1-1-2011

Functional Characterization Of The Ocfc Gene And Optimization Of Medium Components And Culture Condition For Occidiofungin Production By Burkholderia Contaminans Strain Ms14

Kuan-Chih Chen

FUNCTIONAL CHARACTERIZATION OF THE *OCFC* GENE AND OPTIMIZATION OF MEDIUM COMPONENTS AND CULTURE CONDITION FOR OCCIDIOFUNGIN PRODUCTION BY *BURKHOLDERIA CONTAMINANS* STRAIN MS14

By

Kuan-Chih Chen

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master
in Life Sciences
in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology

Mississippi State, Mississippi

December 2011

FUNCTIONAL CHARACTERIZATION OF THE *OCFC* GENE AND OPTIMIZATION OF MEDIUM COMPONENTS AND CULTURE CONDITION FOR OCCIDIOFUNGIN PRODUCTION BY *BURKHOLDERIA CONTAMINANS* STRAIN MS14

By

Kuan-Chih Chen

Approved:

Shi-En Lu Associate Professor of Plant Pathology (Director of Thesis)	Sead Sabanadzovic Associate Professor of Plant Pathology (Committee Member)
James Smith Assistant Professor of Biology Texas A&M University (Committee Member)	
Michael A. Caprio Professor of Entomology (Graduate Coordinator)	George M. Hopper Interim Dean, College of Agriculture and Life Sciences

Name: Kuan-Chih Chen

Date of Degree: December 9, 2011

Institution: Mississippi State University

Major Field: Life Sciences

Major Professor: Shi-En Lu

Title of Study: FUNCTIONAL CHARACTERIZATION OF THE *OCFC* GENE AND OPTIMIZATION OF MEDIUM COMPONENTS AND CULTURE CONDITION FOR OCCIDIOFUNGIN PRODUCTION BY *BURKHOLDEIA CONTAMINANS* STRAIN MS14

Pages in Study: 55

Candidate for Degree of Master

The 56-kb *ocfC* gene cluster is required for occidiofungin production by *Burkholderia contaminans* strain MS14. However, the function of the *ocfC* gene remains unknown. Sequence analysis showed the putative protein encoded by *ocfC* shares significant identities to glycosyltransferase. Plate bioassays showed that occidiofungin production by the *ocfC* mutant (*ocfC*::*nptII*) was significantly reduced as compared with strain MS14. The occidiofungin produced by the *ocfC* mutant is xylose-free, which suggests the *ocfC* gene encodes a xylosyltransferase to add a xylose to the peptide backbone. Single variant optimization of culture condition and medium compositions was also performed in this study. The results indicated that occidiofungin production was promoted with higher cell density inoculum, additional casamino acid, xylose, urea, zinc ions, and at pH 5. The findings have provided insights into development of pharmaceutical drug and agricultural biofungicide.

DEDICATION

I would like to dedicate this research to my lovely family: my parents, Ming-Jen Chen and Tsui-Hui Lee, and my brother, Kuan-Wei.

ACKNOWLEDGEMENTS

I would like to express my appreciation to my major professor, Dr. Shi-En Lu, for his patience, invaluable advice, encouragement, friendship, fanatical support and spending time and effort in reviewing the thesis. I also own thanks to my committee members, Dr. Sead Sabanadzovic (EPP-MSU) and Dr. James Leif Smith (Department of Biology, Texas A&M University) for their assistance and guidance during my study.

My sincere appreciation is to all the faculty members, especially Dr. Richard Baird, Dr. Alan Henn, and Dr. Zenaida Magbanua staff and graduate students, Mr. Mark Alexander, Mr. Paul Scott, Mr. Christian Stephenson, and Ms. Alicia Wood-Jones in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology for the friendship and assistance. Many thanks go to my dedicated lab mates, Dr. Ganyu Gu, Ms. Sonya Baird, Ms. Nisma Mujahid, Mr. Zirui Gu, and Mr. Deng Peng for their friendship and assistance.

