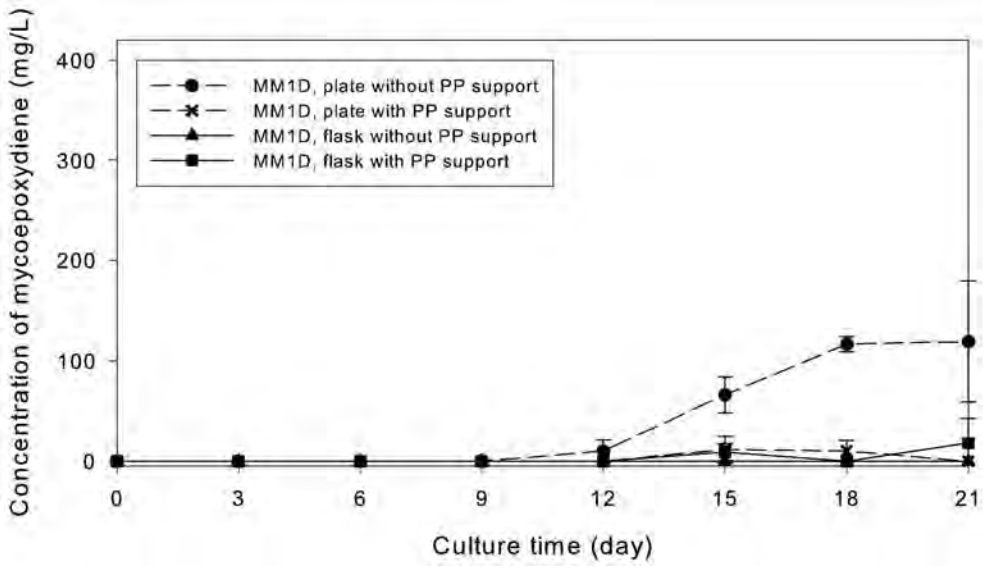


Figure 29 Mycoepoxydiene concentrations in fermented MM1D broths obtained from floating solid support fermentations under static condition

4.3.5.2 Static/shaking condition

Static/shaking fermentation of *Penicillium* sp. *LL*-WF159 in potato dextrose broth containing PP floating solid support was previously found to increase yields of anthraquinones and flavomannin (Bigelis *et al*., 2006). In this experiment, productions of mycoepoxydiene by *Phomopsis* sp. Hant25 grown in media containing PP floating solid support under static/shaking fermentations were studied. Anti-*C. albicans* activity of fermented MM1D was found to be higher than that of fermented MCzB, as shown in Figure 30. These results were in agreement with the results of static/shaking fermentation without PP support (Figure 17).

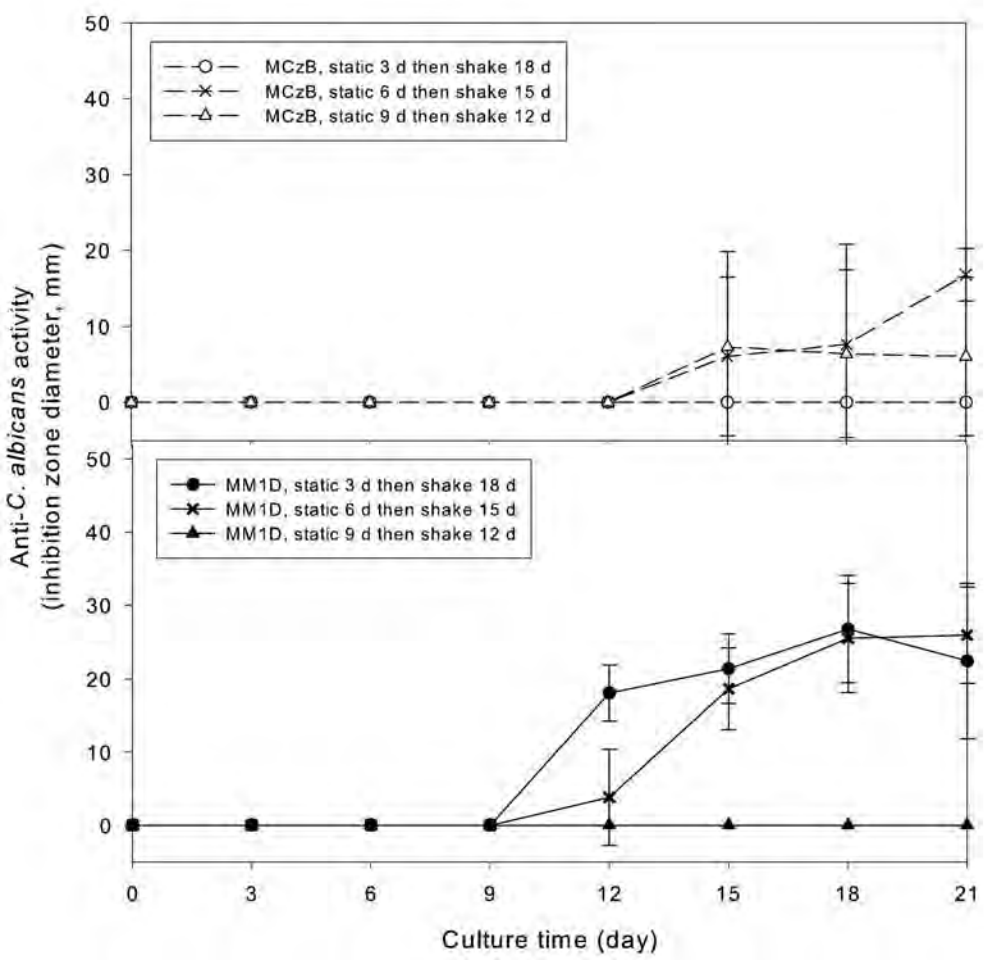


Figure 30 Anti-*C. albicans* activity of fermented broths obtained from static/shaking fermentation conditions containing PP floating solid support

Fermented broths with the highest anti-*C. albicans* activity were determined for the amount of mycoepoxydiene by HPLC analysis. Corresponding with the results observed in static/shaking fermentations without PP support (Figure 18), mycoepoxydiene yields from fermented MM1D were much higher than those from fermented MCzB, as shown in Figure 31. All of the MM1D samples showing the clear inhibition zone were further examined for mycoepoxydiene yields by HPLC analysis. The maximum amount of mycoepoxydiene was found in MM1D fermented with PP support under static/shaking condition of being static for 6 days and then shaken at 120 rpm for

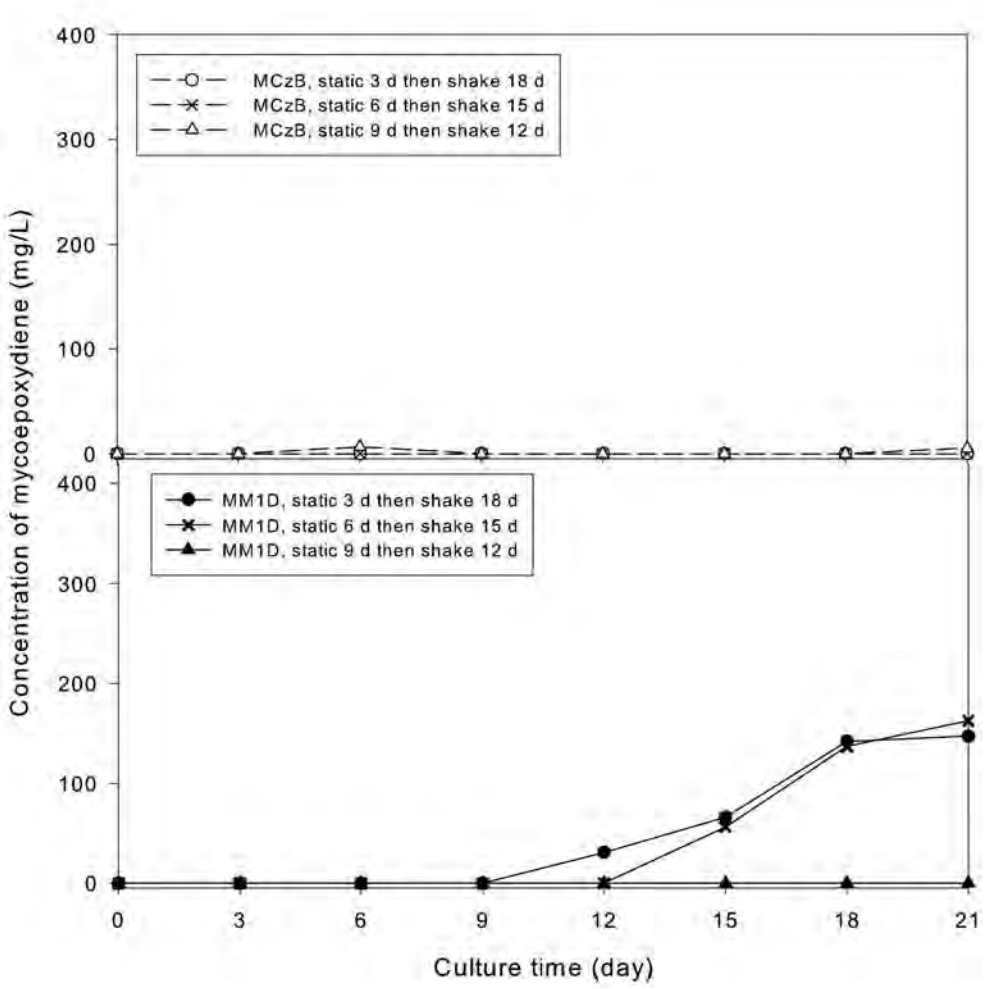


Figure 31 Mycoepoxydiene concentrations in fermented broths obtained from static/shaking fermentation conditions containing PP floating solid support

15 days at 25°C, as shown in Figure 32. This was 96 mg/L, which was 3.91 times higher than that from the submerged fermentation under static condition (24.56 mg/L). Mycoepoxydiene yield produced by *Phomopsis* sp. Hant25 grown under static/shaking condition with PP floating support was lower than that produced by fungus grown under static/shaking condition with filter paper and gauze solid support. It decreased approximately 71.17% and 65.59% when compared with filter paper and gauze support, respectively. This might be arisen from the effect of oxygen transfer limitation by the floating polymer which concealed surface area of liquid medium. The dissolved oxygen tension of the medium is important to the production of various metabolites by filamentous fungi (Kubicek *et al*., 1980; Du *et al*., 2003). In contrast, fermentation of *Penicillium* sp. *LL*-WF159 in potato dextrose broth under static/shaking condition (static 7 days, then shaken 7 days at 200 rpm) with PP support could increase flavomannin and anthraquinones yields (Bigelis *et al*., 2006).

