Efficacy of chitosan nanoparticles loaded bioactive substance obtained from *Bacillus subtilis* B03 cultures for controlling flower rusty spot disease in *Dendrobium*

ประสิทธิภาพของสารสกัดชีวภาพที่เตรียมจาก *Bacillus subtilis* B03 ตรึงลงบนอนุภาคนาโนไคโตซานสำหรับควบคุมโรคดอกจุดสนิมในกล้วยไม้สกุลหวาย

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Abstract

Bioactive substance (Bioactive substance; BS) isolated from *Bacillus subtilis* B03 were immobilized on chitosan nanoparticles with adsorption technique (Bioactive substance immobilized on chitosan nanoparticles with adsorption technique; BSAd) and encapsulation technique (Bioactive substance immobilized on chitosan nanoparticles with encapsulation technique; BSEn). They were evaluated for their inhibition against the spore germination of *Curvularia eragrostidis.* BS, BSAd and BSEn were tested for their effectiveness on fungal spore germination by using hanging drop technique which later fungal spore was measured after incubation for 24 hours. The results showed that BS was able to inhibit 91.4% of spore germination of *Curvularia eragrostidis* at 1000 ppm. BSAd and BSEn were able to be against 99.1% and 98.6% of spore germination at 1000 ppm. The efficacy of antifungal compounds was showed some similarities with the activity of Mancozeb which is chemical fungicide. The results confirmed that the antifungal compound from *Bacillus subtilis* B03 has potential to be antifungal agent against the flower rusty spot disease in *Dendrobium*.

Key words: Bioactive substance, BSAd, BSEn

บทคัดย่อ

สารสกัดทางชีวภาพ (สารสกัดทางชีวภาพ; BS) ที่เตรียมจากเชื้อ *Bacillus subtilis* B03 ถูกตรึงบนอนุภาคนาโนไคโตซานด้วยวิธีการดูดซับ (สารสกัดทางชีวภาพที่ตรึงบนอนุภาคไคโตซานด้วยวิธีการดูดซับ; BSAd) และสารสกัดทางชีวภาพที่ตรึงบนอนุภาคไคโตซานด้วยวิธีการห่อหุ้ม (สารสกัดทางชีวภาพที่ตรึงบนอนุภาคไคโตซานด้วยวิธีการห่อหุ้ม; BSEn) เพื่อใช้ในการยับยั้งการงอกของสปอร์รา *Curvularia eragrostidis* BS, BSAd and BSEn ถูกนำมาทดสอบประสิทธิภาพในการยับยั้งการงอกของสปอร์โดยใช้วิธี Hanging drop โดยวัดอัตราการเจริญของสปอร์ภายหลังจากการบ่มเชื้อไว้ 24 ชั่วโมง ผลการศึกษาพบว่าประสิทธิภาพของ BSที่ความเข้มข้น 1000 ppm สามารถยับยั้งการงอกของสปอร์ได้ 91.4% และประสิทธิภาพของ BSAd และBSEn ที่ความเข้มข้น 1000 ppm เช่นเดียวกันสามารถยับยั้งการงอกของสปอร์ได้ 99.% และ 98.6% ซึ่งมีประสิทธิภาพใกล้เคียงกับ Mancozeb ซึ่งเป็นสารเคมีที่ใช้ในการควบคุมเชื้อราในปัจจุบัน จากผลการทดลองทำให้ทราบว่าสารสกัดทางชีวภาพที่เตรียมจากเชื้อ *Bacillus subtilis* B03 มีศักยภาพในการควบคุมเชื้อราซึ่งเป็นสาเหตุก่อโรคดอกจุดสนิมในกล้วยไม้สกุลหวาย

คำสำคัญ : สารสกัดชีวภาพ, สารสกัดชีวภาพตรึงบนอนุภาคนาโนไคโตแซนด้วยวิธีการดูดซับ, สารสกัดชีวภาพตรึงบนอนุภาคนาโนไคโตแซนด้วยวิธีการห่อหุ้ม

INTRODUCTION

Orchidaceae is the most commercial orchid genus according to the Convention on International Trade in Endangered Species of wild Fauna and Flora (CITES) statistics. Orchid is one of the important exported plants of Thailand, which is popular in both Thailand and international markets. In 2014, orchids were exported up to the amount of 33 million kilograms and export revenue was accounted for 760 million Baht (Department of agricultural economics, 2015). *Dendrobium* is one of orchid genus is the most exported of Thailand which is accounted for 67% of the total orchid exports in the country (Hossain, 2011).

