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Utilization of Sweat Potato Starch Wastewater and Monosodium Glutamate Wastewater for Cultivation of an Anti-Fungal Biocontrol Agent *Paenibacillus Polymyxa*

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1. Introduction

Effluents from monosodium glutamate manufacturing plants possess a high strength of COD (10,000--30,000 mg/l), ammonium (15,000--25,000 mg/l), sulphate (15,000--30,000 mg/l) and very low pH (< 2) (Yang *et al.*, 2005). Effluents from sweat potato starch industry contains a high load of protein, pectin, and starchy materials with the COD of 10,000 - 35,000 mg/l (Mishra *et al.*, 2004). Due to the low pH, high concentration of COD, sufate and NH_3-N, the treatment of such wastewaters by conventional activated sludge processes consumes a lot of energy, resulting in high treatment costs (Bai *et al.*, 2004).

Phyllosphere bacteria often have a positive influence upon plant, where they may be involved in the fixation of nitrogen, promoting the growth of plants, or the control of plant pathogens. However, some high infectivity fungi may damage many economically important crops and trees, and bring a significant risk and safety concerns for the food supplies. Although chemical control agents have been used world widely, the biological control agents have attracted a great R&D interest because of their potential for long-term application as environmental friendly agents (Ten Hoopen and Krauss, 2006). Biocontrol agents have been used to protect plants against foliar diseases in several crops (Yuen and Schoneweis, 2007; Alvindia and Natsuaki, 2008; Perello *et al.*, 2007). However, many of them are poor competitors for leaf surface nutrients compared with indigenous phyllosphere microbes (Zhang *et al.*, 2008).

Strains of *Paenibacillus polymyxa* have been isolated from different soils (Ash *et al.*, 1993), rhizospheres and roots from plants cultivated all over the world, and many of them have been described as effective plant growth promoting rhizobacteria (Pichard *et al.*, 1995; Petersen *et al.*, 1996; Lorentz *et al.*, 2006). Untill now, *P. polymyxa* has been seldom discovered in the phyllosphere. In the present work, a strain of *P. polymyxa* EBL06 was isolated from wheat phyllosphere, which could restrain the growth of the filamentous fungi. *P. polymyxa* strains

have been proved to produce a wide variety of secondary metabolites, including different antibacterial and/or antifungal compounds (von der Weid *et al.*, 2000). Therefore, the antagonistic effect of these strains upon microbial growth suggests a potential application as biological control agents. The aims of this study were to characterize the newly isolated strain of *P. polymyxa* EBL06 and optimize the production of the bacterium using the sweat potato wastewater (SPW) and the monosodium glutamate wastewater (MGW).

2. Materials and methods

2.1 Isolation and *in vitro* antagonist assays

The experiment was conducted in a native field located at the Tongzhou near Beijing City, China. Wheat cultivars were planted in the field in October, 2006, watered and fertilized in accordance to local cultivation practices. Wheat phyllosphere microbes were collected in May, 2007 according to the methods described by Zhang *et al.* (2008). An anti-fungi bacterial strain EBL06 was isolated on Potato-dextrose Agar (PDA) medium Petri dish, and maintained on the PDA slant tubes. The antagonistic activity of the strain was evaluated by the method of confronting cultures with the filamentous fungi (Foldes *et al.*, 2000; Bai *et al.*, 2008), such as *Trichoderma harzimum*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium* spp., *Macrophom* spp., which was modified as the following. A point of filamentous fungi was inoculated onto the center of PDA Petri dish; three points of isolation were inoculated at 2 cm distance from the center of the Petri dish symmetrically. The Petri dish was then incubated at 28 ^{o}C for a few days. The isolated strain was considered to be antagonistic to the filamentous fungus if it restrains the fungi growth with inhibition zone (Fig. 1).

2.2 Resistance to antibiotics of the Bacteria

Resistance to antibiotics was determined using standard antibiotic disks. Inhibition diameters were recorded after 24 h of incubation at 30 ^{o}C under aerobic conditions. The classification of the strain, as sensitive, not sensitive or intermediate sensitive to the antibiotics, was done according to the inhibition diameters. Tests were performed in triplicate.