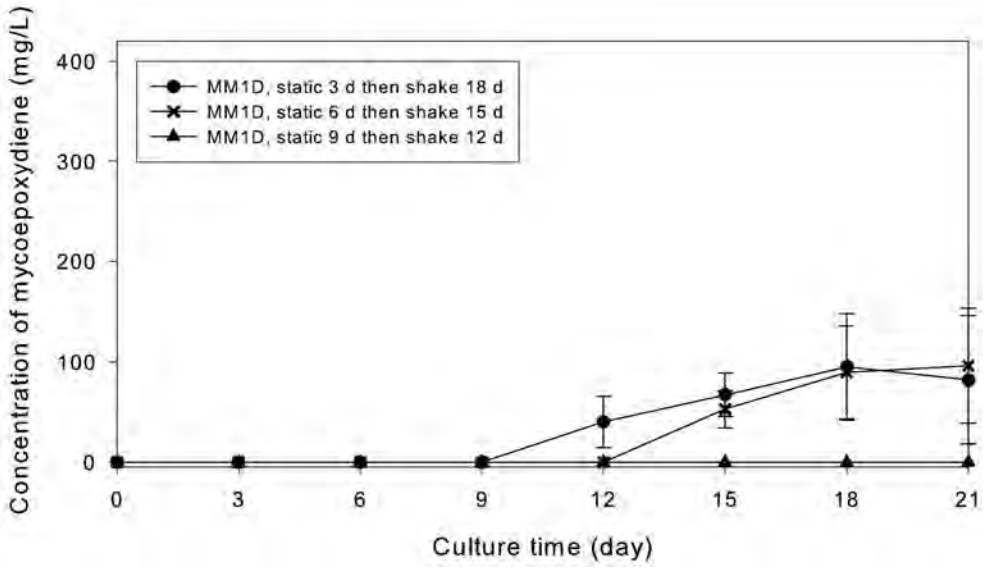


Figure 32 Mycoepoxydiene concentrations in MM1D fermented broths obtained from static/shaking fermentation conditions containing PP floating solid support

4.3.6 Fed-batch fermentation method on mycoepoxydiene production

In fed-batch fermentation, only MM1D was used as culture medium for mycoepoxydiene production because previous data revealed that MCzB could produce low yield. Fed batch fermentations were studied under static/shaking conditions without solid support and with filter paper, gauze, and PP solid supports.

4.3.6.1 Filter paper solid support

Anti-*C. albicans* activity and mycoepoxydiene concentration of fermented MM1D samples were determined by agar diffusion assay and HPLC analysis, respectively. The high value of anti-*C. albicans* activity was found in samples taken from fed-batch fermentation under static/shaking conditions with filter paper support of being static for 3 days and then shaken for 12 days, and being static for 6 days and then shaken for 18 days, as shown in Figure 33.

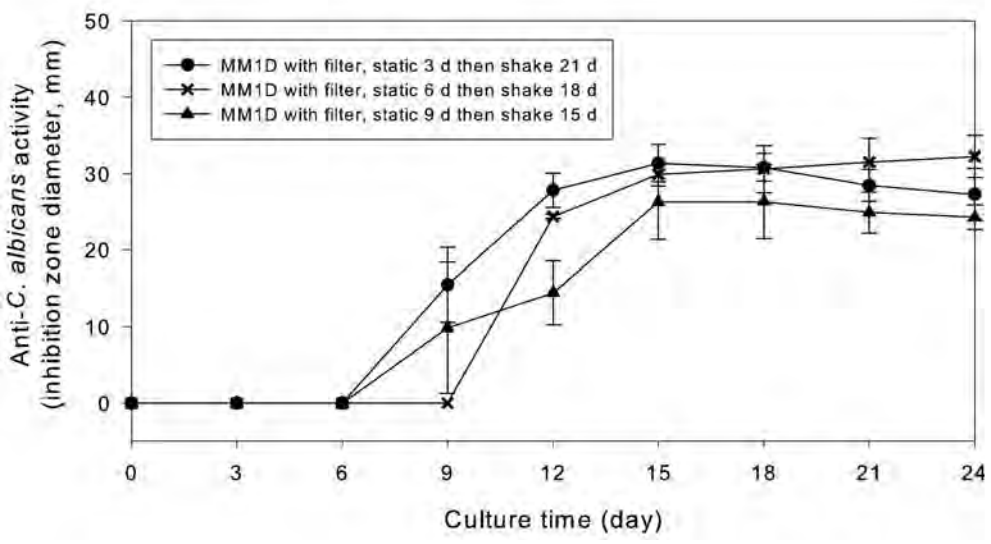


Figure 33 Anti-*C. albicans* activity of fermented MM1D broths obtained from fed-batch fermentation conditions containing filter paper solid support

It was apparent that maximum production of mycoepoxydiene in fed-batch fermentation with filter paper support at day 21 of cultivation reach 191 mg/L

when cultured in MM1D at 25°C with static for 6 days, then agitation rate at 120 rpm for 15 days, as shown in Figure 34. It was 7.78 times increase in productivity over that from preliminary submerged fermentation culture.

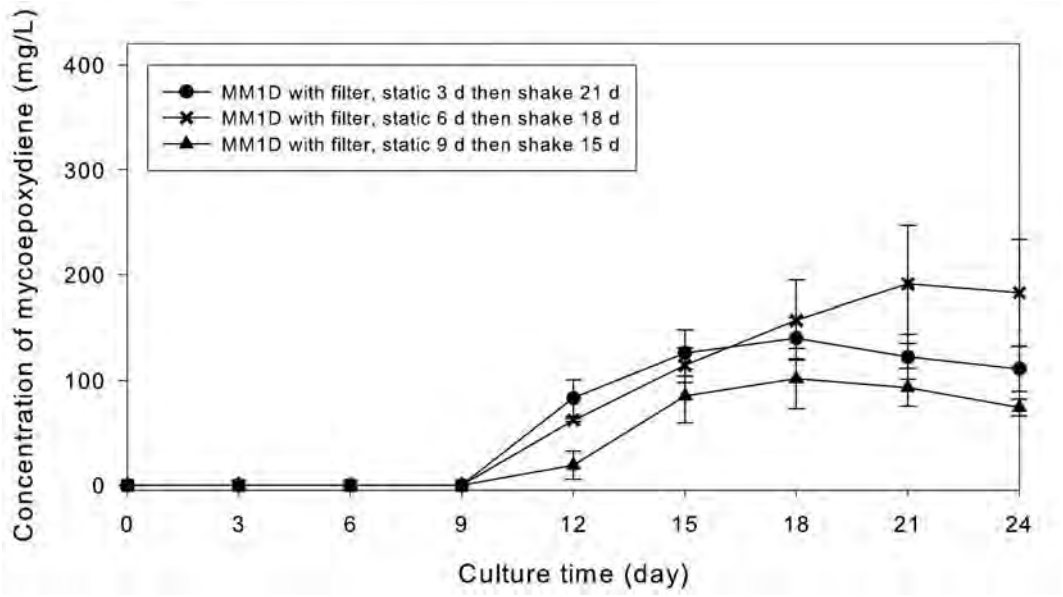


Figure 34 Mycoepoxydiene concentrations in fermented MM1D broths obtained from fed-batch fermentation conditions containing filter paper solid support

4.3.6.2 Gauze solid support

MM1D samples from fed-batch fermentation under three static/shaking conditions with gauze support showed comparable anti-*C. albicans* activity, as shown in Figure 35. Maximum production of mycoepoxydiene was observed in MM1D broth fermented under fed-batch fermentation in static/shaking method of being static for 9 days and then shaken for 15 days, as shown in Figure 36. It reached 301 mg/L which was 12.26 times increase in productivity over that from preliminary submerged fermentation culture.

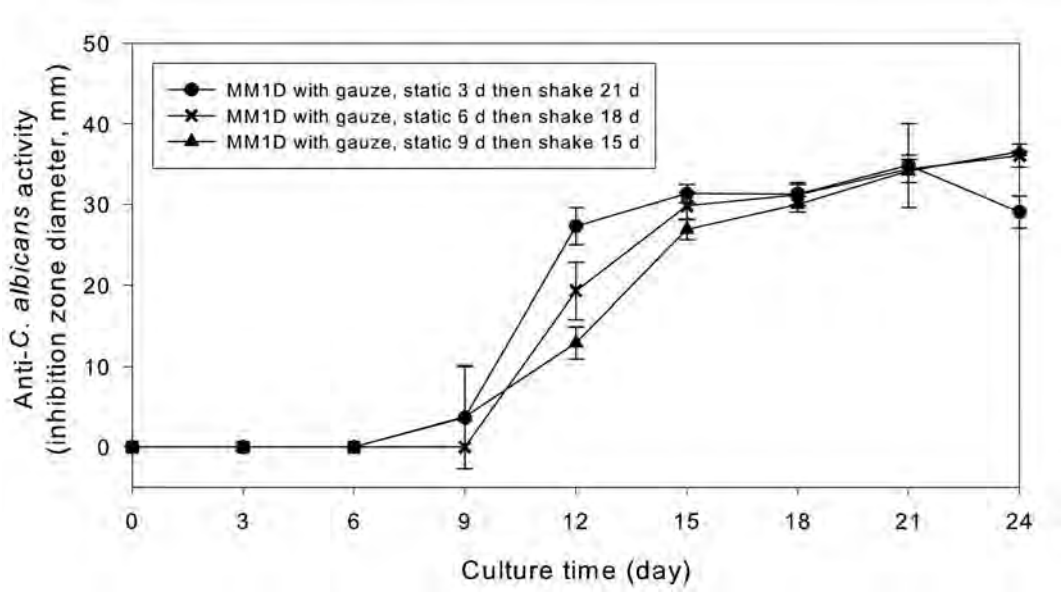


Figure 35 Anti-*C. albicans* activity of fermented MM1D broths obtained from fed-batch fermentation conditions containing gauze solid support

