Biosurfactant as a microbial pesticide

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Abstract

Soil-borne infectious disease including *Pythium aphanidermatum* and *Rhizoctonia solani* causes severe damage to plants, such as cucumber. This soil-borne infectious disease was not controlled effectively by chemical pesticide. Since these diseases spread through the soil, chemical agents are usually ineffective. Instead, biological control, including antagonistic microbe can be used as a preferred control method.

An efficient method was developed to select an antagonistic strain to be used as a biological control agent strain. In this new method, surface tension reduction potential of an isolate was included in the 'decision factor' in addition to the other factors, such as growth rate, and pathogen inhibition rate. Considering these 3 decision factors by a statistical method, an isolate from soil was selected and was identified as *Bacillus* sp. GB16. In the pot test, this strain showed the best performance among the isolated strains. The lowest disease incidence rate and fastest seed growth was observed when *Bacillus* sp. GB16 was used. Therefore this strain was considered as plant growth promoting rhizobacteria (PGPR). The action of surface tension reducing component was deduced as the enhancement of wetting, spreading, and residing of antagonistic strain in the rhizosphere. This result showed that new selection method was significantly effective in selecting the best antagonistic strain for biological control of soil-borne infectious plant pathogen.

The antifungal substances against *P. aphanidermatum* and *R. solani* were partially purified from the culture filtrates of *Bacillus* sp. GB16. In this study, lipopeptide possessing antifungal activity was isolated from *Bacillus* sp. GB16 cultures by various purification procedures and was identified as a surfactin-like lipopeptide based on the Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), high performance liquid chromatography mass spectroscopy (HPLC-MS), and quadrupole time-of-flight (Q-TOF) ESI-MS/MS data. The lipopeptide, named GB16-BS, completely

inhibited the growth of *Pythium aphanidermatum, Rhizoctonia solani, Penicillium* sp., and *Botrytis cineria* at concentrations of 10 and 50 mg/L, respectively.

A novel method to prevent the foaming and to provide oxygen was developed. During the production of surface active agent, such as lipopeptide (surfactin), large amount of foam was produced by aeration. This resulted in the carryover of cells to the outside of the fermentor, which leads to the significant loss of cells. Instead of using cell-toxic antifoaming agents, low amount of hydrogen peroxide was added. Catalase produced by cells converted hydrogen peroxide into oxygen and water. Also addition of corn oil as an oxygen vector as well as antifoaming agent was attempted. In addition, Ca-stearate, a metal soap, was added to enhance the antifoam activity of corn oil. These methods could prevent the foaming significantly and maintained high dissolved oxygen in spite of lower aeration and agitation. Using these methods, high cell density, could be achieved with increased lipopeptide productivity.

In conclusion to produce an effective biological control agent for soil-borne infectious disease, following strategies were attempted

i) effective screening of antagonist by including surface tension as an important decision factor

ii) identification of antifungal compound produced from the isolated strain

iii) novel oxygenation by H_2O_2-catalase with vegetable oil for antifungal lipopeptide production.

Introduction

The excessive use of chemical pesticides induce the occurrence of pesticide-resistant microorganisms (1), and reduction of agricultural producing potentials due to acidification of soil and decrease of indigenous antagonists in soil (2). Especially, soil-borne infectious disease is hard to control with chemical pesticides because it can be drained from soil by rain. Also there are many other side-effects such as promotion of eutrophication and bioaccumulation.

As a results, needs for biological control agent to produce nonpolluting crops have been increased. Biological control can be defined as the directed, accurate management of common components of ecosystems to protect plants against pathogens. It preserves environmental quality by a reduction in chemical inputs, and is characteristic of sustainable management practices. Also registration of commodity of biological control agent is relatively easy for chemical pesticide due to safety to non-target living organisms and environmental suitability. A lot of studies present the variety of biological control agents

comprehensively.

In 1995, world biopesticide sales (including microbial pesticides, entomopathogenic nematodes, baculoviruses, plant-derived pesticides and insect pheromones) were estimated to be around US $380 million, representing approximately 1.3% of the total market for pesticides. A recent survey on the European market alone for biopesticides predicts biopesticides sales to reach US 167 million by year 2004. The growth rate for biopesticides over the next 10 years has been forecast at 10-15% per annum, in contrast to 2% for chemical pesticides.

Pythium aphanidermatum and *Rhizoctonia solani*, which cause damping-off of cucumber seedling are the limiting factors in production of cucumber, pepper, and tomato etc. *Bacillus* sp. GB16, which showed substantial antagonistic activity against soil-borne phytopathogens, *Pythium aphanidermatum* and *Rhizoctonia solani*. The antifungal substances active against *P. aphanidermatum* and *R. solani* were semi-purified from the culture supernatant of *Bacillus* sp. GB16. However, the antifungal substances had a high surface active properties so that excessive foam was generated during conventional cultivation in jar fermentor.

