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ORIGINAL PAPER

# Optimization of culture conditions for mycoepoxydiene production by *Phomopsis* sp. Hant25

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**Abstract** Culture media and fermentation conditions for cultivation of an endophytic fungus *Phomopsis* sp. Hant25 were investigated in order to improve the yield of mycoepoxydiene, a novel fungal metabolite having potent cytotoxic activity against many cancer cell lines. Mycoepoxydiene accumulated in the culture broth during the stationary phase of fungal growth. Modified M1D medium was superior to malt Czapek, and Czapek yeast autolysate broths in supporting mycoepoxydiene production. Pellet growth was the morphological form that favored biosynthesis of mycoepoxydiene. This could be achieved by incubating the culture statically for 6 days before shaking at 120 rpm. Incorporation of a cellulose paper disc into the culture flask promoted fungal growth at the liquid surface, which accelerated mycoepoxydiene production and maximized the final yield to a level of 354 mg $l^{-1}$, though fungal attachment to the solid support was not required. Since the peak concentration of mycoepoxydiene in the culture broth was followed by a steeply declining phase, the harvest time had to be precisely determined for maximum product yield. Understanding the factor(s) involved in rapid degradation of mycoepoxydiene could lead to improved final yields.

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## Introduction

Mycoepoxydiene (Fig. 1) was first isolated in 1999 from solid-state fermentation culture of a sterile dematiaceous fungus isolated from twig litter collected in a Brazilian forest and designated OS-F66617 [2]. Because the compound contained a rare oxygen-bridged cyclooctadiene and a functional $\delta$-lactone, it was a novel fungal secondary metabolite, but there was no report of its biological activity at that time. Later in 2003, a culture-broth extract of an unidentified endophytic fungus (strain 1893) from a mangrove tree (*Kandelia candel*) from the South China Sea coast was found to be cytotoxic for human non-small cell lung cancer-derived (NCI-4460) and human hepatoma (BEL-7402) cell lines. It was also highly lethal to the lipidopteran bollworm pest *Heliothis armigera* (Hübner) and to parasitic copepods *Sinergasilus* spp. [3]. Purification of the extract revealed two new oxygen-bridged cyclooctadiene-containing lactones similar to mycoepoxydiene and probably rearranged metabolites of it, together with three other compounds. Since it was the crude extract that was tested, it was not certain which of these compounds exerted the biological activities. Large-scale (170 l) cultivation of the 1893 fungus in an industrial fermenter was reported to yield 50 mg of mycoepoxydiene after solvent extraction and chromatographic purification [4]. However, the chemical structure of the compound referred to as mycoepoxydiene in that report (compound **2**) was not exactly mycoepoxydiene.

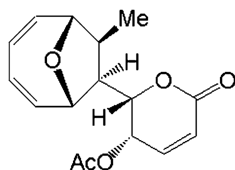


**Fig. 1** Chemical structure of mycoepoxydiene

Mycoepoxydiene was re-isolated from a marine lignicolous fungus *Diaporthe* sp. and its biological activity in pure form was demonstrated for the first time as cytotoxic against the KB (human mouth epidermal carcinoma) cell line with an $IC_{50}$ of less than 6.25 μg $ml^{-1}$ [6].

Because of their unique structural features, rarity in nature, and biological activities, routes for total chemical synthesis of mycoepoxydiene and related natural products were developed. The process leading to (±)-mycoepoxydiene consisted of 24 steps with a 1.6% overall yield [12]. In subsequent development, asymmetric total synthesis of the natural (+)-mycoepoxydiene and unnatural (−)-mycoepoxydiene were accomplished [13]. The two compounds displayed similar in vitro cytotoxicity toward K562 (human chronic myelogenous leukemia) and HepG2 (human hepatocellular carcinoma) cell lines but no detectable inhibitory activities against Gram-positive and Gram-negative bacteria.