My special gratitude goes to all my friends in Taiwan and the United States, especially to Dr. Chuan-Yu Hsu, Dr. Chien Yu, Mr. Jeng-Yang Wu, Mr. Chia-Ching Chu, Ms. Hui-Wen Tsai and Ms. Yu-Ting Chen. They always give me their care, support, friendship to help me go through everything. Finally, I want to thank to my parents and brother for their support and endless love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

GENERAL INTRODUCTION

Biological control

Biological control is the use of natural or modified organisms, genes, or gene products to reduce the effects of undesirable organisms such as plant pathogens and to favor desirable organisms such as crops (5). Classical biological control, referred to as bio-control, can be categorized into three strategies (22). First, regulation of pest population is to manage pest population at or under an acceptable population level. Second, exclusionary systems of protection use beneficial microorganisms to prevent infection or pest attack. The third, systems of self-defense, is used when regulation or pest population and exclusionary systems of protection are not possible. This strategy could enhance self- defense in the plant that could limit disease severity or pest damage (22).

Three bio-control agents are described by Cook as follows (22). First, the pest or pathogen is applied against itself. For example, sterile males or modify the behavior of insects with pheromones was released to regulate the population (Strategy 1). Second, antagonists or enemies of pest or disease agents are used to exclude the pest or pathogen. For instance, using rhizobacteria provides an exclusionary system of protection from target pathogens (strategy 2) (5). One well-known example of this is *Agrobacterium*

radiobacter strain K84 for bio-control of crown gall caused by *Agrobacterium tumefaciens* (21). Last, host plants are managed or manipulated to defend themselves. Plants can be managed culturally or genetically in order to maximize the physiological or biological systems of self-defense of the plant (Strategy 3).

According to Herrera- Estrella and Chet, there are several mechanisms to apply bio-control of plant disease (11). The first mechanism is to induce resistance and cross-protection of the plant. de Wit stated that susceptible plant response to challenge by microorganisms or abiotic agents is the way to induce resistance. He also mentioned that cross-protection is to follow the inoculation with an avirulent or weak strain of pathogen (7). After that, both the pathogen and inducing microorganisms will survive in the plants. Application of a weak strain of *Fusarium oxysporum* f. sp. *vasinfectum* is one of the examples of cross-protection against pathogenic *Fusarium oxysporum* f. sp. *vasinfectum* (12). The second mechanism is hypovirulence, defined as a strain of pathogen with reduced virulence. A hypovirulent strain of *Cryphonectria parasitica* was used against chestnut blight (1). Third, competition happens when space or nutrition is limited. For example, there would be bio-control agent near the root may protect it by using space and nutrition resources and making them unavailable to a pathogen. Fourth, an antibiotic is a compound to inhibit the growth of a pathogen. For instance, zwittermicin produced by *Bacillus cereus* UW85 can be used for bio-control agent for *Phytophothora* root blight (24). Fifth, antibiosis is antagonism with metabolites of fungal antibiotics or antibiotic-like compounds or some toxic compounds (10). Last, bio-control has some advantages over chemical pesticides for soilborne disease. *Pseudomonas fluorescens* has been used

for soil-borne disease management because of its ability to colonize roots and produce antifungal metabolites (13,15).

Optimization of antifungal compound production

Antifungal compounds produced by bacteria may be used as biological control agents. However, the production of antifungal compounds in standard culture conditions is relatively low. Optimization of the antifungal compound production is routinely used for increasing the yields of these compounds. For example, iturin A is a kind of lipopeptide antibiotic produced by *Bacillus subtilis* RB14-CS that showed antagonistic activities against some plant pathogens (19). Solid-state fermentation (SSF) with soybean residue was used to optimize the production of iturin A. By using SSF, the production of iturin A reached 3,300 mg/kg, about a ten-fold compared to the original method (19). By using experimental design to find out the combination of 0.998g per wet okara glucose and 1.83g per wet okara soybean meal, the concentration of iturin A was 5,591 mg/kg (20). Cyclosporin A, a cyclic antibiotic, is produced by *Tolypocladium inflatum* MTCC 557 (3). It showed not only immunosuppression but also antifungal activities. Solid state fermentation was used to enhance the production of Cyclosporin A so that the concentration of Cyclosporin A was five times that of the traditional culture method (25). Carnobacteriocins produced by *Carnobacterium maltaromaticum* CP5 are potential food preservative agents (14). Heterologous expression in *Escheridia coli* was used to optimize the production of carnobacteriocins from 100 μg/L to 320 mg/L.

