การเปรียบเทียบกิจกรรมต้านเชื้อราระหว่างส่วนใสปราศจากเซลล์ที่ได้จากการเพาะเลี้ยงแบคทีเรีย *Xenorhabdus stockiae* PB09 ในระดับฟลาสก์และระดับถังหมัก

Comparison Between Antifungal Activities of *Xenorhabdus stockiae* PB09 Cell-free Supernatants Derived from Shake-Flask Cultivation and Fermentation

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บทคัดย่อ

สารเมทาโบไลท์หลายชนิดจากแบคทีเรีย *Xenorhabdus* spp. ซึ่งเป็นแบคทีเรียร่วมอาศัยในไส้เดือนฝอยศัตรูแมลง มีรายงานว่าสามารถต้านเชื้อราก่อโรคพืชได้หลากหลาย โดยเฉพาะเชื้อรา *Colletotrichum gloeosporioides* (Penz.) Sacc. ซึ่งเป็นหนึ่งในเชื้อราก่อโรคพืชที่สร้างความเสียหายมากที่สุดต่อพืชผลไม้เมืองร้อนจำนวนมาก โดยเป็นสาเหตุของโรคแอนแทรคโนส งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาและเปรียบเทียบกิจกรรมต้านเชื้อราระหว่างส่วนใสปราศจากเซลล์ที่ได้จากการเพาะเลี้ยงแบคทีเรีย *Xenorhabdus stockiae* PB09 ในระดับฟลาสก์และระดับถังหมักเพื่อยับยั้งการเจริญของเส้นใยเชื้อรา *C. gloeosporioides* ด้วยวิธีอาหารพิษ จากผลการทดลองแสดงให้เห็นว่าส่วนใสปราศจากเซลล์ที่ได้จากการเพาะเลี้ยงทั้งในระดับฟลาสก์และระดับถังหมักสามารถยับยั้งการเจริญของเชื้อรา *C. gloeosporioides* ได้ โดยกิจกรรมต้านเชื้อราของส่วนใสปราศจากเซลล์ที่ได้จากการเพาะเลี้ยงแบคทีเรีย *X. stockiae* PB09 ในระดับฟลาสก์และระดับถังหมักขนาด 5 ลิตร จะเพิ่มขึ้นตามระยะเวลาการเพาะเลี้ยงและมีค่าสูงสุดที่ 72 และ 48 ชั่วโมง ตามลำดับ นอกจากนี้การหมักในระดับถังหมักยังส่งผลต่อระดับของกิจกรรมการต้านเชื้อราของส่วนใสปราศจากเซลล์จากแบคทีเรีย *X. stockiae* PB09 สูงกว่าการเพาะเลี้ยงในระดับฟลาสก์ งานวิจัยนี้แสดงให้เห็นว่าส่วนใสปราศจากเซลล์ของแบคทีเรีย *X. stockiae* PB09 สามารถนำมาใช้ในการควบคุมเชื้อรา *C. gloeosporioides* ได้ และการเพาะเลี้ยงแบคทีเรียในระดับถังหมักให้ผลดีกว่าการเพาะเลี้ยงในระดับฟลาสก์โดยเพิ่มประสิทธิภาพของส่วนใสปราศจากเซลล์ในระยะเวลาที่สั้นลงถึง 24 ชั่วโมง

คำสำคัญ: *Xenorhabdus stockiae* กิจกรรมต้านเชื้อรา *Colletotrichum gloeosporioides*

Abstract

A variety of metabolites from the entomopathogenic bacterium *Xenorhabdus* spp. have been reported to have antifungal activities, especially against *Colletotrichum gloeosporioides* (Penz.) Sacc., which is one of the most damaging pathogens targeting many tropical fruit plants by causing anthracnose disease. This study aimed to evaluate and compare the antifungal activities of *Xenorhabdus stockiae* PB09 cell-free supernatants derived from cultivations in shake-flask and

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fermenter scales for controlling *C. gloeosporioides* mycelial growth by using poisoned food technique on agar media. The results showed that cell-free supernatants of both shake flask-scale and fermenter-scale productions could inhibit the growth of *C. gloeosporioides*. Antifungal activities of *X. stockiae* PB09 cell-free supernatants derived from cultivations by shake-flasks and 5L-fermenters increased over time and reached their peaks at 72 and 48 h, respectively. Fermentation also resulted in the levels of antifungal activities of *X. stockiae* PB09 cell-free supernatant being higher than that obtained by shake-flask cultivation. This study demonstrated that cell-free supernatant of *X. stockiae* PB09 could be used to control the growth of *C. gloeosporioides* and the large scale production using fermenter was superior to shake-flask cultivation by giving more effective cell-free supernatant in a shorter period of time for up to 24 h.