The major problems of orchids export are the diseases caused by fungi because of high humidity in greenhouses that orchids are grown in. Fungi that often found are *Curvularia eragrostidis* (Peter, 2005).

It can destroy the flowers all parts of the plant within 8-24 hours. Especially during the rainy season in early winter around November - December (Trisri, 2001 and Mongkolsuk, 2006). The fungi can dry out the roots and cause the leaves to be yellow which eventually fall out. *Curvularia eragrostidis* produces small brown spots which appear on the petals of orchid flowers. It was Characteristic symptoms of flower rusty sport disease (Duff, 2002).

Nanotechnology is a multidisciplinary field of applications in the pharmaceutical industry (Gupta, 2006). It is now possible to produce drug nanoparticle for disease treatment in plants. It can be utilized in a variety of innovation ways (Jain, 2008). Chitosan nanoparticles (Chitosan nanoparticles; CsNs) is a nature material with excellent physiochemical properties which is considered to be environment friendly and bioactive (Hu et al., 2004; Qi and Xu, 2004; Shen and Ting, 2005). Recently, many researchers such as Atif et al. reported that CsNs have the potential of becoming a powerful and safe natural antifungal activity (Atif et al., 2012). Therefore, it's essential to find new compounds as bioactive substances obtained from *Bacillus subtilis* B03. Chitosan nanoparticles loaded bioactive substances were prepared using the method of ionic gelation based on the interaction between chitosan cat ion and tripolyphosphate anion. The aim of this research study is to investigate the effectiveness and concentration suitable of chitosan nanoparticles loaded bioactive substances for control flower rusty spot diseases in *Dendrobium* orchid flowers. The results of the present study will be guidance for farmers to control fungi diseases in orchid cultivation. Bioactive substances (Bioactive substances ; BS) immobilize on CsNs itself is biodegradable and considered to be environmental friendly. Therefore, bio control with bioactive substances immobilize on CsNs are applied to minimize the problems in grow areas of orchid and the environment.

OBJECTIVES

1. To observe effects of chitosan nanoparticles loaded bioactive substances obtained from *Bacillus subtilis* B03 on spore germination of *Curvularia eragrostidis* causing agent of flower rusty spots on orchid flowers

2. To compare antifungal properties of chitosan nanoparticles loaded bioactive substances prepared from different immobilization techniques including encapsulation and adsorption techniques in controlling growth of *Curvularia eragrostidis* spores

MATERIALS AND METHODS

Preparation of bioactive substance (bioactive substance ;BS)

Bacillus subtilis B03 was grown in culture media Potato Dextrose Broth (Potato Dextrose Broth; PDB), at 30°C for 96 hours. This was centrifuged to isolate supernatant, which later would be adjusted to the pH 2 using 12 N hydrochloric acid (Hydrochloric acid ; HCl) to precipitate BS. According to procedures described by Mckeen et al. (1986). Bioactive substances were subsequently separated by centrifugation at 4 °C for 30 min with 8000 rpm/min and extracted twice with 50 ml of 80% ethanol. The BS was evaporated and lyophilized. The BS was dried and stored at room temperature in desiccators before further use or analysis (Liu, 2007). The BS was dissolved and diluted with 80% ethanol with vary final concentration at 1,10,25,50,100,250,500, and 1000 ppm. It was prepared for BSAd and BSEn in experiment.

Preparation of chitosan nanoparticles (Chitosan nanoparticles ;CsNs)

According to Maturot (2012), 3:1 chitosan to TPP solutions was the most suitable ratio to form CSNs. Using a peristaltic pump, TPP solution was drop wisely added into chitosan solution at room temperature. The nanoparticles were subsequently separated from the supernatant through centrifugation at high speed (8,000 rpm) at 4°C for 30 min (Wu et.al, 2005).

