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Potential of Marine Chitinolytic *Bacillus* Isolates as Biocontrol Agents of Phytopathogenic Fungi

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Abstract. Phytopathogenic fungi pose a serious problem around the world in the economically important plants. Chemical fungicides are widely used in current agriculture. However, excessive use of chemical fungicides has led to the deterioration of human health, environmental pollution, and development of pathogenic resistance to fungicides. A serious search is required to identify alternative methods for crop protection, which is less dependent on chemicals and more environmentally friendly. Microbial antagonists widely used for biocontrol of plant fungal disease. The success of biocontrol depends on the nature of antagonistic properties and mechanisms of action of the biocontrol agent against the phytopathogenic fungi. In the present study, 83 *Bacillus* isolates isolated from marine samples were screened on the colloidal chitin agar medium. Based on the chitinolytic activity and percentage inhibition against fungal phytopathogens by dual plate technique, isolate B26 is most potent as biocontrol agents. The *Bacillus* B26 inhibited the growth of *Fusarium solani* TISTR 3436 and *Penicillium chrysogenum* with percentage inhibition 69% and 46.6% respectively, while did not inhibit the growth of *Aspergillus niger* and *Aspergillus flavus*. *Bacillus* isolate B26 showed a positive result for the urease production, catalase test, starch hydrolysis, casein hydrolysis with most suitable growth condition at 37°C, pH 7-8, and could grow at 0-5% NaCl concentration.

Keywords: chitinolytic activity, *Bacillus*, phytopathogenic fungi

1. Introduction

For many years, plant pathogenic fungi have caused devastating losses worth in crops worldwide. Synthetic fungicides have effectively controlled plant pathogenic fungi. Their repeated use over decades has disrupted natural biological systems, and sometimes resulted in development of fungal resistance. They had undesirable effects on non-target organisms, and fostered environmental and human health concerns. [1] Because of the worsening problem in the control of fungal diseases, alternative measures have been developed for crop protection in an attempt to reduce the use of synthetic fungicides, intensive investigations into the possible exploitation of biopesticides are safe for humans and environmentally friendly. Biological control, the used microorganisms to control plant diseases, offers an alternative, environmental friendly strategy for controlling the phytopathogens. Recently, biological control has been focused on microorganisms producing mycolytic enzyme especially chitinase, which are known hydrolyze chitin, a major component of the fungal cell wall. Chitinolytic microorganisms are currently being studied as an attractive alternative to synthesis chemicals because of their perceived safety and lower environmental impact.

In aquatic environments, chitin is decomposed primarily by aerobic bacteria include of the genera *Aeromonas*, *Enterobacter*, *Flavobacterium*, *Serratia*, and *Bacillus*. [2] *Bacillus* spp. are widespread in nature, nonpathogenic and harmless to humans and other animals but harmless to plants. These bacteria excrete antimicrobial compounds in vitro, including the lipopeptides antibiotics and antifungal protein. Moreover, *Bacillus* sp. offers advantages over others against of fungal phytopathogens due to endospores and secretion of broad-spectrum antibiotics. The endospores forming ability facilitates resistant to the elevated temperature and high concentrations of chemicals. [3]

The aim of this work was to screen the chitinolytic *Bacillus* isolates which were isolated from marine samples with potential as biocontrol agents against some phytopathogenic fungi.



2. Experimental Method

2.1. Preparation of fungal strains

The fungi including *Aspergillus niger* TISTR 3130, *Aspergillus flavus* TISTR 3041, *Fusarium solani* TISTR 3436, and *Penicillium chrysogenum* were obtained from Thailand Institute of Scientific and Technology Research. The fungi were cultured on potato dextrose agar (PDA) (Himedia, India) plates and incubated at 28°C for 5 days. Stock cultures of fungi were maintained on PDA slants and stored at 4°C.

2.2. Preparation of colloidal chitin

The colloidal chitin was prepared, five grams of shrimp chitin powder (Himedia) added to 60 mL concentrated HCl with mechanical stirring for an hour. [4] The mixture was filtered through glass wool and the filtrate added to a 200 mL of 50% ethanol that stirred during the process. The precipitate was transferred to glass funnel with filter paper Whatman No.1 and washed with sterile distilled water until the colloidal chitin became neutral (pH 7). The prepared colloidal chitin was autoclaved (15 psi, 15 min, 121°C) and stored at 4°C.