Keywords: *Xenorhabdus stockiae*, antifungal activity, *Colletotrichum gloeosporioides*

Introduction

Colletotrichum gloeosporioides (Penz.) Sacc. has been found to cause anthracnose disease which is very devastating to several fruit plants and responsible for serious economic losses.[1] Several chemical fungicides have been used for controlling this fungal pathogen, but they have resulted in the development of resistance and adverse effects on the farmers, consumers, environment and ecosystem.[2] Therefore, it is necessary to develop an alternative approach for effective control of anthracnose disease. The bacterium *Xenorhabdus* spp. has been reported to produce several antimicrobial compounds that are known to have suppressive effects on a variety of plant pathogens.[3,4,5]

Antimicrobial substances from *Xenorhabdus* spp. have been found to differ qualitatively depending on the strains and species of bacteria[4] and their culture conditions.[6,7] Shake flasks have been widely used to study the basic processing conditions, which allowing the experiments to be carried out with minimal costs and materials.[8,9] However, shake flasks have several limitations when comparing to fermenters because they have completely different systems of geometry, mixing and gas regimes.[10] Therefore, scaling-up from shake flasks to fermenters is used to produce large quantities of the final products.

The objective of this study was to evaluate the *in vitro* inhibitory effects of cell-free supernatants of *X. stockiae* PB09 derived from shake-flask and fermenter cultivations on *C. gloeosporioides*, a causative agent of mango anthracnose disease.

Materials and methods

Xenorhabdus stockiae PB09

Xenorhabdus stockiae PB09 was isolated from the infective juveniles (IJ) of *Steinernema siamkayai* Stock, Somsook and Reid nematode obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives Thailand by following the methods described by Kaya and Stock (1997).[11] Seed culture of *X. stockiae* PB09 was prepared by inoculating a loop of phase I colonies growing on a nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) plate into 250 mL-flask containing 100 mL nutrient broth (NB) and cultivated at 28°C on a rotary shaker at 200 rpm for 16-24 h in the dark until the optical density (600 nm) was approximately 2.0.

Cultivation by using shake-flask technique

Tryptic soy broth (TSB, g/L) (17 tryptone, 3 soytone, 2.5 glucose, 5 NaCl and 2.5 K_2HPO_4) was used for *X. stockiae* PB09 cultivation using shake-flask technique. The pH of the medium was adjusted to 7.5 by using 2.0 mol/L NaOH and 2.0 mol/L HCl. The seed culture of *X. stockiae* PB09 (10% v/v) was transferred to this medium (total volume of 100 mL each) in 250 mL-flasks and incubated in the dark at 28°C on a rotary shaker at 200 rpm for 24, 48, 72 and 96 h. A sample (5 mL) was removed every day, then centrifuged (10,000 rpm, 20 min, 4°C) and filtered using 0.22 µm-syringe filters to obtain cell-free supernatant,

which was stored at 4°C until required. The experiments were repeated in triplicates.

Fermentation by using 5L-fermenters

Batch cultivation of *X. stockiae* PB09 was carried out in 5L-fermenters (B. Braun Biotech, Germany) with working volume of 3L. The cultivation temperature was 28°C with agitation speed of 200 rpm and the aeration rate of 2.5 l/min. The seed culture of *X. stockiae* PB09 at 10% v/v was transferred to 3L sterile medium (TSB) in the 5L-fermenters. The pH profile was adjusted to a set pH with 2.0 mol/L NaOH and 2.0 mol/L HCl. The fermenters were incubated according to the cultivation conditions for 72 h. Sample (20 mL) was taken every day, then centrifuged (10,000 rpm, 20 min, 4°C), filtered using 0.22 µm-syringe filter to obtain cell-free supernatant, and stored at 4°C until required.

Assay of antifungal activity

C. gloeosporioides was isolated from the upper surface of infected mango and cultured using potato dextrose agar (PDA) medium at 25°C. Cell-free supernatants of *X. stockiae* PB09 were *in vitro* tested for their efficacy against *C. gloeosporioides* mycelia growth by using the poisoned food technique on agar media.[3,12] Carbendazim was used as positive control and caused the highest mycelia growth inhibition (100%) (data not shown).

Measurement of cell growth

The growth of bacterial cells was measured by optical density (OD) of the cultures at 600 nm with a spectrophotometer. The dry cell weight (DCW) was determined from a calibration curve as described by Wang et al. (2010).[13]

Measurement of glucose concentration

The glucose concentration was measured by the 3,5-dinitrosalicylic acid spectrometric method.[14]

Statistical analysis

The data of percentages of mycelial growth inhibition were analyzed by one-way ANOVA. Significance differences between the treatments were compared using the LSD test at $P<0.05$.