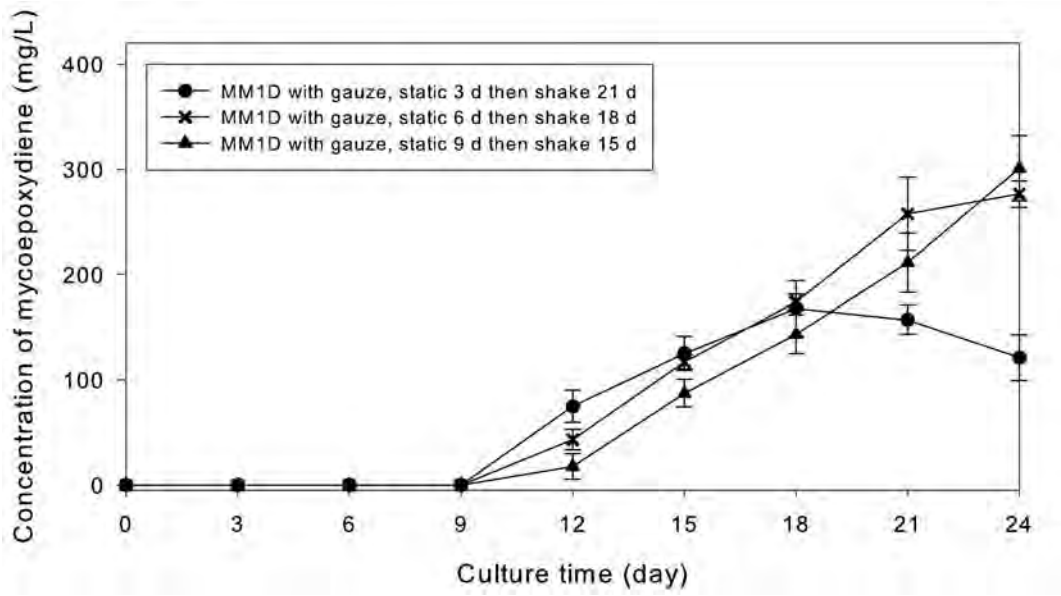


Figure 36 Mycoepoxydiene concentrations in fermented MM1D broths obtained from fed-batch fermentation conditions containing gauze solid support

4.3.6.3 PP floating solid support

MM1D samples from static conditions of 3 and 6 days of fed-batch fermentation under static/shaking conditions with PP floating solid support showed comparable maximum anti-*C. albicans* activity, as shown in Figure 37. Maximum production of mycoepoxydiene was observed in MM1D broth fermented under fed-batch fermentation in static/shaking method of being static for 6 days and then shaken for 18 days, as shown in Figure 38. It reached 127 mg/L which was 5.17 times increase in productivity over that from preliminary submerged fermentation culture.

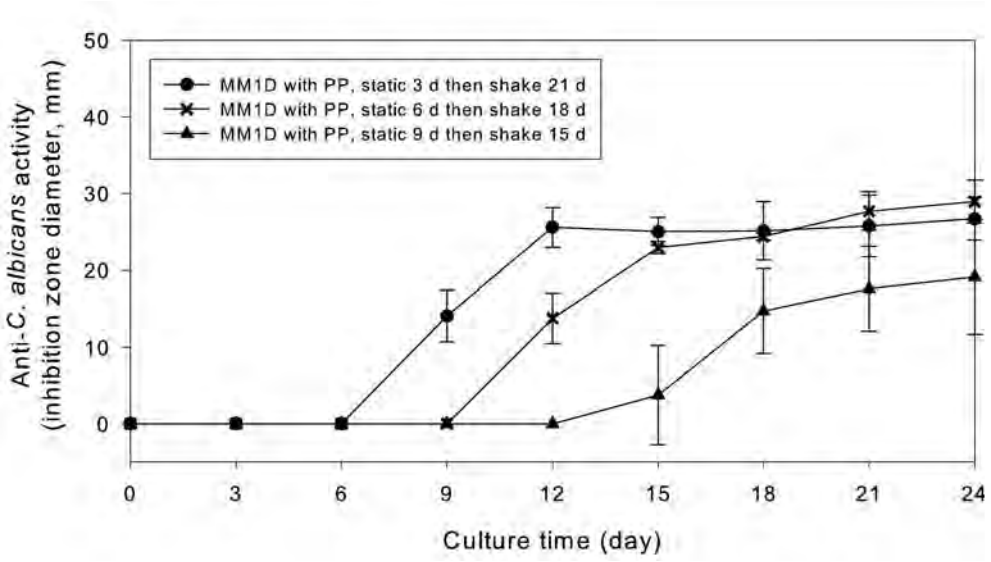


Figure 37 Anti-*C. albicans* activity of fermented MM1D broths obtained from fed-batch fermentation conditions containing PP floating solid support

4.3.6.4 Without support

Anti-*C. albicans* activity was found to be maximum in fermented MM1D of fed-batch fermentation without support under static/shaking conditions of being static for 6 and 9 days, as shown in Figure 39. It was apparent that maximum production of mycoepoxydiene in fed-batch fermentation without support under

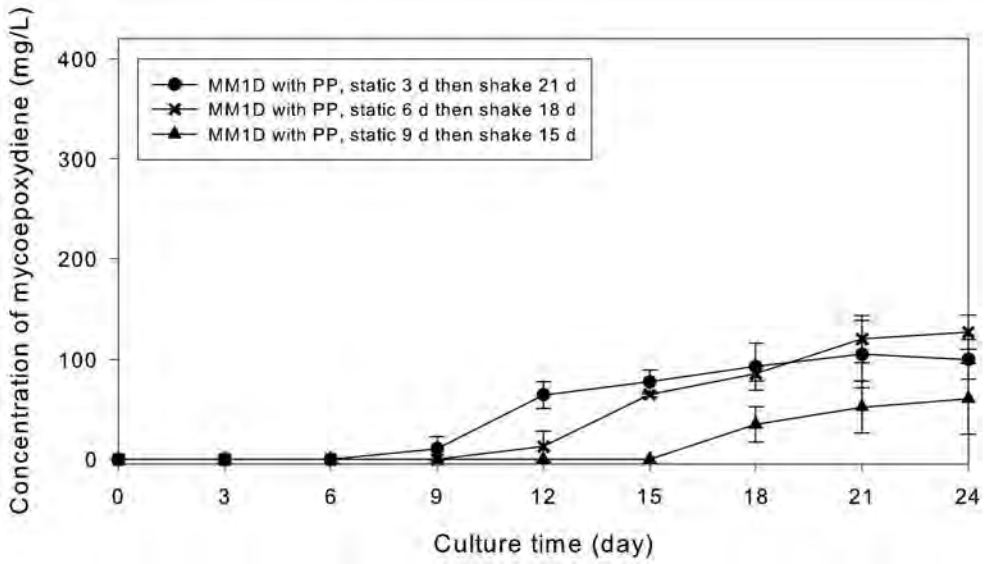


Figure 38 Mycoepoxydiene concentrations in fermented MM1D broths obtained from fed-batch fermentation conditions containing PP floating solid support

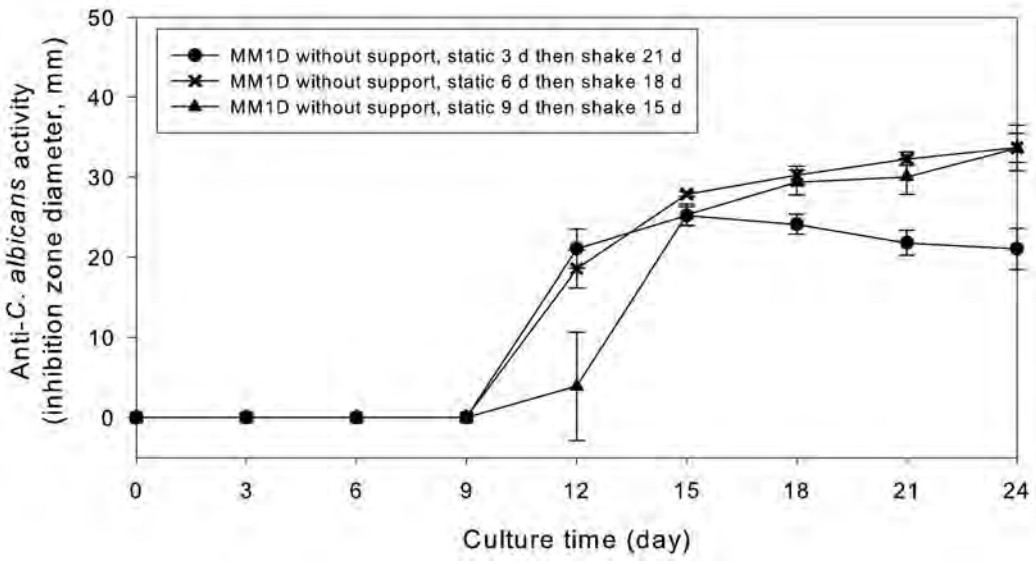


Figure 39 Anti-*C. albicans* activity of fermented MM1D broths obtained from fed-batch fermentation conditions without solid support

static/shaking method of being static for 6 days and then shaken for 18 days, as shown in Figure 40. It reached 211 mg/L, This was 8.59 times increase in productivity over that from preliminary submerged fermentation culture.

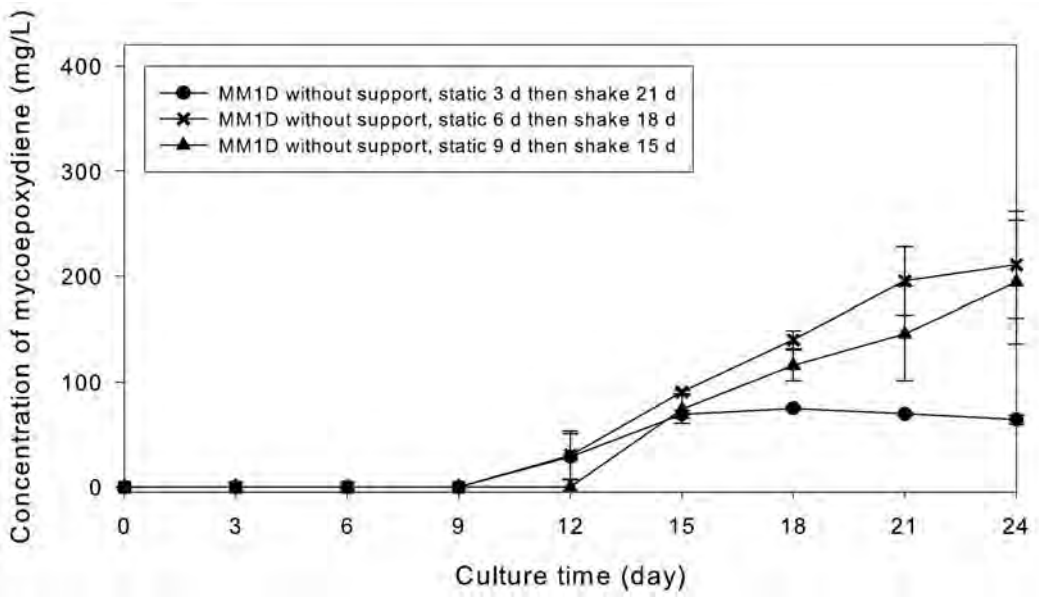


Figure 40 Mycoepoxydiene concentrations in fermented MM1D broths obtained from fed-batch fermentation conditions without solid support

In summary, single fed-batch fermentation method was an effective method to elevate 8.59 folds of mycoepoxydiene production when compared to preliminary submerged fermentation. Interestingly, it was obvious that this operation was highly successful in raising the quantity of mycoepoxydiene production in MM1D with gauze support. The highest yield of mycoepoxydiene was increased approximately 12.26 times from 24.56 mg/L to 301 mg/L when cultured in MM1D with gauze support at 25°C under static/shaking condition (static for 9 days and then shaken at 120 rpm for 15 days). This level of productivity of fed-batch fermentation was 7.89% increase over the batch static/shaking fermentation method with gauze support. Moreover, the fed-batch fermentation with PP support corresponded with gauze support, as both methods

similarly increased the production of mycoepoxydiene. Maximum mycoepoxydiene production of PP support in fed-batch culture was recorded at 127 mg/l, which was increased 32.29% when compared to batch fermentation method.