Preparation of chitosan nanoparticles loaded with bioactive substance using adsorption technique (Bioactive substance immobilized on chitosan nanoparticles with adsorption technique; BSAd)

According to Lifeng (2004), loading of various concentrations of BS on CsNs using adsorption technique, could be done as follows: For the purpose, 200 µl of 20000 ppm bioactive substance solution

was added into 1.5 g of $7.5x10^5$ ppm CsNs solution, which were in a form of opalescent gel. 0.375%, w/v, CsNs suspension was derived from the following method. The mixture was allowed to mix using a vortex mixer until the solution was observed as homogenous. Afterwards, the solution was further shaken at 150 rpm/min, 30°C for 1 hour. The solution was then centrifuged at 10,000 rpm/min, 4°C for 30 min to remove the supernatant. The sediment was later applied for testing fungicidal properties of the prepared samples against growth of *Curvularia eragrostidis* in orchids.

Preparation of chitosan nanoparticles loaded with bioactive substance using encapsulation technique (Bioactive substance immobilized on chitosan nanoparticles with encapsulation technique; BSEn)

Based on the method developed by Liu (2007), this was conducted by mixing 1 ml of 40000 ppm prepared bioactive substance (prepared from 1) into 9 ml of TPP solution before adding chitosan solution (Calvo, 1998). The mixed solution was then drop wisely added into chitosan solution at room temperature and 700 rpm. To remove supernatant, the sample was centrifuged 5-7 times at 10,000 rpm/min at 4°C for 30 min.

Preparation of spore suspension

Curvularia eragrostidis was inoculated on PDA agar plates at 28±2°C. After 7 days incubation, spore culture of the fungal was prepared by scrapping gently of conidia with a spender glass rod to ensure maximum conidia and mix it with 5 ml sterile distilled water (Petlamul, 2012).The conidia suspensions were adjusted the volume to 50 ml with sterile distilled water (Begum, 2010). To separate fiber or mycelia out of the suspension, the suspensions were shaken with a vortex mixer for approximately 1 min (Thaochan, 2014). After that, spores were aseptically filtered off the mycelia in a funnel containing sterile cotton cloth. Each sample was then added with sterile distilled water to give a final spore concentration of approximately 10^5 spores/ml (Thieankhao, 2007). The concentration was determined using a haemacytometer under the light microscope at a 400x magnification (Soundarapandian, 2007).

Inhibitory effects of tested compounds against spore germination of *Curvularia eragrostidis*

Spore germination study was modified from Hanging drop technique (Chandel, 2014). This was done by adding antifungal compounds at different concentrations on a glass slide to test spore germination of the concerned fungi. For the present study, various concentrations, i.e. 1, 10, 25, 50, 100,250,500 and 1000 ppm, of antifungal compounds, including CsNs, chitosan nanoparticles loaded bioactive substance, as well as Mancozeb were prepared and tested. This was done by mixing 25 µl of spore suspensions, containing 10^5 spores/ml, with 25 µl of the three tested antifungal compounds at various concentrations as mentioned earlier. Spore suspension mixed only with sterile distilled water was used as the control.

The prepared slides were then kept in a moisture chamber, which prepared by placing two moist filter papers inside the base and lid of sterilized Petri dishes (Singh, 2009). They were incubated at 28 ± 2° C for 24 hours. The samples were subsequently covered with a glass cover lid. Germinated spores of each sample were observed by count under microscope 100 spores on each slide. Spore and germ tube elongation percentage was calculated as compared to the control (Singh, 2009). All the experiments were conducted in triplicate.

RESULT AND DISCUSSION

Bioactive substance (Bioactive substance ; BS) Production

In this experiment, *Bacillus subtilis* B03 was cultivated in PDB. Bioactive substances were produced from secondary metabolite of *Bacillus subtilis* B03 in PDB culture media. 4 liters of them were prepared each time. The bioactive substances activities were extracted with 80% ethanol solution and were stabilized with lyophilization. They were kept at room temperature with desiccators. After lyophilization, a dark brown powder was character of bioactive substance.