2.3 PCR amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene

The primer set 27F-1492R was used in PCR amplification of the 16S rRNA gene fragment of isolate EBL06 under that conditions as described by Kuklinsky *et al.* (2004). The 16S rDNA was sequenced by Shanghai Sangon Co. Ltd., China. Sequence similarity searches were conducted using the National Center for Biotechnology Information BLAST network service (nucleotide blast). Similar 16S rRNA gene sequences, from previously cultured bacteria, were downloaded from GenBank and manually checked for ambiguous sites using bioedit 7.0.1 software. Alignments were then performed against the 16S rRNA gene sequence of isolate EBL06, where the pair-wise deletion option for gaps was employed. The alignment data were then used for neighbour-joining analysis with 1000 bootstrap replicates (MEGA version 4.0; Arizona State University, USA) (Li *et al.*, 2007).

2.4 Optimization of culture conditions

The SPW samples were collected from a sweat potato starch process waste stream in Changsha, China, mainly containing COD 16000 mg/l. The pH of SPW was 6.2; it was adjusted to 7.0 by NaOH when the SPW was used as the culture medium. The starch, pectin

and sugars in the SPW were used as the main carbon source throughout the investigation. The MGW samples were obtained from Henan Lianhua Monosodium Glutamate Co., Ltd., which located in Zhoukou, Henan Province, China. The MGW consisted of 1.20% total Kjeldahl-N, 1.17% NH_4^+, 4.06% SO_4^{2+} and 0.275% reducing sugar. Trace element components in the MGW were given as follows (mg/l): Ba, 0.932; Ca, 389; Co, 0.0123; Cu, 0.605; K, 259; Mg, 79.0; Pb, 0.588; Sr, 0.869; Zn, 1.80; Mn, 2.66; Fe, 4.28; Na, 794; Cr, 0.862; P, 81.4; V, 0.0082. The pH of raw MGW 1.5 and was adjusted to 6.7 by NaOH when the MGW was used as the culture medium. Ammonium in the MGW was used as the main nitrogen source.

The isolate EBL06 was grown on the PDA slants at 30 ºC for 2 days. A single clone was inoculated into 250-ml flask containing 100 ml of the PDA medium at 30 ºC for 24 h. This seed culture was used to initiate the growth in fermentation medium used in this study. The basic medium (BM) is composed of as follows (g/l): $MgSO_4 \cdot 7H_2O$ 0.10, KH_2PO_4 0.50, NaC1 0.50, and K_2HPO_4 1.50. The fermentation was conducted in 250 ml Erlenmeyer flasks containing 100 ml medium inoculated with 5 ml of seed culture. Unless otherwise was stated, the agitation rate and incubation temperature were 200 r/min and 30 ºC, respectively.

All experiments were conducted in duplicate and the average values are reported. Key results were repeated three times to establish their validity.

3. Results

3.1 Morphology and antagonistic fungus activity of the isolate EBL06

Microscopic observation of the isolate EBL06 is a mesophilic, Gram-positive and motile bacterium, and cells are rod-shaped with peritrichous flagella in overnight culture in PD medium at 30 ºC and 150 r/min using Light-microscophy. The cells are found singly, double, and chains. Colonies of the strain on PDA are slightly yellow, circular, smooth, convex, semi-transparent and 2--3 mm in diameter with an entire margin after incubation for 48 h at 30 ºC.

EBL06 showed a significant antagonistic activity towards fungal species of *Cladosporium cucumerinum*, *Trichoderma harzimum*, *Botrytis cinerea*, *Fusarium* spp. and *Macrophom* spp. on PDA plates. Figure 1 shows two images of the test process.