Results

Antifungal activities of *X. stockiae* PB09 grown by shake-flask technique

Figure 1 shows the dry cell weight (DCW, g/L) and antifungal activity (%) of *X. stockiae* PB09 cultivated in shake-flasks for 0, 24, 48, 72 and 96 hours. The maximum dry cell weight was found when the bacteria were cultured for 72 h. An increase of bacterial growth led to simultaneous increase of its antifungal activity, which reached its peak at 72 h. All the concentrations of *X. stockiae* cell-free supernatant from 0.25 to 1.50% v/v were found to have significant differences ($P<0.05$ as compared by LSD test) in the levels of antifungal activities against the mycelial growth of *C. gloeosporioides* and the highest activities (67.63±1.16%) were found in the 72h cell-free supernatant at the concentration of 1.50% v/v. Moreover, their activities increased continually from 24 to 72 h of cultivation, and then began to drop at 96 h.

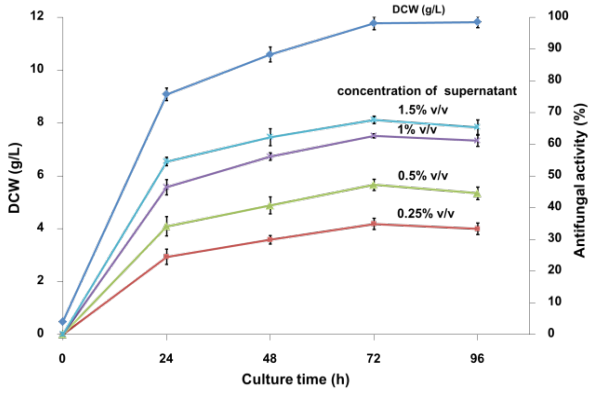

Figure 1 Time profiles of dry cell weight and antifungal activity of *X. stockiae* PB09 grown by using shake-flasks for 96 h and applied at different concentrations (0.25 to 1.50%v/v)

Antifungal activities of *X. stockiae* PB09 grown by using 5L-fermenters

Figure 2 shows the antifungal activities of *X. stockiae* PB09 cell-free supernatants grown by using 5L-fermenters for different cultivation periods. Similar to the results of shake-flask setting, all the concentrations of cell-free

supernatant (0.25 to 1.50% v/v) grown by using 5L-fermenters had significantly different levels of antifungal activities (P<0.05 as compared by LSD test) against the mycelial growth of *C. gloeosporioides*, and their levels of activities were rather higher than that grown by shake-flasks. In addition, the maximum antifungal activities (89.60±1.33%) were also found when applying with 1.50% v/v cell-free supernatant grown in the 5L-fermenter for 48 h. However, the antifungal activities began to drop at 72 h of fermentation. When comparing between shake-flask cultivation and 5L-fermentation, the antifungal activities of cell-free supernatant from shake-flask cultivation reached its peak at 72 h, while that from 5L-fermenation reached a peak at 48 h with higher antifungal activities.

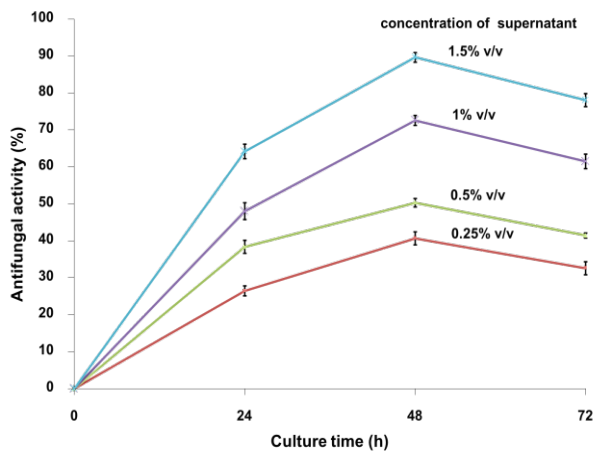

Figure 2 Antifungal activities of cell-free supernatant of *X. stockiae* PB09 grown by using 5L-fermenters for 72 h and applied at different concentrations (0.25 to 1.50% v/v)

Time profile of *X. stockiae* PB09 growth and its antifungal activity during fermentation

To investigate the antifungal activities of *X. stockiae* PB09 cell-free supernatant grown by using the 5L-fermenter, the bacterium was cultured in 5L-fermenter for 72 h during which dry cell weight (DCW, g/L), residual glucose concentration (g/L), and antifungal activity (%) were measured (Figure 3). The results showed that *X. stockiae* PB09 greatly consumed the glucose during the first 24 h which resulted in its sharply exponential growth. An increase of bacterial growth led to simultaneous increase of its antifungal activity, which reached its peak at 48 h when used at the concentration of 1.5% v/v. However, *X. stockiae* PB09 entered the stationary phase after 48 h of fermentation which was similar to the results in Figure 2.