The *Burkholderia* bacteria

The genus *Burkholderia* used to be part of the genus *Pseudomonas*. In 1992, Yabuuchi et al. split a few species in *Pseudomonas* by rRNA group II to form the new genus *Burkholderia* with *B. cepacia* as the type species (28). In general, the 16S rDNA sequence is used to differentiate the bacteria (27). However, the sequence for the *recA* gene is used to differentiate the *Burkholderia* bacteria to species level. This is because 16S rDNA could not provide enough sequence variation to differentiate some genomovars of *Burkholderia* (23).

The bacteria *Burkholderia* bacteria exist in water, soil, and the rhizosphere. These *Burkholderia* could be potential bio-control, bio-mediation, and plant-growth-promoting agents. However, some of the *Burkholderia* bacteria are also opportunistic pathogens of human cystic fibrosis patients (23). For this reason the U.S. Environmental Protection Agency (EPA) had prohibited the further application of *Burkholderia* (23). Therefore, understanding these bacteria at the molecular level might provide important clues to applying antifungals as potential human and animal drugs.

Burkholderia contaminans

Burkholderia contaminans is a species discovered as a sample contaminant (18). *B. contaminans* strains are Gram-negative, aerobic, non-sporulating rods. Colonies on the plates are moist and most strains show yellow pigment. It is reported that this bacterium could grow at 30, 37 and 42 °C and its G+C content is 67% (26). These strains are mostly isolated from clinical samples and some strains are from natural environments. The type strain J2956 was found from sheep milk in Spain (2).

The *ocf* gene cluster

The *ocf* gene cluster was identified from *Burkholderia contaminans* strain MS14 and also existed in other strains such as *Burkholderia ambifaria* strain AMMD (8). The size of whole gene cluster is 56 kb and 16 ORFs (or genes) were identified (Fig. 1.1) (8). The genes *ambR1* and *ambR2* encode two LuxR regulatory proteins (9). The genes *ocfD*, *ocfE*, *ocfF*, *ocfH*, and *ocfJ* encoded non-ribosomal peptide synthetases and are required for biosynthesis of occidiofungin (9). The *ocfA* gene was predicted to code for an ATP-binding cassette (8). The *ocfC*, *ocfK*, *ocfL*, *ocfM*, and *ocfN* genes were predicted to be involved in modification of occidiofungin (8).

Occidiofungin

Occidiofungin (fungus killers) is an antifungal compound produced by *Burkholderia contaminans* strain MS14 (16). It was a cyclic glycopeptide made up of eight amino acids and one xylose (16). Four variants, named as occidiofungin A, B, C and D have been identified from the MS14 strain culture (Fig. 1.2) (8). Occidiofungin inhibits the growth of a broad range of fungal pathogens and its mode of action was predicted to be disruption of biosynthesis of the fungal cell wall (17). The compounds have shown the great potential for pharmaceutical and agricultural applications.

Glycosyltransferase

Glycosyltransferase is a group of enzymes which catalyzes the transfer of a monosaccharide unit from a glycosyl donor to a glycosyl acceptor molecule.