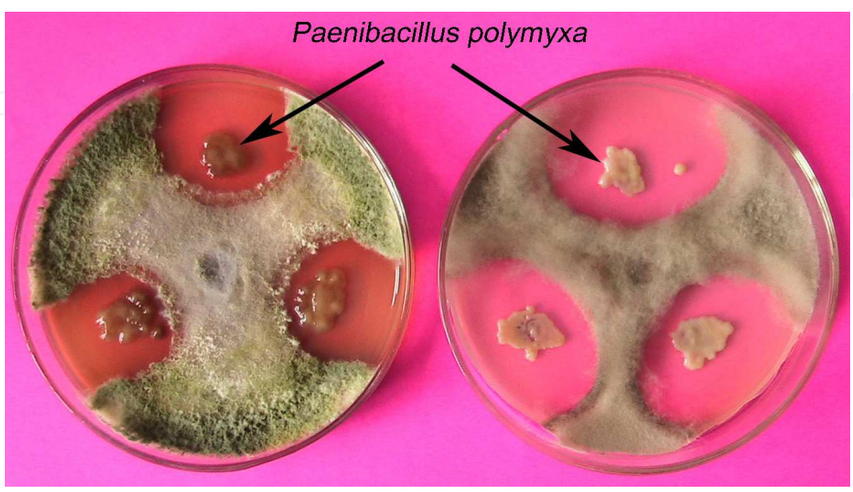

Fig. 1. Growth impact of the isolated EBL06 on the *Trichoderma harzimum* (left) and *Botrytis cinerea* (right) at the PDA medium.

3.2 Resistance to antibiotics of the isolate EBL06

The growth behaviour of the isolate EBL06 was studied in the presence of a range of antibiotics. The strain was susceptible to penicillin, streptomycin, kanamycin and tetracycline, and was weakly susceptible to chloramphenicol, and resistant to polymyxine and colistine.

3.3 Phylogenetic analysis

To analyze the phylogenetic position, the 16S rDNA sequence of the isolate EBL06 was determined, and a phylogenetic tree was constructed (Fig. 2). The sequence was deposited in the GenBank database under the accession number EF545556. The phylogenetic analysis indicated that the isolate EBL06 is most closely related to species of *P. polymyxa*.

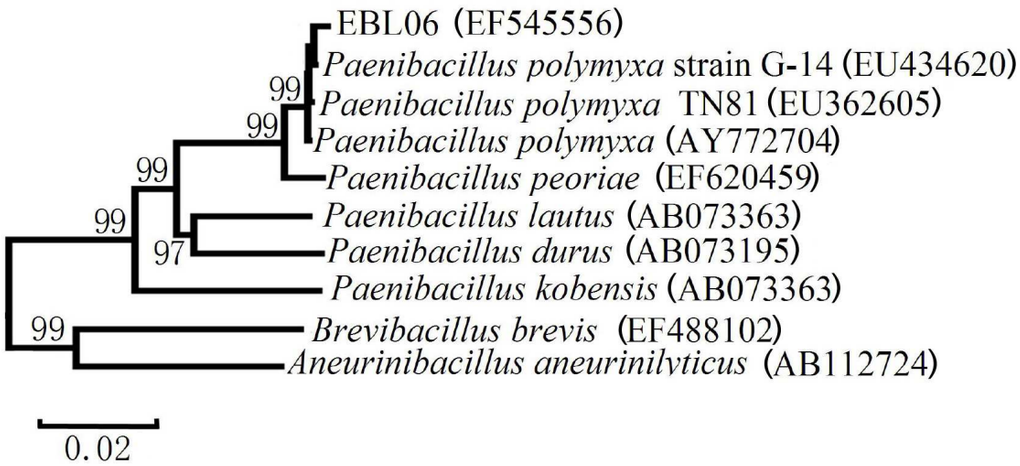

Fig. 2. Neighbor-joining trees showing the phylogenetic position of the isolate EBL06 and its related species based on 16S rRNA gene sequences. The GenBank accession number for each microorganism used in the analysis is shown after the species name. Bootstrap values (expressed as a percentage of 1000 replications) greater than 50% are shown at the branches.

3.4 Effect of carbon sources on the isolate EBL06 growth

The fermentation medium contained 10 ml MGW, 90 ml BM, and 2% each carbon sources, including sugar, D-glucose, soluble starch, and SPW (90 ml, the carbon source equal to 2 g starch, no BM). After inoculation with 2 ml of inoculum, the medium was incubated at 30 ^{o}C for 20 h. The effect of carbon sources on the production of the isolate EBL06 are presented in Fig. 3. It was found that SPW are the most suitable carbon source.