2.3. Screening of chitinase producing bacteria

A total of 83 *Bacillus* strains, which were isolated from marine samples such as seawater and sand of marine beaches located in Songkhla and Pattani provinces, were obtained from the culture collection of Department of Science, Faculty of Science and Technology, Prince of Songkla University, Thailand. Point inoculation was performed on three replications for all the bacterial isolates on colloidal chitin agar medium. The medium consists of (g/L): colloidal chitin 10; yeast extract 0.5; (NH₄)₂SO₄ 1.0; MgSO₄•7H₂O 0.3; KH₂PO₄ 1.36; NaCl 30; agar 15; and pH 7.0. The colonies showing clearance zones calculated the chitinolytic index after three days of incubation. The chitinolytic index was calculated using the equation:

$$\text{Chitinolytic index} = \frac{\text{Diameter of the clear zone} - \text{diameter of the colony}}{\text{Diameter of the colony}}$$

Five potential isolates with highest chitinolytic activity were tested for their activity against fungi.

2.4. Antagonistic activity of isolates against fungi

The activity of five chitinolytic *Bacillus* isolates against fungi was studied by dual culture technique. [5] Briefly, a culture of the bacterial isolate was a streak at the middle of the PDA plate and incubated at 28°C for 24 hours. A mycelial plug of 5 mm diameter from 5 days old of each fungus was cut and transferred a cm from *Bacillus*-pregrown PDA plate. The experiments were performed on three replications. The growth of fungi on plates was observed after 5 days of incubation at 30°C. Percentage of mycelial growth inhibition of fungi was calculated using the equation:

$$\text{Percentage inhibition (\%)} = 100 \times \frac{R1 - R2}{R1}$$

Where R1 is the radial fungal growth measured from the center of the mycelial disc to direction away from the bacterial streak and R2 is the radial of fungal growth measured from the center of the mycelial disc towards the bacterial streak.

2.5. Characteristics of *Bacillus* isolates

The selected *Bacillus* isolate was characterized by its morphological, physiological and biochemical features according to Bergey's Manual of Systematic Bacteriology [6].

3. Results and Discussion

3.1. Screening of chitinase producing bacteria

Based on the chitinolytic index, 14 isolates or 16.9% from 83 samples of isolates showed chitinolytic activity with isolate B26 as highest chitinolytic activity (**Table 1**). As a comparison, 4% marine bacterial isolates reported exhibited clear zones from a flake-chitin medium. [7] 74.07% bacteria isolated from soil samples was reported as chitinolytic bacteria which produced clear zone in chitin medium. [8] The low genomes diversity of chitinase-producing bacteria found in the marine environment is due to the abundance of sources of dissolved organic and organic materials. [9] A total of 256 bacterial genomes

from terrestrial habitats, 74 genomes fulfilled the criteria to be considered as chitinase-containing. For aquatic habitats, obtained 36 chitinase-containing bacterial genomes out of a total of 401 bacterial genomes. The difference in relative abundance of chitinase-containing genomes between aquatic and terrestrial bacteria could indicate that the possession of chitinase genes is more common for terrestrial bacterial species than for aquatic one. [10]

Table 1. Chitinolytic bacterial isolates with their chitinolytic index values

Isolates	Chitinolytic index
B26	0.63
B07	0.59
B19	0.58
B10	0.48
B20	0.44
B18	0.40
B02	0.38
B01	0.36
B09	0.28
B12	0.26
B04	0.24
B13	0.18
B16	0.12
B40	0.06

3.2. Antagonistic activity of *Bacillus* isolates against fungi

Table 2 showed the antagonistic activity of selected *Bacillus* isolates. Results showed that the maximum growth inhibition exhibited by B26 and B10 was 69% and 65.5% respectively, was recorded against *F. solani* TISTR 3436 after 5 days of incubation. The other three bacterial strains i.e. B19, B20, and B07 caused 63.6%, 63.3%, and 56.2% growth inhibition of *F. solani* TISTR 3436 respectively. Five bacterial strains showed positive antagonistic activity against *P. chrysogenum* with maximum growth inhibition was 46.6% and none of the bacterial strains were positive against *A. niger* and *A. flavus*. Antagonistic activity of *Bacillus* sp. B26 against *F. solani* and *P. chrysogenum* are shown in **Figure 1**. Some bacteria have been known to have antagonistic activity by producing antibiotic compounds, such as iturin, bacillomycin, surfactin, fengycin, and bacteriocins [11] and extracellular hydrolytic enzymes such as chitinase. Chitinase production by *Bacillus cereus* [12] and *Bacillus subtilis* [13] reported able to inhibit the growth of *F. solani* and *P. chrysogenum* respectively. In addition, the activity of bacterial antagonism can also be through the competition of nutrients, direct contact with pathogenic hyphae, and producing enzymes that can break down cell walls. [14]