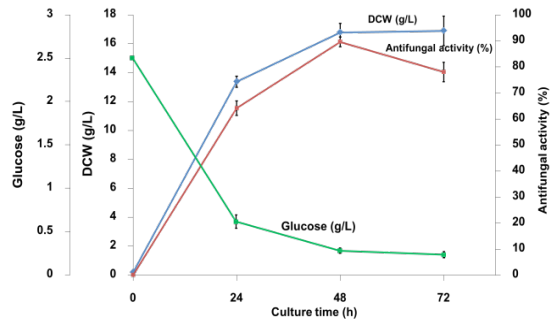

Figure 3 Time profiles of dry cell weight, glucose concentration and antifungal activity of *X. stockiae* PB09 grown by using 5L-fermenters.

Discussion

In this study, cell-free supernatants of *X. stockiae* PB09 cultivated by fermenters and shake flasks were shown to have maximum antifungal activities against *C. gloeosporioides* at 48 and 72h, respectively. The time profile of *X. stockiae* PB09 grown in the fermenter suggested that it entered the stationary phase at 48 h post inoculation. Remarkably, high levels of antifungal activity and cell growth in 5-L fermenter scale could be achieved faster than that in the shake-flask scale. This may be due to the addition of different neutralizing agents into various voluminal bioreactive systems of the fermenter. Similar results were obtained in the study of Wang and Zhang (2007)[6] and Wang et al. (2010)[13] that reported the aeration and agitation on fermenter had great influences on cell growth and the production of antibiotic by *X. nematophila* YL001. Isaacson and Webster (2002)[5] reported that the level of antimicrobial activity of *Xenorhabdus* sp. RIO followed a pattern similar to that of the growth curve to enter its stationary phase, whereby its antibacterial activity reached a maximum level at 48 h, while the maximum antifungal activity reached at 72 h. Furthermore, previous report showed that the cell-free supernatants of stationary-phase cultures of *X. szentirmaii* and *X. budapestensis* at 6.25, 25 and 50 ppm doses could

inhibit the growth of *Phytophthora nicotianae* colonies at approximately 56.2, 77.1 and 84.0%, respectively.[15]

The cell-free supernatant of *X. stockiae* PB09 culture exhibited an inhibitory effect on *C. gloeosporioides*, a causative agent of mango anthracnose disease. Previous studies have reported the variation in antimicrobial activities of different *Xenorhabdus* spp. and strains against plant pathogenic fungi and oomycetes.[3,4,5,12,15,16] For example, the cell-free supernatants (10% v/v) of *X. bovienii* YL002[12] and *X. nematophila* TB[3] grown by using 5L-fermenters for 72 h were shown to have high inhibitory effects (>90%) on mycelial growth of *Botrytis cinerea, Phytophthora capsici, Bipolaris maydis, Bipolaris sorokiniana, Dothiorella gregaria* and *Sclerotinia sclerotiorum*, but exhibited low inhibitory effect (<15%) on *Colletotrichum lagenrium*.

Although the mode of action of *X. stockiae* PB09 on fungi is unknown, in a previous study, Isaacson and Webster (2002)[5] found that the antimicrobial activity from *Xenorhabdus* sp. RIO was due to its exo- and endo-chitinases as well as other proteinaceous and some small molecule compounds. Furthermore, *X. nematophila* var. *pekingensis* has been known to produce Xenocoumacin 1 which was highly active against *P. infestans, P. boehmeriae, P. melonis, P. capsici, B. cinerea* and *Alternaria alternata.*[16] Moreover, compounds, such as xenorhabdins[17], xenocoumacin[18], indole compounds[19] and nematophin (which have particularly high antifungal activities)[20] from other *Xenorhabdus* spp. have been shown to have antibacterial and antifungal activities. To the best of our knowledge, this study is the first to describe the influences of method and period of cultivation on the antifungal activities of *X. stockiae* PB09 and this information may be useful for the future development of *X. stockiae* PB09 as products for biological control of fungal anthracnose disease.

Conclusions

The results in this study demonstrated that cell-free supernatants of *X. stockiae* PB09 had high antifungal activities for controlling *C. gloeosporioides*, a causative agent of fungal anthracnose disease. *X. stockiae* PB09 cultivation by fermentation was superior to shake-flask cultivation by giving more effective cell-free supernatant in shorter time for up to 24 h. Further studies are will be needed to determine the optimum conditions for cultivation of *X. stockiae* PB09, particularly in a large scale settings.

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