In contrast, mycoepoxydiene yields obtained from fed-batch cultures with filter paper and without support were significantly lower when compared with batch method. The productions of mycoepoxydiene in both conditions were observed to decrease approximately 42.64% and 18.85% when compared to static/shaking batch fermentation with filter paper support and without support, respectively.

The fed-batch fermentation for culture *Phomopsis* sp. Hant25 should be determined further because the experiment had many interesting issues to study e.g.

1. Specific needs of nutrients or other factors to add during fermentation
2. Finding of the suitable time to add the specific nutrients
3. The best conditions for fed-batch fermentation method such as medium, temperature, agitation, pH, etc.
4. Harvested time to collect mycoepoxydiene
5. Effect of substrate concentrations as inducer or inhibitor

4.3.7 Solid-liquid two phase partitioning fermentation method on mycoepoxydiene production

4.3.7.1 Determination of adsorption of mycoepoxydiene on Amberlite XAD-7 beads and elution in small scale

Amberlite XAD-7 was applied to remove mycoepoxydiene from culture media because it can remove non-aromatic compounds from polar solvents (Rohm and Haas Singapore Pte. Ltd., 2002). Mycoepoxydiene structure possesses the ester functional group that can bind with Amberlite XAD-7.

Before adding Amberlite XAD-7 bead, fermented MM1D contained mycoepoxydiene in a concentration of 247 mg/L as analyzed by HPLC. After

mixing the beads with fermented MM1D for 1 h, HPLC analysis could not detect mycoepoxydiene left in the medium adsorbed with 1, 2, 3 and 4% of beads. This indicated that all mycoepoxydiene in the medium was completely adsorbed by Amberlite XAD-7. Elution of mycoepoxydiene adsorbed on 1% of bead by methanol for 2 times could completely elute mycoepoxydiene, as shown in Figure 41. Therefore, 1% of beads were suitable to adsorb all of mycoepoxydiene from culture broth and extraction with methanol for 2 times was an effective method.

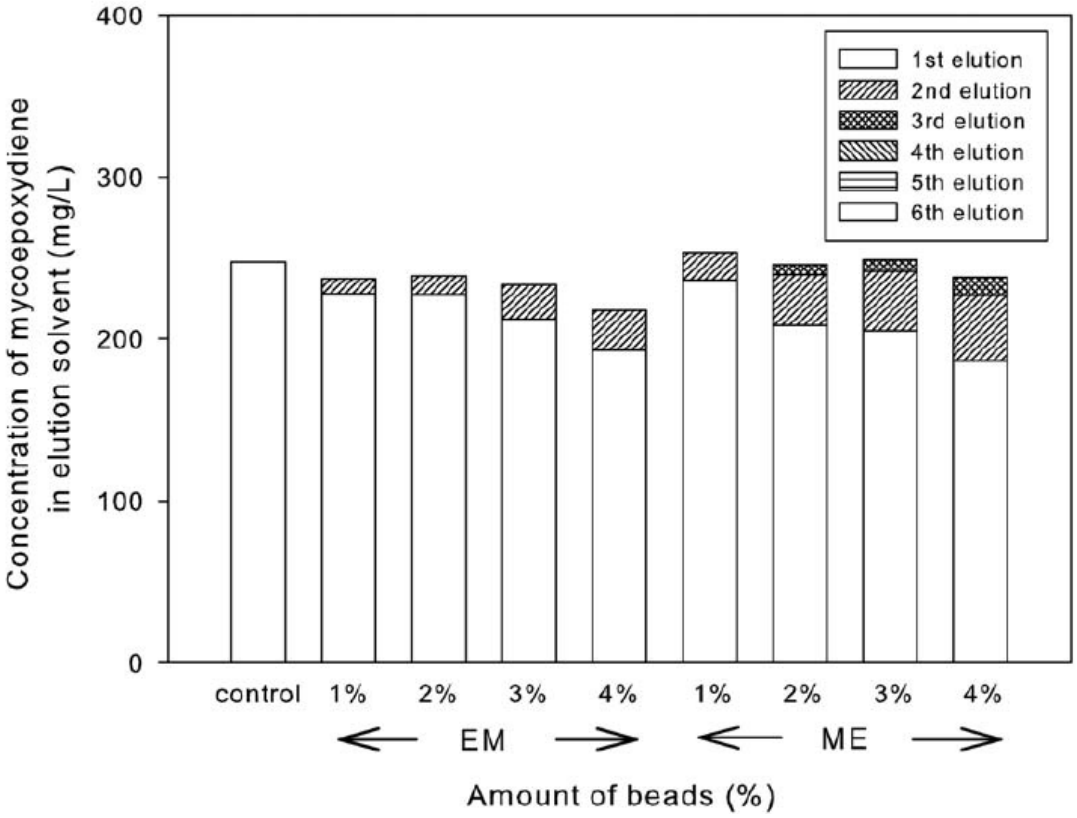


Figure 41 Effectiveness of Amberlite XAD-7 beads for adsorption of mycoepoxydiene in fermented MM1D and elution efficiency of mycoepoxydiene from beads, in small scale. EM means that elution of compound by 3 times of ethyl acetate and then 3 times of methanol. ME means that elution of compound by 3 times of methanol and then 3 times of ethyl acetate.

4.3.7.2 Determination of adsorption of mycoepoxydiene on Amberlite XAD-7 beads and elution in actual scale

According to the results in small scale, adsorption of mycoepoxydiene with 1% Amberlite XAD-7 beads and 4 times of methanol elution was used in actual scale. It was found that 1% of beads added to the fermented MM1D in culturing condition could adsorb completely mycoepoxydiene from fermented broths containing mycoepoxydiene in a concentration of less than 300 mg/L, as shown in Figure 42. For sample containing mycoepoxydiene in a concentration of 300 mg/L, only small amounts of mycoepoxydiene were remained in the broth sample. This suggested that Amberlite XAD-7 beads could effectively adsorb mycoepoxydiene produced in static/shaking fermentation under culturing condition, and adsorbed mycoepoxydiene in beads could be subsequently eluted off by methanol.

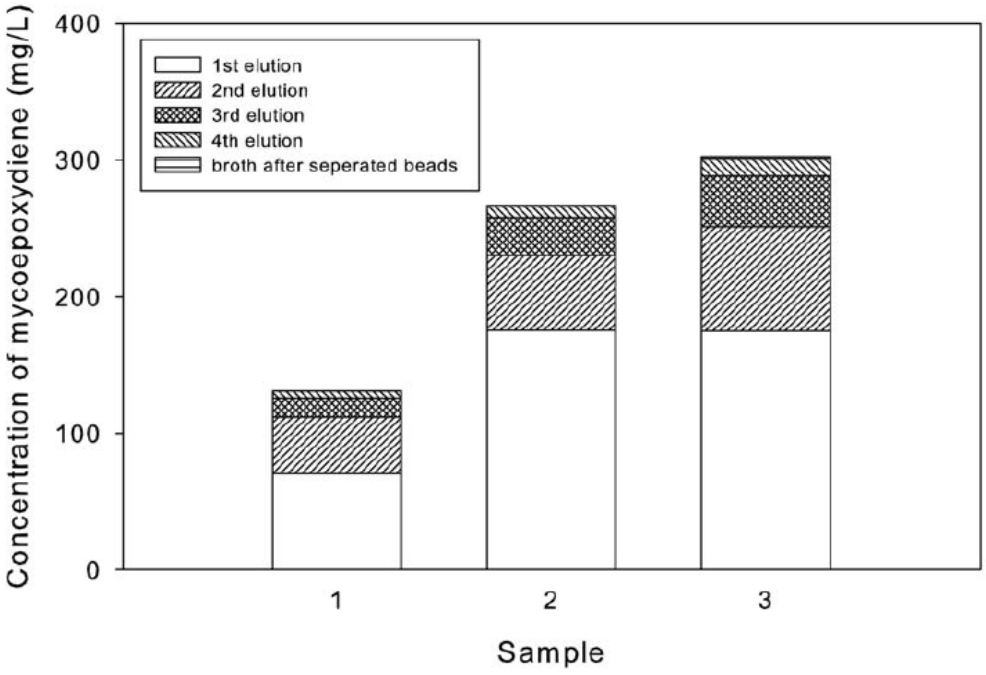


Figure 42 Effectiveness of Amberlite XAD-7 beads for adsorption of mycoepoxydiene in fermented MM1D under cultivating condition and elution efficiency of mycoepoxydiene from beads by methanol, in actual scale

4.3.7.3 Solid-liquid two phase partitioning fermentation method

In secondary metabolite biosynthesis, feedback regulation by secondary metabolites is a common factor which inhibits or represses their own biosynthesis (Betina, 1994). In order to increase mycoepoxydiene yield produced by *Phomopsis* sp. Hant25, Amberlite XAD-7 beads was used as a solid adsorbent to adsorb mycoepoxydiene in the culture medium to prevent the feedback regulation that might be occurred.

4.3.7.3.1 Static condition with 1% Amberlite XAD-7 beads

Amberlite XAD-7 beads in a final concentration of 1% were added into MM1D medium before sterilization. This medium containing beads was used in cultivation of *Phomopsis* sp Hant25 under static condition, as described in Section 3.5.7.4.1. Normally, *Phomopsis* sp. Hant25 cultured in MM1D medium with static condition could not produce mycoepoxydiene. Hence, production of this compound in a few amount shown to be feasible, as shown in Figure 43. This experiment was performed to compare with other methods using Amberlite XAD-7 beads.