In the preparation of bioactive substance, wet weight from the starter which was 4L crude extract was approximately 13.0 g. After lyophilization, Yields of BS were decreased because of its water composition which were around that 46.1, 48.8, and 53.1%, respectively.

Antifungal activity of BSAd and BSEn in aqueous phase

Chitosan was precipitated in acetic acid occurred upon loaded to bioactive substance. The ability of chitosan nanoparticles loaded bioactive substances obtained from *Bacillus subtilis* B03 which are BSAd and BSEn for growth inhibition of *C. eragrostidis.* Germinated spores of each experiment compound in each concentration of BS, BSAd, and BSEn on slides and incubated at 25±2°C for 24 hours were counted under microscope.

Fungi used in the present study were chosen primarily on the basis of their important as pathogens in economically important plants, especially in orchids. In this study, *Curvularia eragrostidis* was selected.

Inhibition of spore germination by antifungal compound

Antifungal compound at higher concentration had given better result in the inhibition of spore germination. In experiment, there are 5 chemical compounds used in the controlled treatment which are chitosan nanoparticles, ethanol, acetic acid HCl, and Mancozeb. Positive control*, Curvularia eragrostidis* spore suspension are not effective in inhibiting the growth of spores when used. The data presented in Table 1.

Table1 Inhibitory effects of various compounds on spore germination and germ tube elongation of *Curvularia eragrostidis* after 24 hours incubation at 25±2°C (n=3)

Treatment	Compound concentration (ppm)	Spore germination inhibition (%)	Germ tube elongation inhibition (%)
Control [1]	0	0.0±0.0	0.0±0.0
CsNs	$7.5x10^5$	7.2±0.4	9.2±0.3
Ethanol	$8x10^5$	4.8±0.3	7.6±0.4
Acetic acid	$1x10^3$	40.4±0.5	48.0±0.3
HCl	$3.7x10^3$	45.6±0.5	51.3±0.4

Mancozeb	$1.5x10^3$	98.8±0.5	100.0±0.0

[1] Samples treated with distilled water

The results revealed that, Efficacy for inhibit spore germination of chitosan nanoparticles are 7.2%. The inhibition rate of ethanol is at 4.8%. Acetic acid and HCl has spore inhibition rate at 40.4 and 45.6%. The acid solution was used in the experiment. It has ability to inhibit the growth of the spores. In comparison, Mancozeb, commonly known chemicals fungicide, is used at concentration of 1500 ppm in the test and the results show its effectiveness in inhibiting spores at 98.8% which considered being very high in preventing the growth of the spore.

The study found that if the inhibiting spore had low inhibition rate, the growth of germ tube will be inversely high. As a reason, the spores can expand its population and as time goes by germ tube will be produced in the process of spore propagation. If the spores were inhibited by antifungal compound, the growth of spore will be disrupted and the germ tube production will also be decreased.

In laboratory fungal growth inhibition values coincided with previous results shown by other researchers. Inhibition of *Curvularia* species by *Bacillus subtilis* ATCC 6633 with radial growth inhibition has been reported to be between 66 to 100% (Castellanos et al. 2002). In 2014, Rakdee has been reported that effectiveness of bioactive substances immobilized on chitosan nanoparticles against growth of *Curvularia eragrostidis* at 10 mg/ml was 97±0.5%.