Recently, our group has isolated an endophytic fungus identified as *Phomopsis* sp. Hant25 from a Thai medicinal plant, *Hydnocarpus anthelminthicus*. Mycoepoxydiene together with two derivatives, deacetylmycoepoxydiene and 2,3-dihydromycoepoxydiene could be purified from fermentation broth of the Hant25 fungus [9]. Mycoepoxydiene and the deacetyl derivative exhibited potent cytotoxic activities toward HepG2 and KB cells and a wider variety of cancer cell lines, including those from human lung carcinoma (A549), human hepatocellular carcinoma (HCC-S102), human cholangiocarcinoma (HuCCA-1), cervical adenocarcinoma (HeLa), human breast cancer (MDA-MB231), human mammary adenocarcinoma (T47D), human promyelocytic leukemia (HL-60) and murine leukemia (P388). The fact that the dihydro derivative was inactive indicated that the $\alpha,\beta$-unsaturated lactone moiety in mycoepoxydiene played a crucial role in its biological activity. The cytotoxicity profile of mycoepoxydiene against the cell lines test panel was distinct from that of etoposide, a standard anticancer drug. It was thus of interest to investigate in more detail the mechanism of mycoepoxydiene cytotoxicity toward cancer cells and the structure–activity relationship. To support such investigations, a good supply of mycoepoxydiene is needed.

Previously reported fungal fermentation which utilized submerged-culture produced low amount of mycoepoxydiene. Shake-flask culture of *Diaporthe* sp. [6] and industrial-scale fermenter culture of strain 1893 fungus [4] yielded mycoepoxydiene at only 0.8 and 0.3 mg $l^{-1}$, respectively. With *Phomopsis* sp. Hant25, no mycoepoxydiene was detectable in malt Czapek broth shake-flask culture but 26 mg $l^{-1}$ was obtained from static culture [9] where the fungus grew at the medium surface. We hypothesized that surface culture enhances mycoepoxydiene production in the Hant25 fungus. In the present study, further improvement of mycoepoxydiene titer in the Hant25 culture was attempted. But instead of employing the straight shake or static culture method as commonly performed, the static/shake fermentation [1] was adopted and optimized to maintain fungal surface growth while mixing the medium sufficiently during incubation. This together with culture medium selection and solid support addition highly improved the *Phomopsis* sp. Hant25 production of mycoepoxydiene.

## Materials and methods

### Microorganism and inoculum preparation

*Phomopsis* sp. Hant25 was obtained from our previous study [9]. Inoculum was prepared by growing Hant25 on potato dextrose agar (PDA) plates at 25°C for 7 days. Agar plugs cut from the outer zone of the colony using a sterile No. 4 cork borer were use as inocula.

### Culture media

Media were prepared by dissolving the ingredients in distilled de-ionized water and autoclaving at 121°C for 15 min. Czapek yeast autolysate broth (CzYB) contained (per liter) 30 g sucrose, 2 g $NaNO_3$, 1 g $K_2HPO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4{\cdot}7H_2O$, 5 g yeast extract, 0.01 g $ZnSO_4{\cdot}7H_2O$ and 0.005 g $CuSO_4{\cdot}7H_2O$. Malt Czapek broth (MCzB) contained (per liter) 30 g sucrose, 2 g $NaNO_3$, 1 g $K_2HPO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4{\cdot}7H_2O$, 40 g malt extract, 0.01 g $ZnSO_4{\cdot}7H_2O$ and 0.005 g $CuSO_4{\cdot}7H_2O$. Modified M1D (MM1D) medium contained (per liter) 0.28 g $Ca(NO_3)_2{\cdot}4H_2O$, 0.08 g $KNO_3$, 0.065 g KCl, 0.74 g $MgSO_4{\cdot}7H_2O$, 1.1 mg $NaH_2PO_4{\cdot}2H_2O$, 30 g sucrose, 5 g ammonium tartrate, 1.2 mg $FeCl_3$, 5.1 mg $MnSO_4$, 2.5 mg $ZnSO_4{\cdot}7H_2O$, 0.136 mg $H_3BO_3$, 0.747 mg KI, 0.5 g yeast extract and 1 g peptone from soymeal. The pH was adjusted to 5.5 with 0.1 M HCl [8].