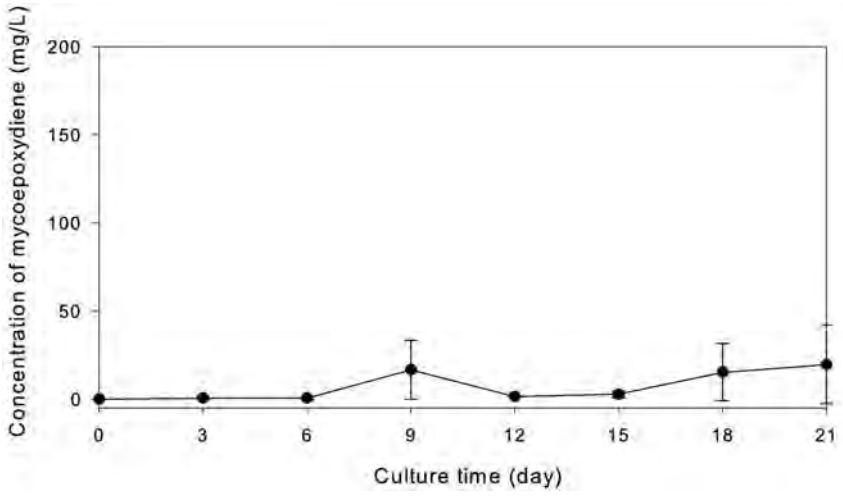


Figure 43 Mycoepoxydiene production by *Phomopsis* sp. Hant25 grown in MM1D containing 1% of Amberlite XAD-7 beads under static condition

4.3.7.3.2 Static/shaking condition with 1% Amberlite XAD-7 beads

Fermentation of *Phomopsis* sp. Hant25 under static/shake condition was conducted in MM1D containing 1% Amberlite XAD-7 beads. As shown in Figure 44, mycoepoxydiene produced by the fungus was significantly decreased in contrast to the result from static/shaking fermentation method without beads. Amberlite XAD-7 beads might adsorb some nutrients which importance for growth or production of mycoepoxydiene in the culture medium because the morphology of fungus with 1% beads absolutely distinguished from the static/shaking fermentation method, as shown in Figure 45. Therefore, it led to later experiment which Amberlite XAD-7 beads were added after culturing the fungus for 18 days. The fungal morphology in this condition was the same as previous static condition with beads.

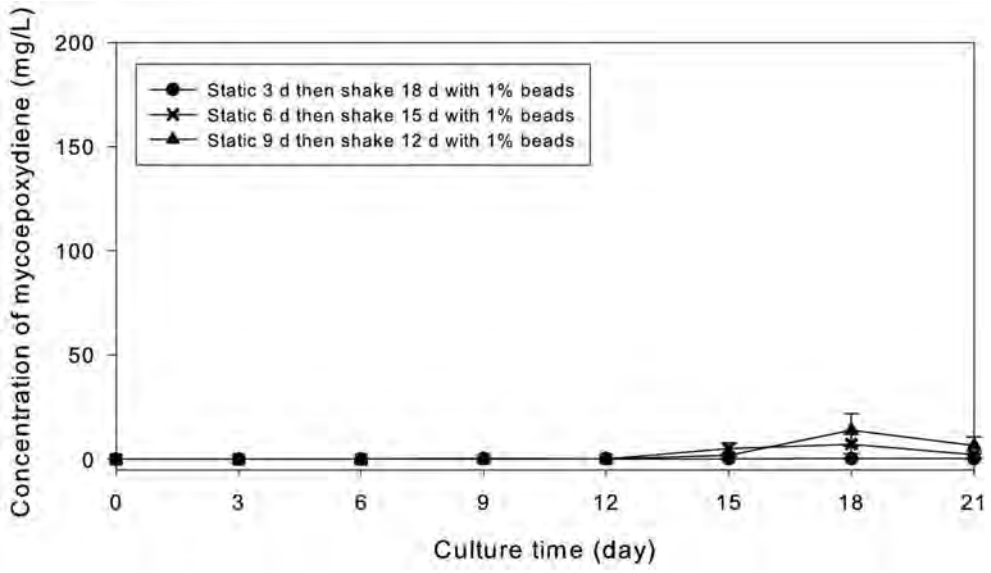


Figure 44 Effect of static/shaking fermentation in medium containing 1% beads on mycoepoxydiene production

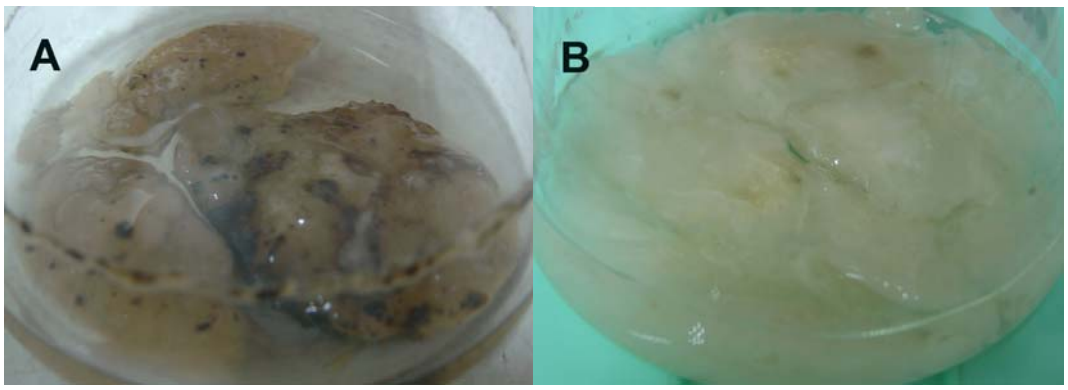


Figure 45 Morphology of *Phomopsis* sp. Hant25 cultivated in static/shaking fermentation methods (A) without beads and (B) with beads

4.3.7.3.3 Static/shaking condition with 1% Amberlite XAD-7 beads addition on day 18

As shown in Figure 46, addition of 1% Amberlite XAD-7 beads on day 18 of static/shaking cultivation affected mycoepoxydiene production. After addition of beads on day 18 and further incubation for one hour and three days (day 21) amount of mycoepoxydiene eluted from beads diminished 50.36% and 29.14%, respectively, when compared with mycoepoxydiene in fermented broth on day 18 before adding beads. In return, if the beads were harvested on day 24 and day 27 of cultivation, the yields of mycoepoxydiene enhanced 23.53% and 15.62%, respectively. Increase of mycoepoxydiene yield by addition of Amberlite XAD-7 beads suggested that biosynthesis of mycoepoxydiene might be controlled by negative feedback mechanism. Mycoepoxydiene has been found to be cytotoxic (Prachya *et al*., 2007, Sommart *et al*., 2009). When the quantity of this compound reached to toxic level on fungus, it might lead to inhibition of compound production. Amberlite XAD-7 beads made the reduction of mycoepoxydiene in system. Finally, the fungus could further produce mycoepoxydiene to the optimum level.

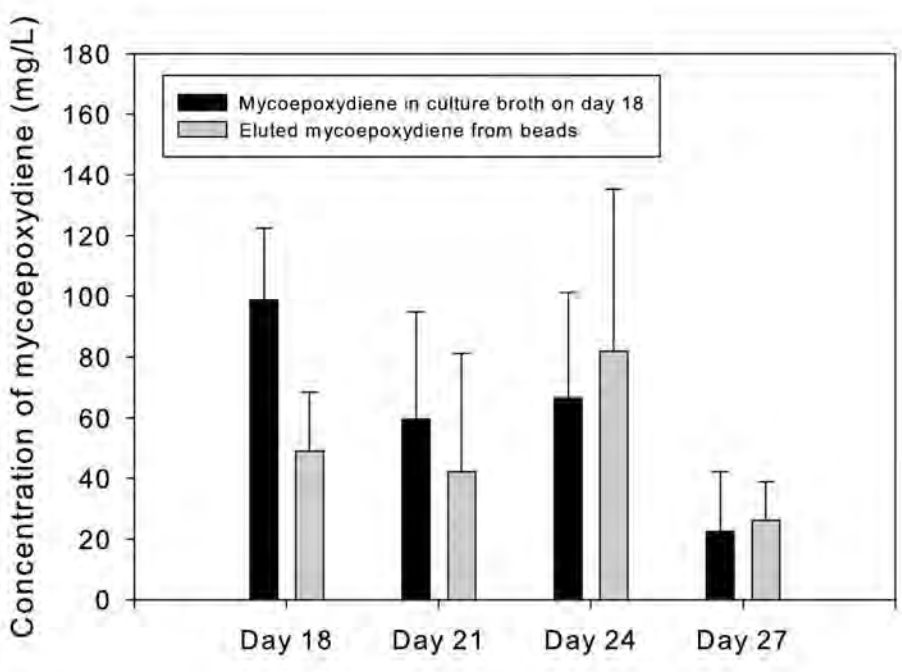


Figure 46 Effect of addition of 1% beads on day 18 of static/shaking fermentation on mycoepoxydiene production

Amberlite XAD-7 are used in the adsorption of secondary metabolites from the microbial fermentation cultures, polymeric adsorbent normally adsorbs the organic matter from aqueous broths like organic solvent extraction, and can also adsorb polar compounds from non-polar solvents. The presence of a resin during the fermentation affected significantly on the production of metabolites. That effect was unpredictable, maybe increasing or decreasing. The advantages of resin were explainable from some reports. When the metabolites were produced in the early stage and released into the culture broth, the resin captured the product and prevented formation of other derivatives. When the metabolites were produced in the late stage and reached inhibitory level, the resin removed them from the medium and enhanced production (Jarvis *et al*., 1990). Conversely, our experiment of using the resin XAD-7 in *Phomopsis* sp. Hant25 fermentation showed decreasing of mycoepoxydiene production especially adding the resin at initial phase of growth. It was surmised that the resin might adsorbed some important compositions in the medium. Thus, fungal growth and

metabolite production were inhibited. Not only the inhibition of fungal growth, but also the alteration of fungal morphology was observed. In group of static for 3 days then shake 18 days was absolutely adapted. Conclusion of results from the experiment which beads were added on day 18 could not be done owing to the variation of morphology of fungus. The morphology in each flask was quite different. However, improvement of this method has been still interesting due to the ability of beads to adsorb mycoepoxydiene and complete elution of mycoepoxydiene from beads. Moreover, this method was able to reduce the quantity of solvent used for isolation of the compound from the culture broth because the amount of solvent required for the elution of mycoepoxydiene from beads was only 13.33% of direct extraction method. Generally, ethyl acetate is used for separation of mycoepoxydiene from culture media (Prachya *et al.*, 2007). Results found in this study by adsorption of mycoepoxydiene from culture broth on Amberlite XAD-7 bead and then extraction of mycoepoxydiene from beads provided alternative method to extract compound with significantly reduced amount of solvent.