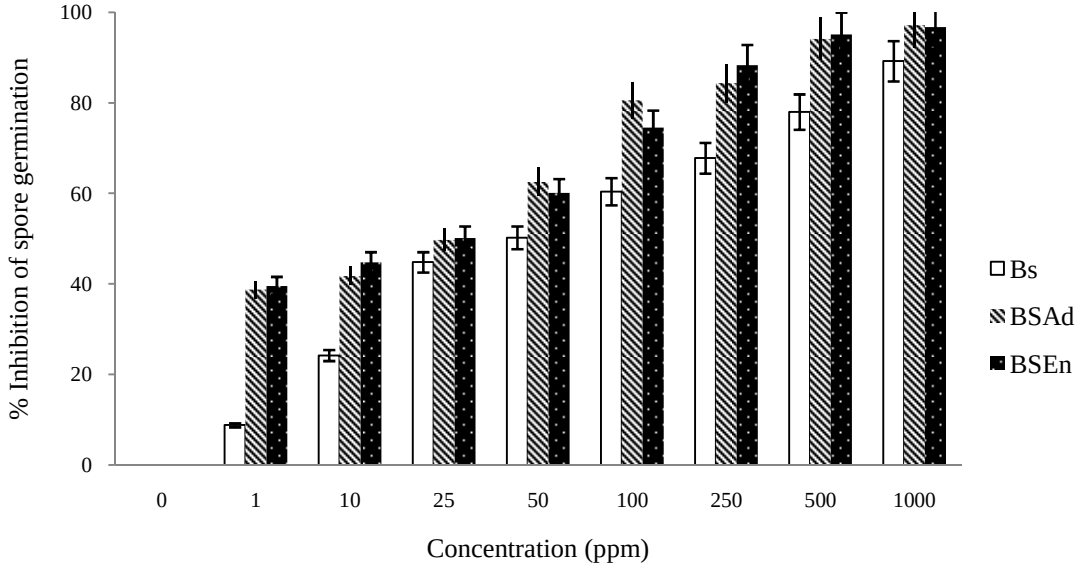

Figure 1 Inhibitory effects of three different antifungal compounds, i.e. BS, BSAd and BSEn on spore germination of *Curvularia eragrostidis* after 24 hours incubation at 25±2°C (n=3 treatments)

In figure 1, Each antifungal compounds which are BS, BSAd and BSEn were then applied to make a dilution and germination of fungal was observed at 24 hours after incubation and repeated of each concentration at 3 times. The efficacy of BS, BSAd and BSEn were evaluated and compared with Mancozeb which is a chemical fungicide. The inhibition spore germination percentages of antifungal compounds were observed and percentage was calculated. The results showed in percentage inhibition in spore germination of antifungal compounds of *Curvularia eragrostidis* in relation to control experiment. The highest inhibition in the spore germination was observed in 1000 ppm concentration of BS, BSAd and BSEn that the effectiveness of the antifungal compound were increasing when the concentration were also increasing. The lowest concentration at1 ppm of BS, BSAd and BSEn were showed percentage inhibitions of spore germination at 8.8%, 38.8% and 39.6%.The highest efficacy for inhibit spore germination at concentration 1000 ppm was found 91.4%, 99.1% and 98.6% respectively. The antifungal compounds at 1000 ppm showed highest inhibitory effect and concentration at 1ppm inhibited the lowest spore germination.

Germ tube elongation inhibition

According to the results given in the figure 2, the elongation of the germ tube from the early experiment after 24 hours of incubation at 25±2°C would give similar results to spore inhibition rates. In order to test the efficiency of antifungal compound, the experiment on the inhibition of germ tube's elongation had been conducted. As a result, it showed the positive relationship between these two tests. The elongation of germ tube was depended on the concentration of BS, BSAd, and BSEn. If the concentration of the antifungal compound are around 1000 ppm, germ tube is unable to elongate or slowly in progress. However, if the concentration of antifungal compound is less than 1000 ppm, germ tube can elongate normally. In comparison with Mancozeb at 1500 ppm which was the concentration of chemical

fungicide control, the efficiency is 0% germ tube; it had similar properties in inhibition with the antifungal compound at 1000 ppm.