### Fermentation and sampling

A 50-ml volume of culture broth was distributed in 250-ml Erlenmeyer flasks (Pyrex® 4980), closed with cotton wool plugs, covered with aluminum foil and sterilized by

autoclaving. In the experiments with solid supports, the following materials were used: spunbonded high-density polyethylene (SHDPE) sheets with perforations (Tyvek® type 16, DuPont), absorbent cotton gauze (Medigauz®, Thailand) or cellulose paper (Whatman chromatography paper, No. 1 Chr). The sheets were cut into 7-cm diameter circular discs, cleaned by autoclaving in water and dried at 60°C. A disc made of one layer, or four layers in the case of absorbent cotton gauze, was placed in each culture flask and autoclaved together with the medium.

Throughout this study, fermentation was started by inoculating the culture flask with 2 agar plugs containing mycelia punched from agar plates as described above. Incubation was at 25°C under static conditions, with continuous shaking at 120 rpm or with a combination of stasis followed by shaking at 120 rpm. At pre-determined times, the culture flasks were swirled gently to ensure homogeneous distribution of the contents in the fermentation broth and, aseptically, 1-ml samples of the broth were taken for HPLC analysis (see below). All the experiments were conducted in triplicates.

### Analytical methods

Mycoepoxydiene yields in fungal fermentation broth were analyzed by high performance liquid chromatography (HPLC) using an Agilent LC 1100 HPLC system with the following conditions.

Column: Hewlett-Packard ODS Hypersil, 5 μm, 125 × 4 mm with LiChrosphere 100 RP-18, 5 mm, 4 × 4 mm guard column
Column temperature: Ambient (~25°C)
Mobile phase: 0.1% AcOH in $H_2O$: acetonitrile (75:25)
Flow rate: 1.5 ml $min^{-1}$
Injection volume: 20 μl
Detection: UV-photodiode array detector monitored at 260 nm
Standard compound: Purified mycoepoxydiene obtained in the previous study [9] kindly provided by Dr Prasat Kittakoop.

Mycoepoxydiene was eluted at ca. 9.8 min and the run time was 12 min for each sample. The concentration of mycoepoxydiene in the culture broth was calculated by comparing peak area of the sample with the standard calibration curve constructed from peak areas of pure mycoepoxydiene solutions.

### Fungal biomass determination

Fungal mycelium was collected from cultures by vacuum filtration with a Buchner funnel using pre-weighed filter paper discs (Whatman No. 1). It was washed 3 times with 30 ml of distilled water and dried to constant weight at 80°C.

## Results and discussion

### Effect of culture media on mycoepoxydiene production in static cultures

In our previous report, 26 mg of mycoepoxydiene could be obtained from *Phomopsis* sp. Hant25 culture in 1 l of MCzB fermentation broth [9]. In the present study, two additional culture media (CzYB and MM1D) were tested. CzYB was similar to MCzB except that 5 g of yeast extract in the former was replaced by 40 g of malt extract in the latter. This was to test whether or not fungal components in the yeast extract would promote or induce mycoepoxydiene, since mycoepoxydiene has been shown in our lab to have a mild inhibitory effect against *Candida albicans*. MM1D medium was included because it was known to enhance secondary metabolites production in fungi [5, 11].

HPLC analysis of fermented broth revealed that *Phomopsis* sp. Hant25 culture in MCzB produced mycoepoxydiene at a concentration of 25 mg $l^{-1}$ at day 12, but that this declined to an undetectable level by day 15 and remained so until the end of incubation at day 21 (graph not shown). Similarly, in MM1D medium, mycoepoxydiene was detected at 28 mg $l^{-1}$ at day 15 but decreased to 22 and 17 mg $l^{-1}$ at days 18 and 21, respectively. A representative HPLC chromatogram from the analysis of mycoepoxydiene in fungal culture broth is shown in Fig. 2. A plot of mycoepoxydiene concentration versus fungal biomass showed that it was produced during the stationary growth phase (Fig. 3). Mycoepoxydiene was found in the culture broth but could not be detected in mycelium extracts. Investigations with CzYB were discontinued since it did not yield measurable amounts of mycoepoxydiene.