CHAPTER V

CONCLUSION

According to the interesting cytotoxic activities of mycoepoxydiene, its mechanism of action and related studies should be conducted further. These require high amount of mycoepoxydiene. However, the conventional shaken or stationary liquid fermentation of the endophytic fungus *Phomopsis* sp. Hant25 could produce mycoepoxydiene in low yield. It is interesting to increase yields of this bioactive metabolite by alternative fermentation methods. The simple agar diffusion assay based on anti-*C. albicans* activity of mycoepoxydiene in combination with ketoconazole was developed to determine relative amount of mycoepoxydiene in fermentation broths. HPLC analysis using Hypersil ODS column with 0.1% acetic acid in H_2O: acetonitrile (75:25) as mobile phase was developed to detect directly the actual quantity of mycoepoxydiene in fermentation broth. Figure 47 summarizes mycoepoxydiene yields obtained from alternative fermentation methods studied.

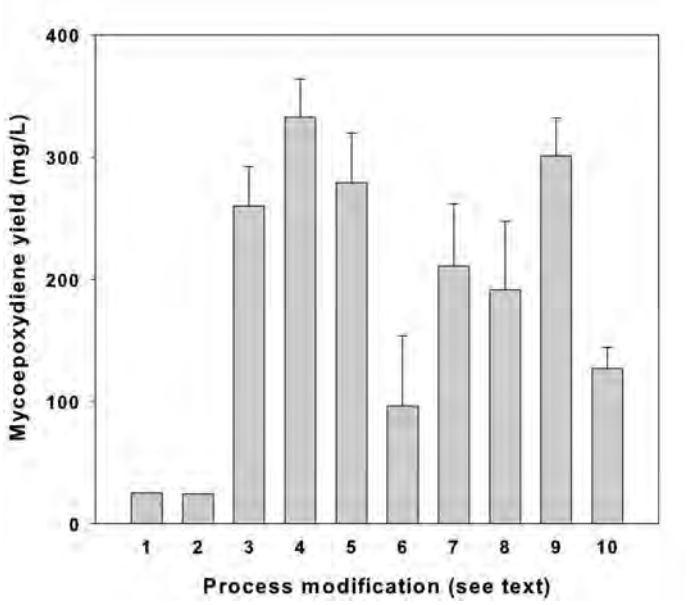


Figure 47 Summary of improved mycoepoxydiene yields obtained by alternative fermentation methods

Process 1 was the submerged fermentation in a 1000-ml Erlenmeyer flask containing 200 ml of MCzB and incubated at 25°C for 21 days, previously studied by Prachya *et al.* (2007). Mycoepoxydiene was obtained after purification of a broth extract and afforded 25.36 mg/L.

Process 2 was preliminary submerged fermentation in a 250-ml Erlenmeyer flask containing 50 ml of MCzB and incubated at 25°C for 21 days, performed in this study. Production yield of mycoepoxydiene obtained is 24.56 mg/L.

Processes 3 to 6 are batch fermentation with static/shaking fermentation method. Processes 7-10 are fed-batch fermentation with static/shaking fermentation method.

The maximum production of mycoepoxydiene concentration in static/shaking fermentation method on day 18 of cultivation reach 260 mg/L when cultured in MM1D at 25°C with static for 3 days, then agitation rate at 120 rpm for 15 days (Process 3). This result was 10.59 times increase in productivity over that obtained from preliminary submerged fermentation.

The highest yield of mycoepoxydiene was increased approximately 13.56 times from 24.56 mg/L to 333 mg/L when cultured in MM1D with filter paper support at 25°C on day 18 of cultivation, with static for 6 days, then shaken at 120 rpm for 12 days (Process 4).

Maximum mycoepoxydiene production of gauze support in MM1D at day 18 of cultivation was recorded at 279 mg/L, which is 11.36 times compared to the preliminary submerged fermentation method, with static for 6 days, then agitation rate at 120 rpm for 12 days at 25°C (Process 5).

Cultures cultivated with PP support for 21 days, with static for 6 days and then shaken at 120 rpm for 15 days at 25°C in MM1D, produced the maximal amount of mycoepoxydiene 96 mg/L, which was 3.91 times higher than the value from preliminary submerged fermentation (Process 6).

The maximum production of mycoepoxydiene concentration in fed-batch with static/shaking fermentation method on day 24 of cultivation reach 211 mg/L when cultured in MM1D at 25°C with static for 6 days and then shaken at 120 rpm for 18 days.

This result was 8.59 times increase in productivity over that obtained from preliminary submerged fermentation (Process 7).

The highest yield of mycoepoxydiene was increased approximately 7.78 times from 24.56 mg/L to 191 mg/L when cultured in fed-batch with filter paper support in MM1D at 25°C on day 21 of cultivation, with static for 6 days and then shaken at 120 rpm for 15 days (Process 8).

Maximum mycoepoxydiene production of fed-batch with gauze support in MM1D on day 24 of cultivation with static for 9 days and then shaken at 120 rpm for 15 days at 25°C was recorded at 301 mg/L, which is 12.26 times compared to that from the preliminary submerged fermentation method (Process 9).

Fed-batch culture with PP support for 24 days of cultivation, with static for 6 days and then shaken at 120 rpm for 18 days at 25°C in MM1D, produced the maximal amount of mycoepoxydiene 127 mg/L, which was 5.17 times higher than that from the preliminary submerged fermentation method (Process 10).

REFERENCES

Barrios-González, J., J. Baños, A. Covarrubias and A. Garay-Arroyo. 2008. Lovastatin biosynthetic genes of *Aspergillus terreus* are expressed differentially in solid-state and in liquid submerged fermentation. Applied Microbiology and Biotechnology 79(2): 179-186.

Barrios-Gonzalez, J. and A. Mejia. 1996. Production of secondary metabolites by solid-state fermentation. Biotechnology Annual Review 2: 85-121.

Barrios-González, J. and A. Mejýa. 2008. Production of Antibiotics and other Commercially Valuable Secondary Metabolites. Current Developments in Solid-state Fermentation: 302-336.

Betina, V. 1994. Bioactive secondary matabolite of microorganisms. Amsterdam, Ister science press, Bratislava, Slovak republic.

Bigelis, R., H. He, H. Yang, L.-P. Chang and M. Greenstein. 2006. Production of fungal antibiotics using polymeric solid supports in solid-state and liquid fermentation. Journal of Industrial Microbiology and Biotechnology 33(10): 815-826.

Bigelis, R., H. He, H. Y. Yang, L. P. Chang and M. Greenstein. 2006. Production of fungal antibiotics using polymeric solid supports in solid-state and liquid fermentation. Journal of Industrial Microbiology Biotechnology 33(10): 815-826.

Blanch, H. T. and D. S. Clark. 1997. Biochemical Engineering. New York, Marcel Dekker, Inc.

Bulder, C. J. E. A., J. W. M. Rivière and W. Verhoeven. 1989. Kluyver's work in retrospect: Wisdom of foresight? Antonie van Leeuwenhoek 56(2): 109-126.

Cai, P., A. T. McPhail, E. Krainer, B. Katz, C. Pearce, C. Boros, *et al*. 1999. Mycoepoxydiene represents a novel class of fungal metabolites. Tetrahedron Letters 40(8): 1479-1482.

Collins, L. D. and A. J. Daugulis. 1996. Use of a two phase partitioning bioreactor for the biodegradation of phenol. Biotechnology Techniques 10(9): 643-648.

Costerton, J. W. 1984. The role of bacterial exopolysaccharides in nature and disease. Arlington, VA, ETATS-UNIS, Society for Industrial Microbiology.

Dreyfuss, M. M. and I. H. Chapela. 1994. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. Biotechnology 26: 49-80.

Du, L. X., S. J. Jia and F. P. Lu. 2003. Morphological changes of *Rhizopus chinesis* 12 in submerged culture and its relationship with antibiotic production. Process Biochemistry 38: 1643-1646.

Gloer, J. B. 2007. Applications of Fungal Ecology in the Search for New Bioactive Natural Products. Environmental and Microbial Relationships: 257-283.

Guo, B., Y. Wang, X. Sun and K. Tang. 2008. Bioactive natural products from endophytes: a review. Applied biochemistry and microbiology 44(2): 153-158.

Hafizuddin, W. M., W. Yussof and M. R. Sarmidi. 2003. Equilibrium and Kinetic Modeling of the Adsorption of Stevioside on Amberlite XAD-7 Beads. Proceedings of the International Conference on Chemical and Bioprocess Engineering (ICCBPE), Kota Kinabalu, Malaysia.

Hawksworth, D. L. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycological Research 95(6): 641-655.

Hawksworth, D. L. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycological Research 105(12): 1422-1432.

Hekmat, D., R. Bauer and J. Fricke. 2003. Optimization of the microbial synthesis of dihydroxyacetone from glycerol with *Gluconobacter oxydans*. Bioprocess and Biosystems Engineering 26(2): 109-116.

Hesseltine, C. W. 1972. Biotechnology report: Solid state fermentations. Biotechnology and Bioengineering 14(4): 517-532.

Hesseltine, C. W. 1987. Solid state fermentation--An overview. International Biodeterioration 23(2): 79-89.

Irving, J. D., H. Professor Dr. Elmar, I. Dr. John, J. Dr, rcaron, P. E*, et al.* 2005. Basic Bioreactor Concepts. Biological Reaction Engineering (Second Edition): 55-66

Iwazaki, R., E. Endo, T. Ueda-Nakamura, C. Nakamura, L. Garcia and B. Filho. 2009. In vitro antifungal activity of the berberine and its synergism with fluconazole. Antonie van Leeuwenhoek 97(2): 201-205.

Jackson, M. A. 1997. Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. Journal of Industrial Microbiology and Biotechnology 19(3): 180-187.

Jarvis, B. B., C. A. Armstrong and M. Zeng. 1990. Use of resins for trichothecene production in liquid cultures. The journal of Antibiotics 43: 1502-1504.

