



# OPTIMIZATION OF ENDOPHYTIC FUNGUS BUEN830 FOR THE HIGHEST ANTIFUNGAL ACTIVITY AGAINST PLANT PATHOGENIC FUNGUS *Colletotrichum gloeosporioides*

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## Abstract

Endophytic fungus strain BUEN830 showed strong antagonistic effect against plant pathogenic fungus *Colletotrichum gloeosporioides* on potato dextrose agar (PDA) while the extract prepared from stagnant potato dextrose broth (PDB) revealed only weak inhibition activity (Inhibition distance 0.1 cm). The fungus was subjected to the stepwise optimization in liquid medium under various cultural conditions, began with salinity then followed by medium type, initial pH, shaking rate, temperature and incubation time. After each optimizing step, the extracts were tested for antifungal activity by disc diffusion method and the best condition was selected for using in the next experiment. After final optimization, strong antifungal activity against *C. gloeosporioides* was observed. The maximal antifungal activity (Inhibition distance 0.30 cm) was obtained after this fungus was fermented in 10 practical salinity unit, Sabourauds dextrose broth (SDB), pH 7 after shaking 150 rpm at 25 °C for 4 to 7 days.


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**Keywords:** endophytic fungus, extract, plant pathogenic fungi, antifungal activity

## Introduction

Anthraxnose diseases caused by *Colletotrichum gloeosporioides* are serious problems in wide range of plants in tropical and subtropical regions. *Colletotrichum gloeosporioides* can cause post harvest fruit rots in papaya, mango, avocado and strawberry (Johnson et al., 1993; Bernstein et al., 1995; Timudo-Torrevilla et al., 2005; Gupta et al., 2010) and are the major limiting factors for production, resulting in serious yield losses (Legard, Whidden, & Chandler, 1997). To date, large numbers of chemicals utilization and the development of resistance in plant pathogens results in the growing of the hazardous side effects (Mondali et al., 2009). Alternative biological and non-chemical plants protection have come to an interest. Biological control of post harvest diseases is one of the options to overcome these problems. These included the utilization of antagonistic and natural products from microorganisms in management of post harvest diseases to replace the chemical pesticides (Pal & Gardener, 2006).

Many marine organisms have been shown to be able to synthesize structurally unique secondary metabolites with the chemistry and biology unlike those found in terrestrial ones (Davidson 1995). Mangrove fungi and endophytic fungi are fungal groups which have been known as important sources of new bioactive compounds, moreover several bioactive substances have been isolated from endophytic fungi from mangrove plants (Lin et al., 2001). Extracts from mangrove endophytic fungi have been studied to control plant pathogens effectively for example the extract from *Diaporthe* sp. can control *Aspergillus niger* (Lin et al., 2005), *Phomopsis* sp. ZSU-H76 can control *F. oxysporum* (Huang et al., 2008) and *Varicosporina ramulosa* can control *F. solani* (Mabrouk et al., 2008).



In our survey, endophytic fungi were isolated from mangrove plant in Chanthaburi Province Thailand. The fungus BUEN830 was isolated from *Bruguiera sexangula* Poir. This fungus showed strong antagonistic effect against *C. gloeosporioides* in dual culture, but weaker reaction was observed when the extract was tested. In this study we evaluated the optimal physical requirements such as salinity, medium type, initial pH, shaking rate, temperature and incubation time, for applying in liquid fermentation of BUEN830 to obtain high antifungal activity against *C. gloeosporioides*.