3.5 Effect of nitrogen sources on the isolate EBL06 growth

The fermentation medium contained 90 ml SPW as carbon source, 10 ml BM, and 0.5% different nitrogen sources, including corn steep liquor, potassium nitrate, ammonium sulfate and MGW (10 ml, the nitrogen source equal to 0.5 g $(NH_4)_2SO_4$, no BM). A control experiment was conducted without addition of nitrogen source. After inoculation with 2 ml of inoculum, the medium was incubated at 30 ^{o}C for 20 h. The results of impact of nitrogen sources on the production of the isolate EBL06 are presented in Fig. 4. It was found that corn steep liquor and MGW were the most efficient nitrogen sources for production of the strain. Comparing with the corn steep liquor, MGW are the most suitable nitrogen source due to the low cost.

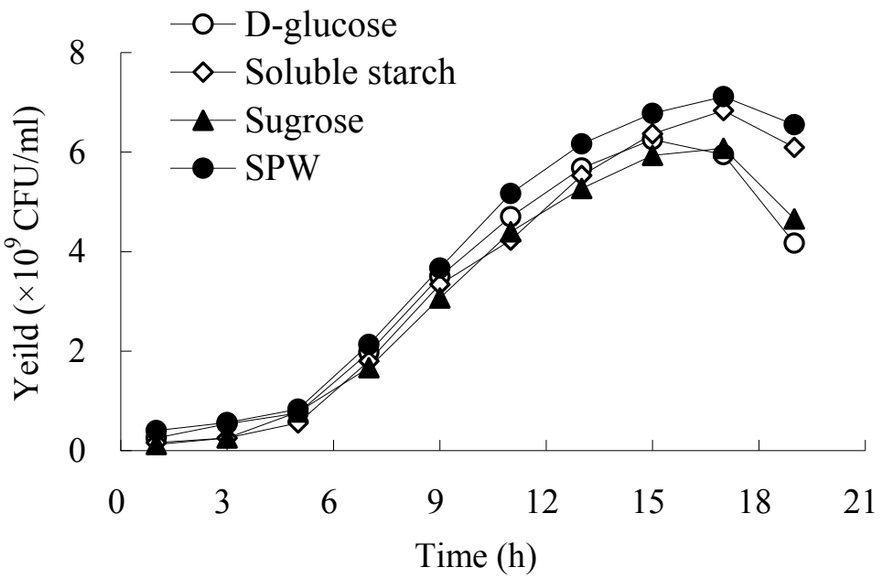

Fig. 3. Effect of different carbon sources on the isolate EBL06 growth.

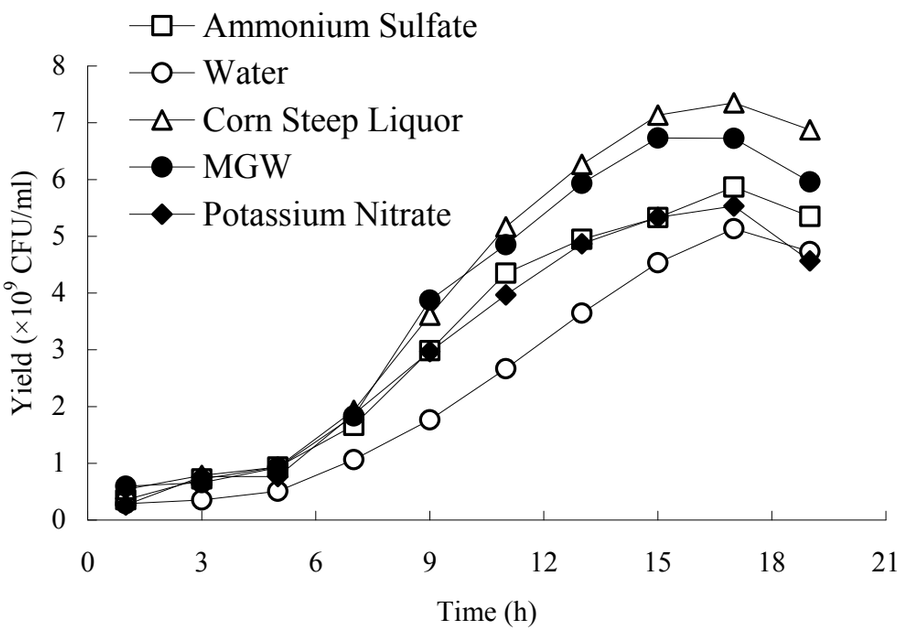

Fig. 4. Effect of different nitrogen sources on the isolate EBL06 growth.