Kinghorn, A. D. 1997. Plant Drug Analysis. A Thin Layer Chromatography Atlas. Journal of Natural Products 60(4): 428-428.

Krishna, C. 2005. Solid-State Fermentation Systems--An Overview. Critical Reviews in Biotechnology 25(1-2): 1-30.

Kubicek, C. P., O. Zehentgruber, H. El-Kalak and M. Röhr. 1980. Regulation of citric acid production by oxygen: Effect of dissolved oxygen tension on adenylate levels and respiration in *Aspergillus niger*. Applied Microbiology and Biotechnology 9(2): 101-115.

Kumar, M. S., S. K. Jana, V. Senthil, V. Shashanka, S. V. Kumar and A. K. Sadhukhan. 2000. Repeated fed-batch process for improving lovastatin production. Process Biochemistry 36: 363-368.

Larroche, C. and J.-B. Gros. 1997. Special transformation processes using fungal spores and immobilized cells. Biotechnology of Aroma Compounds: 179-220.

Li, X.-B., G.-R. Zhao and Y.-J. Yuan. 2005. A strategy of phosphorus feeding for repeated fed-batch fermentation of penicillin G. Biochemical Engineering Journal 27(1): 53-58

Lin, X., Y. Huang, M. Fang, J. Wang, Z. Zheng and W. Su. 2005. Cytotoxic and antimicrobial metabolites from marine lignicolous fungi, *Diaporthe* sp. FEMS Microbiology Letters 251(1): 53-58.

Lotfy, W. A. 2007. The utilization of beet molasses as a novel carbon source for cephalosporin C production by *Acremonium chrysogenum*: Optimization of

process parameters through statistical experimental designs. Bioresource Technology 98(18): 3491-3498.

Mazumdar-Shaw, K. and S. Suryanarayan. 2003. Commercialization of a Novel Fermentation Concept. Biotechnology in India II: 29-42.

Ooijkaas, L. P., F. J. Weber, R. M. Buitelaar, J. Tramper and A. Rinzema. 2000. Defined media and inert supports: their potential as solid-state fermentation production systems. Trends in Biotechnology 18(8): 356-360.

Oriol, E., B. Schettino, G. Viniegra-Gonzales and M. Raimbault. 1988. Solid-State Culture of *Aspergillus niger* on Support. Journal of Fermentation Technology 66(1): 57-62.

Palma, M., A. Pinto, A. Gombert, K. Seitz, S. Kivatinitz, L. Castilho*, et al.* 2000. Lipase production by *Penicillium restrictum* using solid waste of industrial babassu oil production as substrate. Applied Biochemistry and Biotechnology 84-86(1): 1137-1145.

Pandey, A. 1992. Recent process developments in solid-state fermentation. Process Biochemistry 27(2): 109-117.

Panthong, A., D. Kanjanapothi, T. Taesotikul and W. C. Taylor. 1991. Ethnobotanical review of medicinal plants from thai traditional books, part II: Plants with antidiarrheal, laxative and carminative properties. Journal of Ethnopharmacology 31(2): 121-156.

Panthong, A., D. Kanjanapothi and W. C. Taylor. 1986. Ethnobotanical review of medicinal plants from thai traditional books, Part I: Plants with anti-inflammatory, anti-asthmatic and antihypertensive properties. Journal of Ethnopharmacology 18(3): 213-228.

Paterson, R. R. M. and P. D. Bridge. 1994. Biochemical techniques for filamentous fungi. Egham, Surrey, UK, International Mycological Institute.

Pérez-Guerra, N., A. Torrado-Agrasar, C. López-Macias and L. Pastrana. 2003. Main characteristics and applications of solid substrate fermentation. Journal of Environmental Agricultural and Food Chemistry 2(3): 343-350.

Petrini, O. 1991. Fungal endophytes of tree. In: Andrews, J.W., Hirano, S.S. Microbial Ecology of leaves. New york, Spinger-Verlag.

Pfefferle, W., B. Möckel, B. Bathe and A. Marx 2003. Biotechnological Manufacture of Lysine. Advances in Biochemical Engineering/Biotechnology 79: 59-112.

Pietra, F. 1997. Secondary metabolites from marine microorganisms: bacteria, protozoa, algae and fungi. Achievements and prospects. Natural Product Report 14(5): 453-464.

Pinkerton, F. and G. Strobel. 1976. Serinol as an activator of toxin production in attenuated cultures of *Helminthosporium sacchari*. Proceedings of the National Academy of Sciences of the United States of America 73(11): 4007-4011.

Prachya, S., S. Wiyakrutta, N. Sriubolmas, N. Ngamrojanavanich, C. Mahidol, S. Ruchirawat*, et al.* 2007. Cytotoxic mycoepoxydiene derivatives from an endophytic fungus *Phomopsis* sp. isolated from *Hydnocarpus anthelminthicus*. Planta Medica 73(13): 1418-1420.

Prpich, G. P. and A. J. Daugulis. 2005. Enhanced biodegradation of phenol by a microbial consortium in a solid–liquid two phase partitioning bioreactor. Biodegradation 16(4): 329-339.

Prpich, G. P. and A. J. Daugulis. 2007. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnology and Bioengineering 98(5): 1008-1016.

Robinson, T., D. Singh and P. Nigam. 2001. Solid-state fermentation: a promising microbial technology for secondary metabolite production. Applied Microbiology and Biotechnology 55(3): 284-289.

Rohm and Haas Singapore Pte. Ltd. 2002. Amberlite XAD-7, Singapore Technical Centre: Product data sheet.

Rombach, M. 1989. Production of *Beauveria bassiana* [*Deuteromycotina*. *Hyphomycetes*] sympoduloconidia in submerged culture. BioControl 34(1): 45-52.

Saikkonen, K., P. Wali, M. Helander and S. H. Faeth. 2004. Evolution of endophyte-plant symbioses. Trends in Plant Science 9(6): 275-280.

Sekar, C., V. W. Rajasekar and K. Balaraman. 1997. Production of Cyclosporin A by solid state fermentation. Bioprocess and Biosystems Engineering 17(4): 257-259.

Sengupta, S. and J. M. Modak. 2001. Optimization of fed-batch bioreactor for immobilized enzyme processes. Chemical Engineering Science 56(11): 3315-3325.

Shahverdi, A. R., F. Abdolpour, H. R. Monsef-Esfahani and H. Farsam. 2007. A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. Journal of Chromatography B 850(1-2): 528-530.

Shojaosadati, S. A. and V. Babaeipour. 2002. Citric acid production from apple pomace in multi-layer packed bed solid-state bioreactor. Process Biochemistry 37: 909-914.

Silva, R. G., A. J. Cruz, C. O. Hokka, R. L. Giordano and R. C. Giordano. 2001. A hybrid neural network algorithm for on-line state inference that accounts for differences in inoculum of *Cephalosporium acremonium* in fed-batch fermentors. Applied Biochemistry and Biotechnology 91-93: 341-352.

Sommart, U., V. Rukachaisirikul, Y. Sukpondma, S. Phongpaichit, N. Towatana, P. Graidist*, et al.* 2009. A cyclohexenone derivative from Diaporthaceous fungus PSU-H2. Archives of Pharmacal Research 32(9): 1227-1231.

Strobel, G. and B. Daisy. 2003. Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews 67(4): 491-502.

Strobel, G., B. Daisy, U. Castillo and J. Harper. 2004. Natural products from endophytic microorganisms. Journal of Natural Products 67(2): 257-268.

Sun, S., Y. Li, Q. Guo, C. Shi, J. Yu and L. Ma. 2008. In vitro interactions between tacrolimus and azoles against *Candida albicans* determined by different methods. Antimicrobial Agents and Chemotherapy 52(2): 409-417.

Takao, K., G. Watanabe, H. Yasui and K. Tadano. 2002. Total synthesis of (+/-)-mycoepoxydiene, a novel fungal metabolite having an oxygen-bridged cyclooctadiene skeleton. Organic Letters 4(17): 2941-2943.

Takao, K., H. Yasui, S. Yamamoto, D. Sasaki, S. Kawasaki, G. Watanabe*, et al.* 2004. Asymmetric total syntheses of (+)-mycoepoxydiene and related natural product (-)-1893A: application of one-pot ring-opening/cross/ring-closing metathesis to construct their 9-oxabicyclo[4.2.1]nona-2,4-diene skeleton. The Journal of Organic Chemistry 69(25): 8789-8795.

Tan, R. X. and W. X. Zou. 2001. Endophytes: a rich source of functional metabolites. Natural Product Reports 18(4): 448-459.

United States Pharmacopeia 32–National Formulary 27 (USP–NF) 2009. General chapter 81 : Antibiotics-Microbial assay. Rockville, The United States Pharmacopeial Convention Inc.

Wei, P.-l., Z.-n. Xu and P.-l. Cen. 2007. Lovastatin production by *Aspergillus terreus* in solid-state fermentation. Journal of Zhejiang University - Science A 8(9): 1521-1526.

Wiyakrutta, S., N. Sriubolmas, W. Panphut, N. Thongon, K. Danwisetkanjana, N. Ruangrungsi*, et al.* 2004. Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. World Journal of Microbiology and Biotechnology 20(3): 265-272.

Yu, X., S. G. Hallett, J. Sheppard and A. K. Watson. 1997. Application of the Plackett-Burman experimental design to evaluate nutritional requirements for the production of *Colletotrichum coccodes* spores. Applied Microbiology and Biotechnology 47(3): 301-305.

Zhang, H. W., Y. C. Song and R. X. Tan. 2006. Biology and chemistry of endophytes. Natural Product Reports 23(5): 753-771.