Table 2. Antagonistic activity of chitinolytic bacterial isolates against fungi

Bacterial isolates	Mycelial growth inhibition (%*)			
	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Fusarium solani</i>	<i>Penicillium chrysogenum</i>
	TISTR 3130	TISTR 3041	TISTR 3436	
B07	0	0	56.2±5.1	38.2±3.1
B10	0	0	65.5±4.1	42.7±4.0
B19	0	0	63.6±3.2	41.3±3.2
B20	0	0	63.3±5.2	41.2±4.7
B26	0	0	69.0±7.1	46.6±5.9

*Values are expressed as mean ± S.D. from three replications

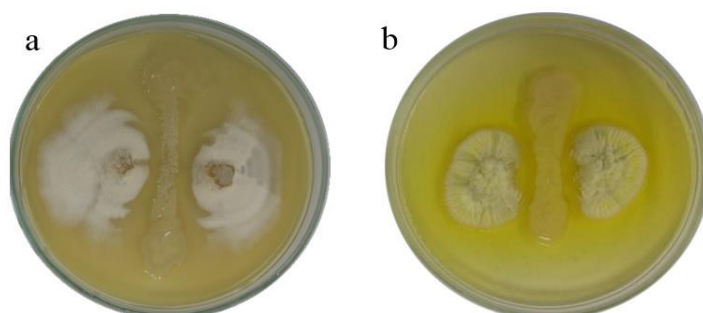


Figure 1. Antagonistic activity of *Bacillus* isolate B26 against a) *Fusarium solani* and b) *Penicillium chrysogenum* after 5 days of incubation at 30°C

3.3. Characteristics of *Bacillus* isolate

Some characteristic of *Bacillus* isolate B26 based morphological, physiological, biochemical tests showed isolate B26 was gram-positive, motile, spore-forming and showed a positive result for the urease production, catalase test, starch hydrolysis, casein hydrolysis with most suitable growth condition at 37°C, pH 7-8, and 2% of NaCl concentration (**Table 3**). The isolate B26 showed negative results for the citrate utilization, oxidase test, and voges-proskauer reaction.

Table 3. Characteristics of *Bacillus* isolate B26

Types of test	Parameters	Colony properties	Isolate B26
Morphology test		Color	Creamish
		Gram's reaction	Positive
		Cellular morphology	Bacilli
		Spore (s)	Positive
		Motility	Positive
Physiological tests	Growth at the temperature (°C)	18	+
		22	++
		37	+++
		50	++
		55	+
	Growth at pH	5	+
		6	++
		7	+++
		8	+++
		9	++
	Growth on NaCl (%)	0-5 %NaCl	Positive
		Optimum (%NaCl)	2
Biochemical tests		Citrate utilization	Negative
		Urease production	Positive
		Oxidase test	Negative
		Catalase test	Positive
		Starch hydrolysis	Positive
		Voges-Proskauer reaction	Negative
		Casein hydrolysis	Positive
Utilization of carbohydrates		Arabinose	Positive
		Lactose	Positive
		Maltose	Positive
		Glucose	Positive
		Sucrose	Positive
		Galactose	Positive
		Mannitol	Positive
		Fructose	Positive

+++ = highest growth, ++ = good growth, + = average growth

4. Conclusions

This study was screened chitinolytic bacteria isolated from marine samples on colloidal chitin agar medium and tested their antagonistic activity against fungi. Based on the chitinolytic index, the *Bacillus* isolate B26 was considered the potent isolate. It produced chitinase and had antagonistic activity against fungal strains, especially phytopathogenic *F. solani* TISTR 3436. Moreover, *Bacillus* isolate B26 could grow at NaCl concentration of 0-5%. This study indicates that *Bacillus* isolate B26 from marine had some useful characteristics supporting the use in further development for biological control.

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