In this study, we screened about 90 strains isolated from healthy soil and compost, based on 2-dimensional selection RPI (Relative Performance Indies) method, considering antagonistic activity against two phytopathogens, growth rate and surface tension reduction by biosurfactant production. As the *Bacillus* sp. GB16 showed the most effective control value and enhancement of seedling growth, using screening method in this study proved its excellence. The antifungal substances, which are active against some phytopathogenic fungi were purified from the culture broth of *Bacillus* sp. GB16 by various purification procedures. By analyzing various spectral and other physicochemical data, their chemical structures were elucidated and the compound was identified as surfactin-like lipopeptide. In addition to in vitro bioassay for antifungal activity, the control efficacy of GB16-BS against phytopathogenic fungi compared to those of commercial fungicides was evaluated. In addition, a novel integrated method, applied in oxygen supplying by hydrogen peroxide and both oxygen holding and foam control by corn oil mixed with calcium-stearate, was used for the control of massive foam occurrence during cultivation.

Materials and Methods

Antagonist screening and cultivation

Microorganisms antagonistic to *Pythium aphanidermatum* and *Rhizoctonia solani* were

isolated according to the modified Herr's method. Soil samples were collected the rhizosphere 10 to 15 cm beneath the soil surface from crop-cultivating fields at different locations in Gyeonggi-do, Korea. Compost samples were collected from several regions. Samples were stored at 4℃ in plastic bags until their use.

About 90 strains isolated from various composts and healthy soils (#1-#87) were used as candidates for antagonist. *Bacillus subtilis* ATCC21332 that produces surfactin, Kodiak (Gustafson, Texas) that is commercially available biological control agent, and *Bacillus* sp. H6 that was previously isolated by our laboratory were used as the positive control. Screening method of antagonist was considered antagonistic activity against *P. aphanidermatum* and *R. solani* causing damping-off, its viable cell number and surface tension reduction by introducing RPI for ranking strains.

Mass spectrometry

HPLC mass spectra were recorded using a triple quadrupole tandem mass spectrometer (Quattro LC, Micromass, Manchester, UK)and ESI-MS/MS data were collected using a quadrupole time-of-flight (Q-TOF) MS using a Q-TOF 2 mass spectrometer (Q-TOF2, Micromass, Manchester, UK).

Results and Discussion

Antagonist screening

Considering growth of candidate in antagonist screening, cell growth is generally presented by optical density due to easy determination. However, the increase of optical density is not proportional to the increase in viable cell number due to the difference in cell size and the presence of extracellular products such as pigments and properties of cell surface. Therefore, cell growth was measured by viable cell count in antagonist screening since the optical density is reduced by cell lysis. Two strains, #75 and #16, were selected by comparing 4 RPI averages of cell growth and inhibition rate to *P. aphanidermatum* and *R. solani* and surface tension reduction (Fig. 1)

Proposed structure

On the basis of LC-MS, ESI-MS/MS and FT-IR, the structure of biosurfactant GB16-BS produced by *Bacillus* sp. GB16 was proposed as shown in Fig. 2. The proposed structure resembled with surfactin produced by other *Bacillus* sp. Phae et al. (1990) reported that though the suppressive effects among the four *B. subtilis* isolates were slightly different, the substances produced may be presumed to be identical, while the fractions of the

components produced or the protease activities excreted may not necessarily be the same (3).

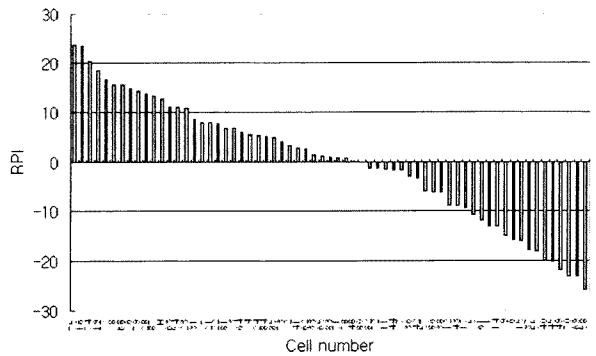

Fig. 1. Use of relative performance indices (RPI) to achieve a 2-dimensional assessment of antagonistic organism based on growth (total cell number), efficacy of cells and surface tension reduction.

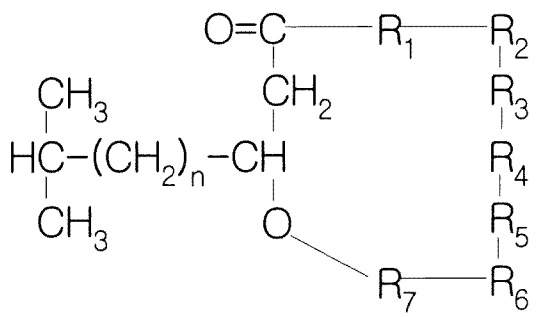

Fig. 2. Proposed structure for lipopeptide isolated from *Bacillus* sp. GB16. n=9, m/z 1022; n=10 m/z 1036; n=11 m/z 1050.

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