### Effect of static/shaking periods on mycoepoxydiene production

*Phomopsis* sp. Hant25 was cultured in MCzB and MM1D medium under different static/shaking programs. In programs I, II, and III the cultures were started with incubation under static conditions for 3, 6, and 9 days, respectively, followed by shaking at 120 rpm for 18, 15, and 12 days, respectively. The resulting mycoepoxydiene concentration profiles in MCzB (Fig. 4a1) revealed no detectable amounts of mycoepoxydiene under any of the static/shaking programs. However, in MM1D medium, relatively large amounts of mycoepoxydiene were produced in cultures under all three static/shaking programs (Fig. 4a2). During the static incubation phase, the fungus grew at the surface

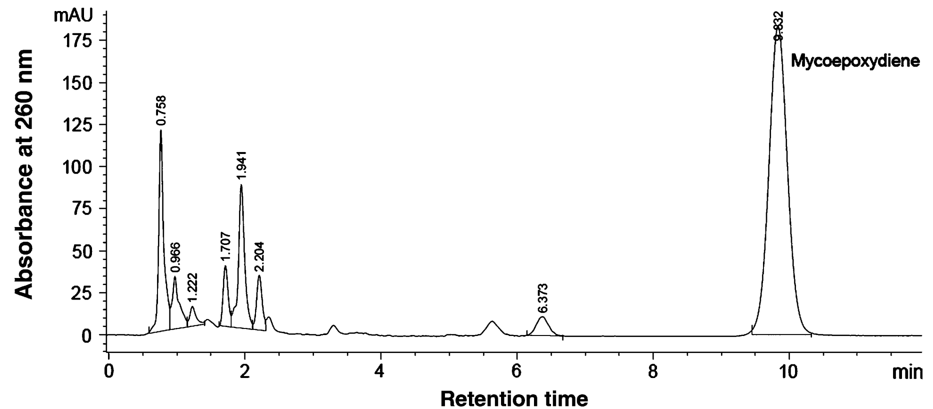


**Fig. 2** Representative HPLC chromatogram of MM1D fermentation broth of *Phomopsis* sp. Hant25 showing the peak corresponding to mycoepoxydiene

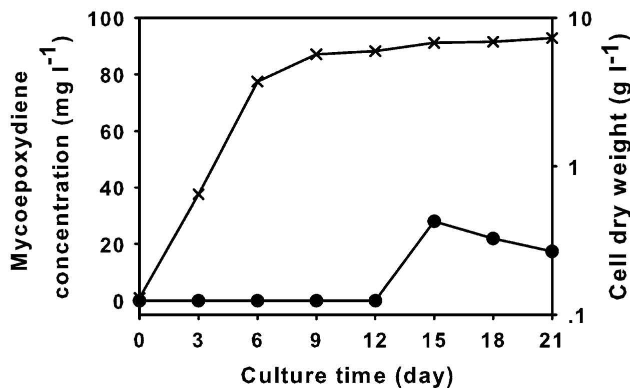


**Fig. 3** Fungal biomass (*cross*) and mycoepoxydiene concentration (*filled circle*) profiles of *Phomopsis* sp. Hant25 static culture in MM1D medium

of the liquid medium forming floating colonies with the top part exposed to the air. In subsequent periods of shaking at 120 rpm, the fungus maintained growth as discrete floating colonies up to 3–4 cm in diameter and well separated from the clear culture broth (Fig. 5a). Cultures having this morphological appearance produced large amounts of mycoepoxydiene. The highest mycoepoxydiene concentration of 288.9 mg $l^{-1}$ was obtained at day 21 with culture program II (6-days of static incubation followed by 15-days of shaking). By contrast, continuous shaking of the culture at 120 rpm from day 1 to 21 resulted in growth of dispersed mycelium that occupied the entire volume of the culture broth (Fig. 5b) and yielded no mycoepoxydiene. Dispersal was probably due to mycelial fragmentation caused by agitation shear force.