## Methodology

Endophytic fungus strain BUEN830 was grown on PDA at 28°C for 7 days and then 5 agar discs (5 mm diameter) of active growing mycelium were transferred to 50 ml of PDB, pH5, contained in 250 ml Erlenmeyer flask and incubated at 28°C for 4 days under stagnant condition (Table 1). The ethyl acetate extracts from broth culture under this conventional condition were tested for antifungal activity against *C. gloeosporioides* DOAC0782 and were designed as before optimization. The stepwise optimizations of antifungal activity began with variation of broth salinity (0-30 practical salinity unit (psu), which corresponds to 30 part per thousand (30 ppt) of salt concentration in seawater), at 150 rpm, while other conditions were fixed. The best salinity was selected for preparation of broth media in the next optimizing experiment. The variation of broth culture, initial pH, shaking rate, temperature and incubation times were subsequent carried out in the similar manner (Table 1). The culture filtrates collected at each step were extracted with twice 50 ml ethyl acetate. The pooled ethyl acetate extracts were evaporated in a rotary evaporator until dry. The crude extracts were dissolved in 1 ml of 50% DMSO and each 20 µl was tested for antifungal activity by disc diffusion method. The inhibition distances (ID) was measured compare to the negative control disc (soaked with 50% DMSO). Results came from three replicated fermentations and three replicated tests conducted in each experiment were expressed as mean ± SD values. Data in each condition were analyzed to select the best condition by one-way analysis of variance test (ANOVA) and post-hoc test with Turkey HSD (significant defined at  $p \leq 0.05$ ) using SPSS version 16.0 software.

## Results

### Before the optimization of antifungal activity

The ethyl acetate extract from culture filtrate of the fungus BUEN830 with stagnant condition in PDB pH 5 incubated at 28°C for 4 days, showed inhibition against *C. gloeosporioides* DOAC 0782 with a distance of 0.10 cm (Table 1).

**Table 1** Optimization condition and antifungal activity of endophytic fungus BUEN830 against *C. gloeosporioides*

Condition optimized		Optimal conditions selected	Inhibition distance <sup>2</sup> (cm)
Before optimization			
	0 psu , PDB, pH 5, 0 rpm, 28°C, 4 days	-	0.10 ± 0.04 <sup>a</sup>
Optimization			
salinity (psu)	0, 10, 15, 20 and 30	10	0.21 ± 0.06 <sup>b</sup>
type of broth culture <sup>1</sup>	0.5xPDB, PDB, LNB, YMB and SDB	SDB	0.28 ± 0.04 <sup>bc</sup>
initial pH	5, 6, 7, 8 and 9	7	0.24 ± 0.06 <sup>bc</sup>
shaking rate (rpm)	0, 50, 100, 150 and 200	150	0.32 ± 0.06 <sup>c</sup>
temperature (°C)	22, 25, 28 and 30	25	0.32 ± 0.07 <sup>c</sup>
incubation period (day)	2, 4, 7, 10 and 14	7	0.30 ± 0.06 <sup>bc</sup>

<sup>1</sup> 0.5xPDB: half concentration of PDB, PDB: potato dextrose broth, SDB: Sabourauds dextrose broth, YMB: yeast malt broth, LNB: low nutrient broth

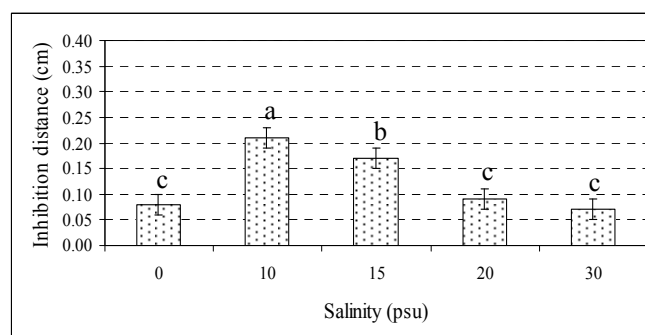
<sup>2</sup> Means ± SD values of 9 replicates

<sup>a-c</sup> Different superscript indicate significant difference letters at p = 0.05 (Turkey HSD)

### Optimization of antifungal activity

#### Salinity concentration

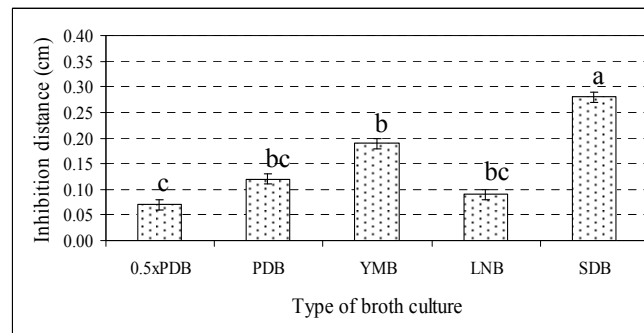
After BUEN830 was cultivated in various salinity (0-30 psu) of broth cultures, the results showed that at the salinity 10 psu, the highest antifungal activity (ID = 0.21 cm) was observed. Although increasing of salinity to 15 psu led to the reduction of inhibition distance, such antagonistic activity was higher than that obtained before optimization (Figure 1).