APPENDICES

APPENDIX A

Media

1. Malt Czapek broth (MCzB)

$NaNO_3$	2.0	g
KCl	0.5	g
$MgSO_4.7H_2O$	0.5	g
$FeSO_4.7H_2O$	0.01	g
K_2HPO_4	1.0	g
Sucrose	30	g
Malt extract	40	g
$ZnSO_4.7H_2O$	0.01	g
$CuSO_4.7H_2O$	0.005	g
Distilled water up to	1	L

2. Czapek Yeast autolysate broth (CzYB)

$NaNO_3$	2.0	g
KCl	0.5	g
$MgSO_4.7H_2O$	0.5	g
$FeSO_4.7H_2O$	0.01	g
K_2HPO_4	1.0	g
Sucrose	30	g
Yeast extract	5.0	g
$ZnSO_4.7H_2O$	0.01	g
$CuSO_4.7H_2O$	0.005	g
Distilled water up to	1	L

3. **Modified M1D medium (MM1D) (Pinkerton and Strobel, 1976)**

$Ca(NO_3)_2.4H_2O$	0.28	g
KNO_3	0.08	g
KCl	0.065	g
$MgSO_4.7H_2O$	0.74	g
$NaH_2PO_4.2H_2O$	1.1	mg
$FeCl_3$	1.2	mg
$MnSO_4$	5.1	mg
$ZnSO_4.7H_2O$	2.5	mg
H_3BO_3	0.136	mg
KI	0.747	mg
Sucrose	30	g
Ammonium Tartrate	5	g
Yeast Extract	0.5	g
Peptone from soymeal	1.0	g
Distilled water up to	1	L

pH = 5.5 with 1 N HCl

APPENDIX B

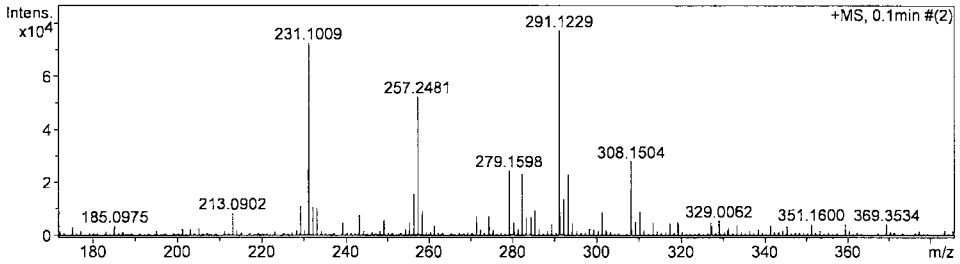
Mass Spectrum List Report

Analysis Info

Analysis Name	TOFCRI005673.d	Acquisition Date	6/10/2009 12:06:46 PM
Method	apci_pos_low-nitirat1.m	Operator	Administrator
Sample Name	Surasak-cpd1-apcipos-M	Instrument	micrOTOF 74

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	56 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	409 V
Scan Begin	80 m/z	Hexapole RF	55.0 V	Set Pulsar Push	409 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	23.3 V	Set Flight Tube	9000 V
				Set Detector TOF	2120 V



#	m/z	Res.	S/N	I	FWHM
1	102.1260	5636	2049.2	32955	0.0181
2	149.0231	6777	1392.4	38158	0.0220
3	165.0879	6969	191.3	6177	0.0237
4	167.0351	7051	289.0	9500	0.0237
5	213.0902	7475	164.3	8415	0.0285
6	229.2158	7952	187.1	10855	0.0288
7	231.1009	8170	1230.9	72318	0.0283
8	232.1049	8062	178.5	10575	0.0288
9	233.1159	8039	166.0	9906	0.0290
10	243.2305	8160	120.5	7745	0.0298
11	256.2630	8241	217.5	15536	0.0311
12	257.2481	8359	726.7	52262	0.0308
13	258.2510	8906	124.9	9066	0.0290
14	271.2617	8703	88.0	7028	0.0312
15	274.2742	8930	88.0	7180	0.0307
16	279.1598	8927	290.8	24455	0.0313
17	282.2794	9002	268.9	23084	0.0314
18	283.2721	6480	75.8	6564	0.0437
19	284.2918	8265	76.3	6649	0.0344
20	285.2806	8286	106.8	9357	0.0344
21	291.1229	8873	850.4	77123	0.0328
22	292.1273	9154	148.9	13605	0.0319
23	293.1383	9036	248.6	22838	0.0324
24	301.2166	9555	89.8	8667	0.0315
25	308.1504	9509	281.4	27849	0.0324
26	310.1652	9552	89.0	8821	0.0325
27	391.2841	9794	7743.3	624290	0.0400
28	392.2873	10204	1663.3	132683	0.0384
29	393.2909	10315	232.3	18354	0.0381
30	481.4000	10735	294.3	12383	0.0448

Figure B1 Mass spectrometry chromatogram of mycoepoxydiene

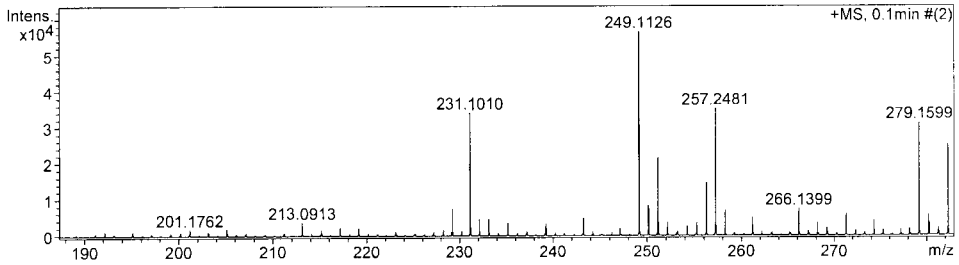
Mass Spectrum List Report

Analysis Info

Analysis Name	TOFCRI005674.d	Acquisition Date	6/10/2009 12:13:18 PM
Method	apci_pos_low-nitirat1.m	Operator	Administrator
Sample Name	Surasak-cpd2-apcipos-M	Instrument	micrOTOF 74

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	56 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	409 V
Scan Begin	80 m/z	Hexapole RF	55.0 V	Set Pulsar Push	409 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	23.3 V	Set Flight Tube	9000 V
				Set Detector TOF	2120 V



#	m/z	Res.	S/N	I	FWHM
1	102.1260	5520	1907.6	30679	0.0185
2	113.1317	6213	500.9	8265	0.0182
3	149.0226	6838	1958.2	51766	0.0218
4	149.0917	6460	238.9	6323	0.0231
5	167.0344	7203	389.3	12238	0.0232
6	229.2164	7829	118.3	7526	0.0293
7	231.1010	8235	526.6	33988	0.0281
8	249.1126	8429	788.1	56506	0.0296
9	250.1170	8429	115.3	8300	0.0297
10	251.1291	8155	298.9	21561	0.0308
11	256.2633	8444	200.8	14688	0.0303
12	257.2481	8063	480.1	35186	0.0319
13	258.2513	8832	95.1	7004	0.0292
14	266.1399	8753	96.9	7287	0.0304
15	279.1599	8921	401.3	31122	0.0313
16	282.2791	8729	320.1	25023	0.0323
17	283.2746	6852	92.8	7287	0.0413
18	285.2785	8116	98.8	7797	0.0352
19	301.2171	8992	97.5	7997	0.0335
20	307.1195	9464	74.1	6181	0.0325
21	313.2751	9372	80.5	6897	0.0334
22	359.2831	8353	63.4	6486	0.0430
23	391.2845	9258	9588.9	876229	0.0423
24	392.2880	10119	2158.5	195747	0.0388
25	393.2911	10077	275.2	24793	0.0390
26	403.1726	10395	615.4	51128	0.0388
27	404.1775	10271	131.3	10844	0.0394
28	419.3162	10103	90.3	6507	0.0415
29	481.3998	10787	1429.4	84953	0.0446
30	482.4035	10606	396.9	23605	0.0455

Figure B2 Mass spectrometry chromatogram of deacetylmycoepoxydiene

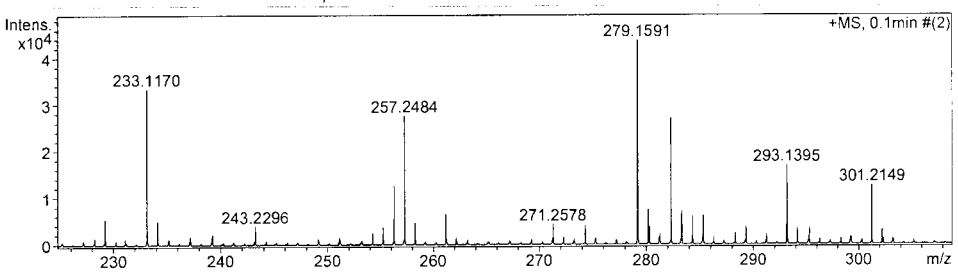
Mass Spectrum List Report

Analysis Info

Analysis Name	TOFCRI005675.d	Acquisition Date	6/10/2009 12:18:03 PM
Method	apci_pos_low-nitirat1.m	Operator	Administrator
Sample Name	Surasak-cpd3-apcipos-M	Instrument	micrOTOF 74

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	56 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	409 V
Scan Begin	80 m/z	Hexapole RF	55.0 V	Set Pulsar Push	409 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	23.3 V	Set Flight Tube	9000 V
				Set Detector TOF	2120 V



#	m/z	Res.	S/N	I	FWHM
1	102.1309	5535	3508.0	69337	0.0185
2	113.1365	6199	606.6	12259	0.0183
3	149.0259	6753	2446.9	75094	0.0221
4	150.0289	6822	205.0	6354	0.0220
5	167.0357	7202	473.6	17032	0.0232
6	233.1170	8174	641.4	33421	0.0285
7	256.2625	8308	196.1	12513	0.0308
8	257.2484	8142	428.9	27614	0.0316
9	261.1499	8118	96.3	6455	0.0322
10	279.1591	8835	557.1	43731	0.0316
11	280.1621	8589	93.7	7433	0.0326
12	282.2778	8804	336.3	27081	0.0321
13	283.2741	6921	87.6	7129	0.0409
14	284.2906	8283	72.4	5937	0.0343
15	285.2777	7687	73.6	6083	0.0371
16	293.1395	8673	192.2	16831	0.0338
17	301.2149	8966	135.3	12559	0.0336
18	313.2723	8869	73.0	7407	0.0353
19	319.2257	9423	70.6	7478	0.0339
20	327.2548	8873	65.1	7285	0.0369
21	333.2417	9118	59.1	6874	0.0365
22	341.2664	8747	59.1	7235	0.0390
23	345.2706	6403	71.5	8956	0.0539
24	359.2794	9562	79.4	10753	0.0376
25	391.2817	9502	11784.0	1363602	0.0412
26	392.2851	9788	2497.3	285634	0.0401
27	393.2887	10073	326.2	36909	0.0390
28	419.3139	10225	124.3	9724	0.0410
29	481.3981	10481	673.3	36023	0.0459
30	482.4020	10639	214.2	11470	0.0453

Figure B3 Mass spectrometry chromatogram of 2, 3-dihydromycoepoxydiene

BIOGRAPHY

Miss. Narukjaporn Thammajaruk was born on August 20, 1985 in Songkhla province, Thailand. She graduated with a Bachelor of Science in Pharmacy (Second Class Honor) from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand in 2008. She has been studying for a Master of Science in Pharmacy Program in Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand since 2008. During studying in Master's Degree, she was supported by Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, Graduate School, Chulalongkorn University.