## Effect of solid supports on mycoepoxydiene production

Inert polymeric solid supports added to liquid medium for cellular attachment are known to enhance secondary metabolite production in fungi [1]. Effects of three different polymeric solid supports on mycoepoxydiene production by *Phomopsis* sp. Hant25 were investigated in this study.

*Spunbonded high-density polyethylene (SHDPE) sheet with perforations*: Culture flasks with perforated SHDPE sheets were inoculated by placing 2 agar plugs from the inoculum plate onto the SHDPE disc that floated on the surface of the culture broth and the 3 static/shake fermentation programs were applied. The fungus grew lightly on the upper surface of the SHDPE disc but penetrated through the pores of the disc forming a mycelial mat underneath. The fungal mycelium was linked firmly to the disc and remained so when shaken, so the culture broth remained clear. SHDPE discs with MCzB produced very little mycoepoxydiene (Fig. 4b1) but with MM1D there was some mycoepoxydiene accumulation (Fig. 4b2), although lower than that produced in MM1D without any solid support (Fig. 4a2). It is possible that the SHDPE disc limited aeration of the medium to the level that retarded growth and secondary metabolite production because it floated on the surface. Since the floating hydrophobic polymeric material had a negative effect on mycoepoxydiene production, solid supports that would be more hydrophilic (and therefore more submerged in the culture medium) were investigated.

*Cotton gauze*: Fungal hyphae penetrated the cotton gauze support and incorporated it into a large single mycelial mat. In MCzB medium, the fungus grew quickly but did not yield any detectable mycoepoxydiene (Fig. 4c1). In MM1D under program II (6-days static and 15-days shaken), mycoepoxydiene was detectable at day 12 and increased rapidly to a maximum level of 307 mg $l^{-1}$ at day 18, after which it declined (Fig. 4c2). Incubating the cultures statically for 3 or 9 days before shaking gave rise to slower rates of mycoepoxydiene accumulation and lower final yields.

*Cellulose paper*: Use of cellulose paper disc supports suspended in the medium promoted surface growth during the static incubation but the mycelium became detached from the support during the subsequent shaking period. However, the disc helped to keep the fungal pellets near the surface of the liquid and reduced the collision force between the pellets and the flask wall. The fungus grew