**Figure 1** Antifungal activity of BUEN830 extract against *C. gloeosporioides* DOAC0782. The fungal extracts were obtained from PDB culture (pH 5) with various salinity. Incubation was performed at 28 °C, 150 rpm, for 4 days.

### Cultivation medium

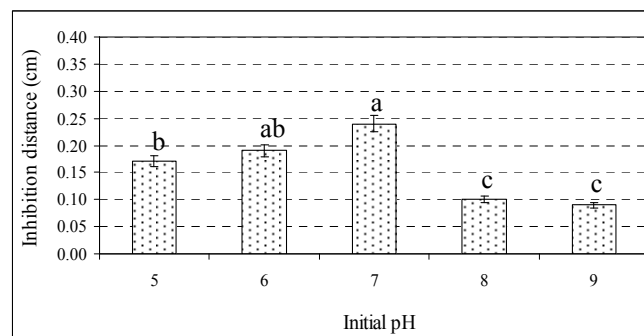
The result in Figure 2 showed the highest antifungal activity of BUEN830 produced in SDB medium. The inhibition distance (ID) of 0.28 cm was significant difference from the ID of 0.12 cm and 0.17 cm of other rich media, PDB and YMG, respectively.



**Figure 2** Effect of medium types (pH 5, 10 psu). Incubation was performed at 28 °C, 150 rpm, for 4 days on antifungal activity of BUEN830 against *C. gloeosporioides* DOAC0782.

### Initial pH

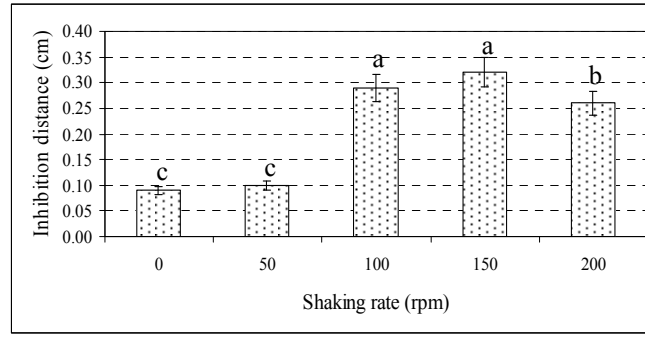
The results of the initial pH of broth culture shown in Figure 3 revealed the maximum activity at pH 7 (ID = 0.24 cm). Higher initial pH (> pH7) led to the conspicuous decreasing of antifungal activity of the extracts.



**Figure 3** Effect of initial pH of culture medium (SDB, 10 psu) on antifungal activity of BUEN830 against *C. gloeosporioides* DOAC0782. Cultures were incubated at 28 °C, 150 rpm, for 4 days.

### Shaking rate

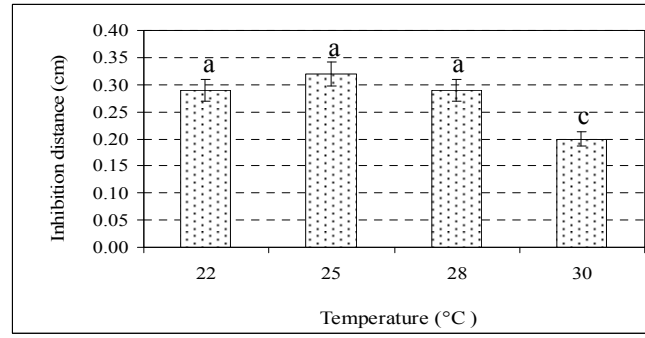
As shown in Figure 4, superior antifungal activities were found to be those of extracts obtained from cultured broth incubated with shaking rate at 100 and 150 rpm. The pronounced ID were 0.29 cm and 0.32 cm, respectively, which were not different significantly.



**Figure 4** Antifungal activities of extracts derived from BUEN830 culture broth (SDB, 10 psu, pH 7) agitated at different speed against *C. gloeosporioides* DOAC0782. All cultures were incubated at 28 °C for 4 days before the extraction to be commenced.