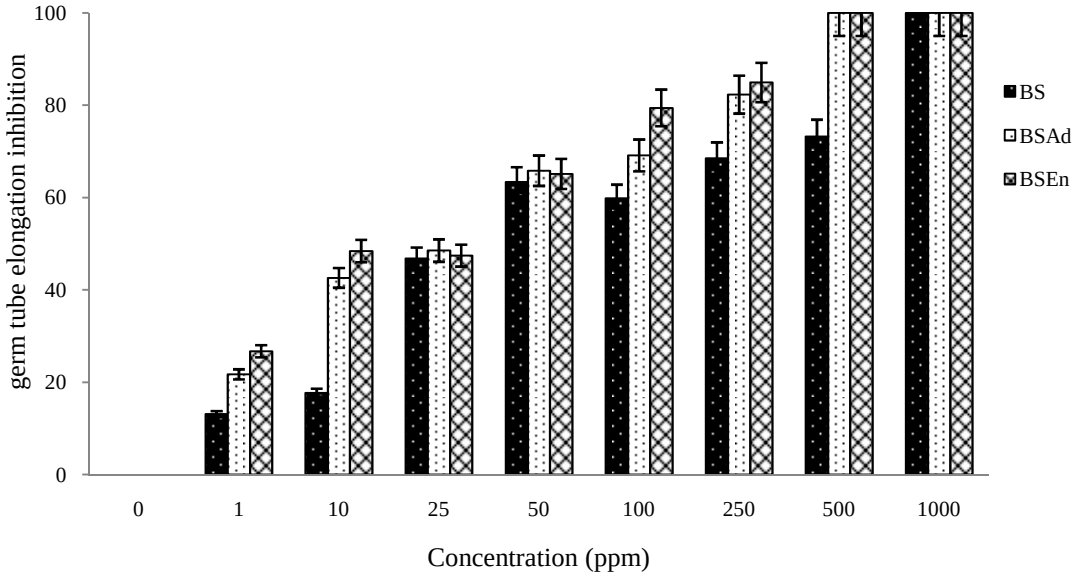

Figure 2 Inhibitory effects of three different antifungal compounds, i.e. BS, BSAd and BSEn on germ tube elongation of *Curvularia eragrostidis* after 24 hours incubation at 25±2°C (n=3 treatments)

The efficiency of BSAd and BSEn at 500 ppm and 1000 ppm were the highest in experiment which spore cannot grow develop into germ tube at all; the growth rate is 0%. For BS, only at the concentration of 1000 ppm was able to generate 0% growth rate. In addition, at the concentration 1 ppm which was the lowest of BS, BSAd and BSEn were not affective in spore and germ tube inhibition. Then spore still can growth and develop into germ tube as normal similar to the positive control condition.

BS, BSAd, and BSEn at different concentrations were tested for the spore germination inhibition. As the results, at 500 ppm, the effective rates were up to 89.0, 94.2 and 95.2% respectively. If the concentrations were increased to 1000 ppm, the rates were raised up to 91.4, 99.1 and 98.6% respectively. And in the inhibition of germ tube elongation, the rates of successful were up to 83.0, 100 and 100 % for BS, BSAd and BSEn at 500 ppm while at 1000 ppm, they were 100 % completely successful in inhibit the germ tube elongation.

Three types of antifungal compound were tested and the results were used to compare with Mancozeb, the chemical fungicide antifungal compound. In this experiment, the highest concentration of antifungal compounds was at 1000 ppm while the highest concentration of Mancozeb was at 1500 ppm. As the results, three types of antifungal compounds were effective in inhibiting spore germination and germ tube elongation similarly to others substances even though they were used in lower concentrations. The antifungal compound is a substance derived from living organisms, which is environmental friendly to all farmers and consumers. In term of effectiveness, it needs to be used continuously in regular basis, which take longer time to see clearly results in controlling the flower rusty spot disease.

CONCLUSION

Chemical fungicide and antifungal compounds that are BS, BSAd and BSEn which extracted from *Bacillus subtilis* B03 were able to inhibit spore germination and germ tube elongation of *Curvularia eragrostidis*. Effects of Mancozeb and antifungal compounds were promising compared to others. Antifungal compounds could be used as a biofungicide to control flower rusty spot diseased in orchids. To be widely used in *Dendrobium* or other species of orchids, BS BSAd and BSEn should be studied the further effects of *Curvularia eragrostidis* inhibition in *Dendrobium* and applied in other orchid species.

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