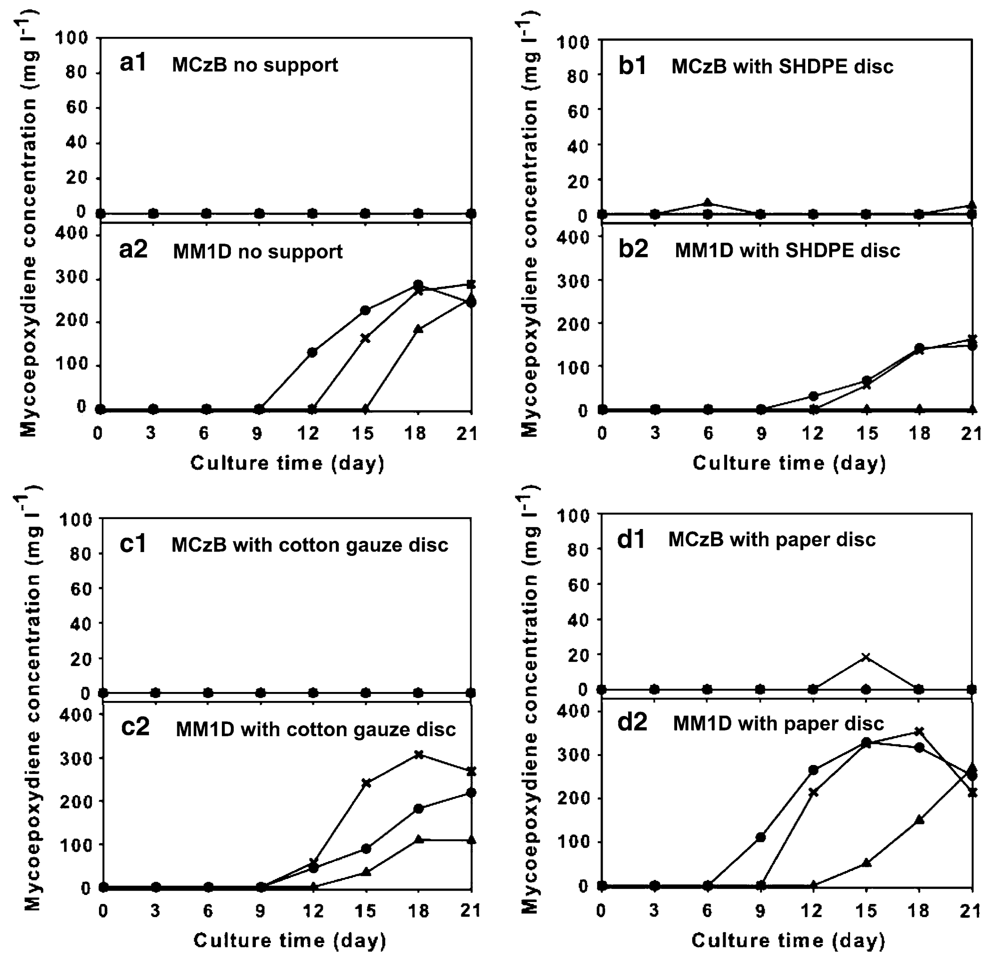


**Fig. 4** Mycoepoxydiene concentration profiles of *Phomopsis* sp. Hant25 cultures in MCzB or MM1D with and without supports as indicated. Cultures started with 3 (*filled circle*), 6 (*cross*), 9 (*filled triangle*) days of static conditions followed by shaking until day 21. For more details, please see text

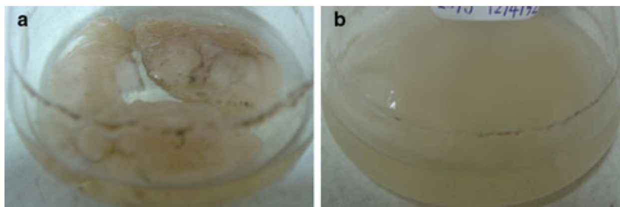


**Fig. 5** Growth morphology of *Phomopsis* sp. Hant25 in MM1D broth **a** as large floating pellets well separated from the clear culture broth and **b** as a dispersed mycelial mass occupying the entire volume of the culture broth

quickly, possibly because of good aeration. MCzB medium did not support good mycoepoxydiene production under any culture program, although a small, transient amount was produced under program II (Fig. 4d1). By contrast, as much as 100 mg $l^{-1}$ mycoepoxydiene could be detected as early as day 9 of culture in MM1D medium under program I (3-days static and 18-days shaking), reaching a maximum of more than 300 mg $l^{-1}$ at day 15, after which it declined (Fig. 4d2). With program II, the highest mycoepoxydiene yield of 354 mg $l^{-1}$ was obtained at day 18. With program III, static pre-incubation for 9 days before shaking resulted in late mycoepoxydiene production.

Secondary metabolite formation in fungi is a complex process usually linked with cellular differentiation and morphological development. Many genes and physiological mechanisms are involved that are influenced, in turn, by chemical and physical environmental factors. Despite substantial accumulation of data from studies of many secondary metabolite biosynthetic pathways, until recently [14] general rules for maximization of secondary metabolite production have not been established [7]. Thus, step-by-step optimization of production processes on a case-by-case basis is an approach that can still improve secondary metabolite production performance.

*Phomopsis* sp. Hant25 grew on laboratory mycological media as mycelia sterilia, i.e., it formed only mycelium that did not produce spores or conidia. Thus, morphological change usually linked with secondary metabolite production was not readily noticeable during the course of its cultivation. This made it difficult to judge when to harvest the product based on visual assessment. However, the time-course study showed that mycoepoxydiene, like many other fungal secondary metabolites, was released into the culture broth in the stationary phase of growth (Fig. 3). It is known that maximum production of secondary metabolites