### Temperature

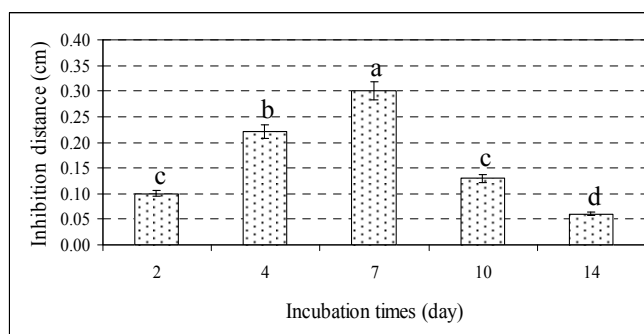
The inhibition activities seem to increase the highest activity when the broth cultures were incubated at 25°C (Figure 5). However, the extracts of cultures incubated at 22-28 °C showed no significant difference in antifungal activities. Regarding the ID, the level of temperature at 30°C was not suitable to be used as culture incubation.



**Figure 5** Effect of incubation temperature on antifungal activity of BUEN830 against *C. gloeosporioides* DOAC0782. BUEN830 was cultured in SDB (10 psu, pH 7) and incubated with shaking at 150 rpm for 4 days. The culture filtrates were extracted and used in the test.

### Incubation time

A linear relationship between incubation time and antifungal activity of the extract was observed during the first 7 days of cultivation. The ID was 0.10 cm when the 2 days culture extract was tested, and increased to the highest value of 0.30 cm by the extract of 7 days culture extract (Figure 6). Longer incubation periods (10-14 days) promising reduced the inhibition efficiency of the culture extracts.



**Figure 6** Effect of incubation times on antifungal activity of BUEN830 against *C. gloeosporioides* DOAC0782. BUEN830 was cultured in SDB (10 psu, pH 7) and incubated at 25 °C, 150 rpm. The cultures were taken at various time intervals, extracted and used in disc diffusion test.

### Discussion and Conclusion

The fungus BUEN830 displayed the highest inhibition activity against *C. gloeosporioides* DOAC0782 when cultured in 10 practical salinity of Sabourauds dextrose broth, pH 7, shaking at 150 rpm, 25 °C for 7 days. The maximal inhibition distance obtained was 0.32 cm, which is three fold increasing compared to the ID of 0.10 cm before optimization. Results indicate the successful optimization in this study. The salinity, type of broth culture and shaking rate are major factors affecting the antifungal activity of BUEN830.

In this study, after the cultivation of mangrove endophyte BUEN830 in PDB with various salinity, the results showed the maximal antifungal activity at 10 psu, while the higher salinity (>15 psu) resulted in decreasing of activity. The salinity was found to be one of critical parameters affecting the antifungal activity. It has been reported that the maximal activity in bioactive compounds from obligate marine fungi can reach in the media containing 25-50% seawater (equivalent to 8-20 psu) (Bugni and Ireland, 2004). Masuma et al. (2001) also reports that the different fungal genera of marine-derived strains showed various behaviors of growth and secondary metabolites activity with the increasing salinity. Shaking rate and temperature were also important factors which affect inhibition activity of BUEN830. Other factors were likely to have only slight effect on the inhibition activity.

The antifungal activity produced in rich media was higher than those of the nutrient deficient medium. Obviously, cultivation in SDB medium, BUEN830 showed the highest antifungal activity. Difference proportions of C:N ratio as well as the compositions of growth factors might be present in the tested media and strongly affect the fungal growth, metabolite production, and subsequent inhibitory antifungal activity. Singh (2003) reports that the accumulations of metabolic products were strongly influenced by the medium compositions such as carbon source, nitrogen source, and other growth factors. The initial pH and incubation period were slightly affected the inhibition activity of BUEN830. At initial pH 7 of SDB medium the maximal antifungal activity of BUEN830 was obtained. The antimicrobial metabolites are produced at neutral pH has been reported previously (Rubini et al., 2005). The highest antifungal activity on 7 days of fermentation in this study seem to correspond to the report of Lin et al. (2005) who indicated that antimicrobial production of some marine-derived fungi was achieved on 7 days of incubation.



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