3.6 Effect of pH on the isolate EBL06 growth

The fermentation medium contained 10 ml MGW, 90 ml SPW. After inoculation with 2 ml of inoculum, the medium was incubated at pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 respectively. The

isolate EBL06 production at different pH is shown in Fig. 5. The highest yield could be given at pH 6.5--7.0 after 15 h fermentation. Consequently, pH 7.0 was selected in the following experiment.

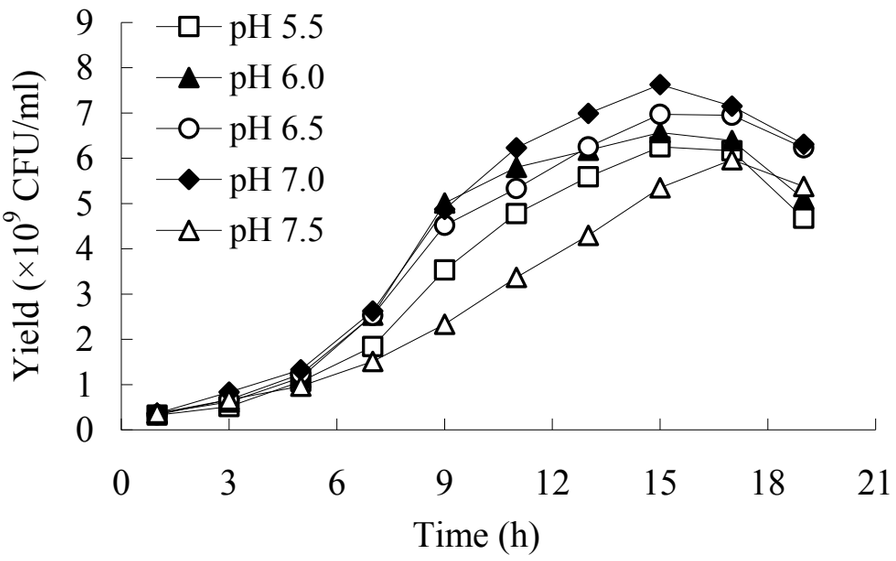

Fig. 5. Effect of initial culture medium pH on the isolate EBL06 growth.

3.7 Effect of culture temperature on the isolate EBL06 production

The fermentation medium contained 10 ml MGW, 90 ml SPW. After inoculation with 2 ml of inoculum, the medium was incubated at 24, 28, 32, 36 and 40 °C, respectively. The time courses of the isolate EBL06 production at different temperature are shown in Fig. 6. The maximum yield of 7.3×10^9 CFU/ml was reached at 32 °C after 15 h fermentation.

4. Disscussion

P. polymyxa endospore was reported to be resistant to desiccation, heat, and UV irradiation, and have excellent biochemical characteristics that allow for further formulation and commercialization procedures. Previous studies have shown that strains of *P. polymyxa* can produce different peptide antimicrobial substances (Rosado and Seldin, 1993; Piuri et al., 1998; Dijksterhuis et al., 1999; Seldin et al., 1999). The peptide metabolites are generally classified into two groups according to their antimicrobial activities. The first group includes the polypeptins, polymyxins, jolipeptin, gavaserin, and saltavalin, which showed antibacterial activity against both gram-negative and gram-positive bacteria. The second group consists of a single family of closely related peptides variously designated gatavalin, fusaricidins, all of which contain an unusual fatty acid side chain, 15-guanidino-3-hydroxypentadecanoic acid (Raza *et al.*, 2009). As a phyllosphere isolate, *P. polymyxa* EBL06 could be suitable to survive in the phyllosphere conditions; the antagonistic fungi ability also give them a growth advantage in the competitive environment. *P. polymyxa* EBL06 showed a significant antagonistic activity towards all the filamentous fungi tested. It will be used as a potential biocontrol agent for protecting plant against fungal disease in further study.