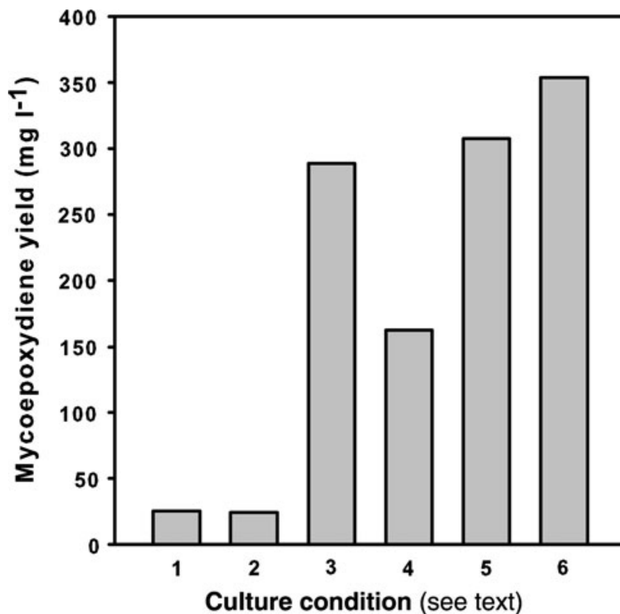


**Fig. 6** Summary of mycoepoxydiene yields obtained during optimization of *Phomopsis* sp. Hant25 culture conditions. (*1–2*) Static culture in MCzB, *1* previous report [9], *2* this report; *3–6* culture in MM1D medium with 6-day static/15-day shaking periods, *3* without solid support, *4* with SHDPE disc, *5* with cotton gauze disc, *6* with cellulose paper disc

depends on the appropriate physiological stage of the organism in an appropriate chemical and physical environment (e.g., culture medium composition, dissolved gases, pH, temperature, agitation, etc.). Factors that promoted mycoepoxydiene production by *Phomopsis* sp. Hant25 were medium composition (MM1D), pellet growth, a combination of static and agitated cultivation and the presence of a solid support. Agitation force during the shaking period was also crucial, and shaking at 120 rpm with the type of flask and volume of the culture medium used in this study was optimal for maintaining pellet growth while giving sufficient mixing. With respect to solid supports, the fact that cellulose paper gave the best mycoepoxydiene production without fungal attachment indicated that adherence to a solid support was not essential for the mycoepoxydiene synthesis but that fungal growth near the liquid surface was more important. It is not known why surface cultivation could significantly enhance mycoepoxydiene productivity in this fungus, since mycoepoxydiene represents a new class of fungal metabolites [2] and its biosynthetic pathway is presently totally uncharacterized. But if a hypothesis is to be made to explain this phenomenon, we would speculate that the process of mycoepoxydiene biosynthesis, particularly the step(s) involving the formation of the 'epoxy' moiety of the molecule, might require the presence of high oxygen concentration, the condition that exists at the liquid/air interface zone.

The decline in mycoepoxydiene concentration in the culture medium after it reached a maximum indicated that precise timing of harvest is essential to obtain the highest product yields. The reason for the decline is currently unknown, but may be the result of such things as self-toxic metablolites [10] or degradative enzyme(s) released from living or lyzed cells. If it could be understood by further investigations, it is possible that appropriate culture or strain manipulation could lead to even higher production yields. Despite these limitations, we have succeeded in increasing the yield of mycoepoxydiene to 354 mg per liter of culture medium, which is 14 times higher than our previous report [9] (Fig. 6).

## Conclusion

We have optimized the culture medium and fermentation condition for mycoepoxydiene production by the endophytic fungus *Phomopsis* sp. Hant25. Among mycological media tested, MM1D medium [8] was the best in supporting mycoepoxydiene production. Unlike other studies reported previously which utilized submerged-cultivation of the producing fungi, we employed liquid surface culture for the *Phomopsis* sp. Hant25 by optimizing the static/agitation fermentation process. Added solid support helped in keeping the fungal mat near the liquid surface and minimizing the collision force against the flask wall. By these manipulations, mycoepoxydiene titer of 354 mg $l^{-1}$ was achieved which is much higher than any other reports known to date. This liquid surface culture with medium mixing can be an alternative fungal fermentation approach when both submerged and static liquid cultures yield low concentrations of target secondary metabolite.

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