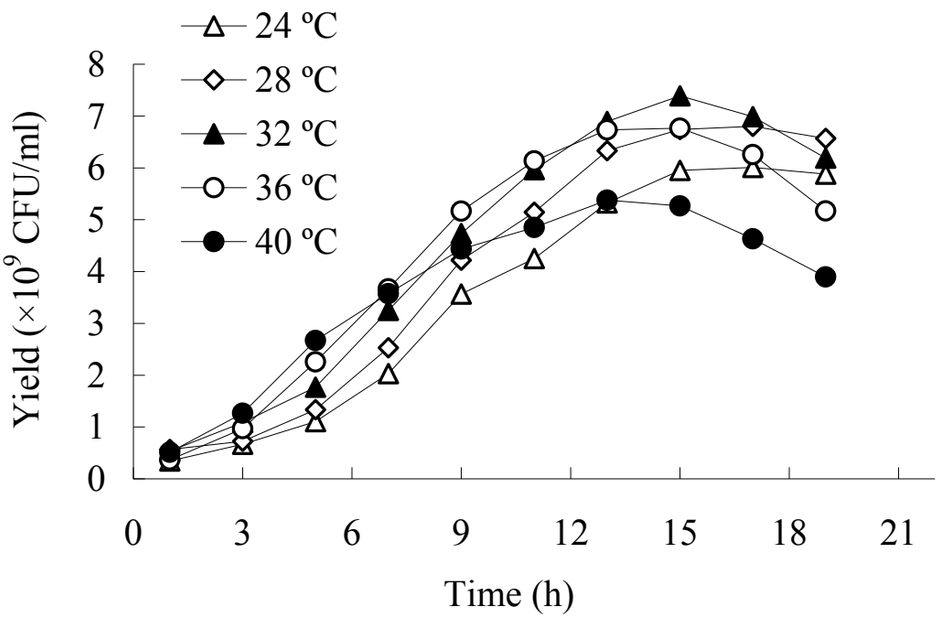

Fig. 6. Effect of culture temperature on the isolate EBL06 growth

MGW is one of the most intractable wastewater because of its high strength of COD, ammonium and sulphate (Yang *et al.*, 2005). SPW also contains high COD and nutrient loadings. A few studies have already focused on the utilization of such high organic loading wastewater as substrates or production media in fermentation processes (Bai *et al.*, 2004; Mishra *et al.*, 2004; Huang *et al.*, 2005). As far as the authors are aware, no papers have been published on utilization of both MGW and SPW in one process. In the present study, the isolate EBL06 could use well the starch in the SPW as the main carbon source, and ammonium in the MGW as the main nitrogen source. In addition, the nutrient and trace element components in the wastewaters are also suitable for the isolate EBL06 growth. Compared with other culture media, the mixture of SPW and MGW is the most suitable for the biocontrol agent production from the economic and environmental point of view.

P. polymyxa has also been isolated from several places such as food, rhizosphere, poultry production environments, soils and most of them can restrain fungal pathogen growth (He *et al.*, 2007; Raza *et al.*, 2009; Svetoch *et al.*, 2005). Further studies will investigate the anti-fungal pathogen activity of the isolate EBL06 both in phyllosphere and in rhizosphere by field trials. Further understanding of the survival strategy of the isolate EBL06 in phyllosphere might improve the efficiency of the biological treatments and also lead to enhanced yields of agricultural crops.

In conclusion, a newly isolated *P. polymyxa* EBL06 from wheat phyllosphere can be used as a nonchemical alternative biocontrol agent against plant disease caused by fungal pathogen. A novel process for economical production of *P. polymyxa* biocontrol agent was developed using MGW and SPW. It is feasible to develop a hybrid biotechnological process, integrating the production of environmental friendly biocontrol agent with treatment of intractable wastewater.

5. Acknowledgments

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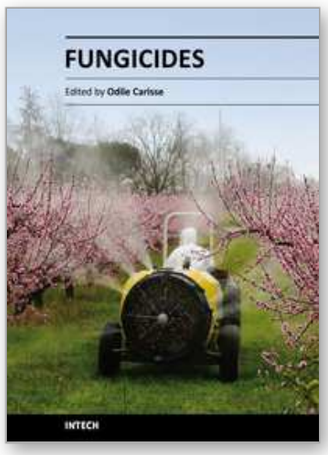

Fungicides

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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