Classification of glycosyltransferase is based on the amino acid sequence similarities except in families 19, 22, 24, 25 and 26 (4). In 1997, 26 glycosyltransferase families were found based on this classification. However, since the sequence of genomes for eukaryotes organism, the growth of glycobiology, and the extension of integrated glycosyltransferase, the number of the glycosyltransferase sequences has increased enormously(6). Currently, there are 94 families for glycosyltransferases based on the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/). Many glycosyltransferases have not been well studied. For *Burkholderia ambifaria* AMMD, there are 79 sequences for glycosyltransferase. Of these there are 19 ORFs in family 2 and 24 ORFs in family 4. For *Burkholderia glumae* BGR1, there are 69 known glycosyltransferase sequences with 21 sequences belonging to families 2 and 4. Thus, for *Burkholderia ambifaria* AMMD and *Burkholderia glumae* BGR1, the majority of the glycosyltransferases belong to families 2 and 4.

Significance of the study

Burkholderia contaminans MS14 isolated from a soil in Mississippi (17) produced the glycopeptide antifungal compound occidiofungin (8, 16).

This study focuses on the function of one of the genes in *ocf* gene cluster, the *ocfC* gene, which is predicted to add xylose to oligopeptide to form occidiofungin. This study also shows that optimization of medium components and culture conditions can promote occidiofungin production by novel strain MS14. Findings of this work provide a solid basis for further studies to enhance the compound production by genetic manipulations and optimization of culture conditions and medium components. Mass

production of the antifungal compound will be extremely useful for further studies of this compound as a pharmaceutical drug and as an agricultural biofungicide.

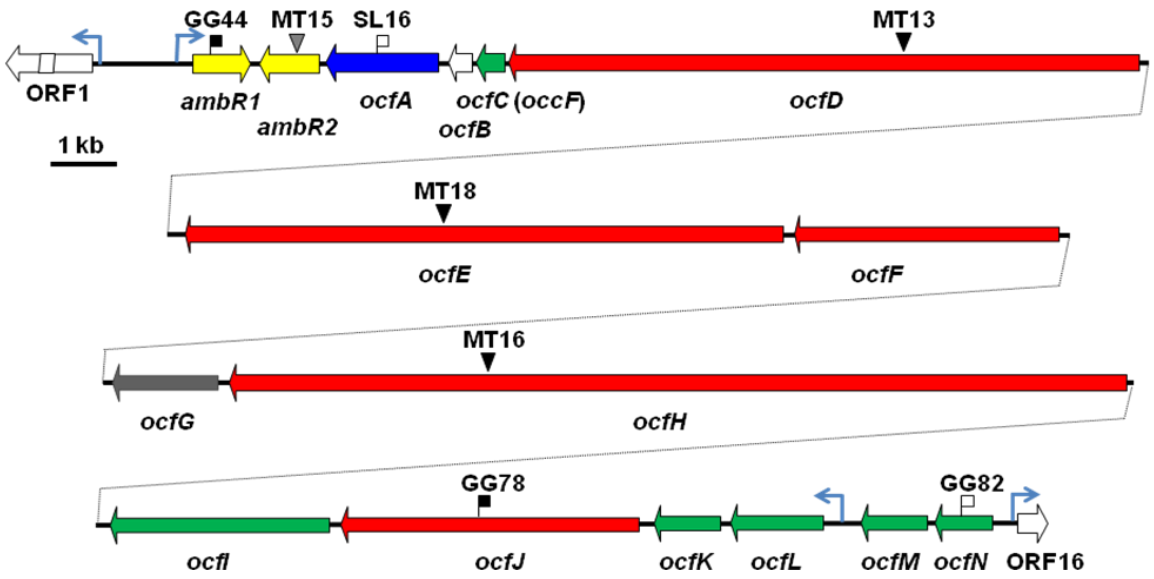

Figure 1.1 Map of 56-kb occidiofungin gene cluster of *Burkholderia contaminans* strain MS14 (8)

Horizontal arrows indicate the positions and orientation of the known genes. The insertion positions of the Tn5 transposon and the *nptII* gene cassette are showed as vertical arrows and flags, respectively. Reduction and elimination of antifungal activity are presented as the open and solid vertical arrows and flags, respectively.

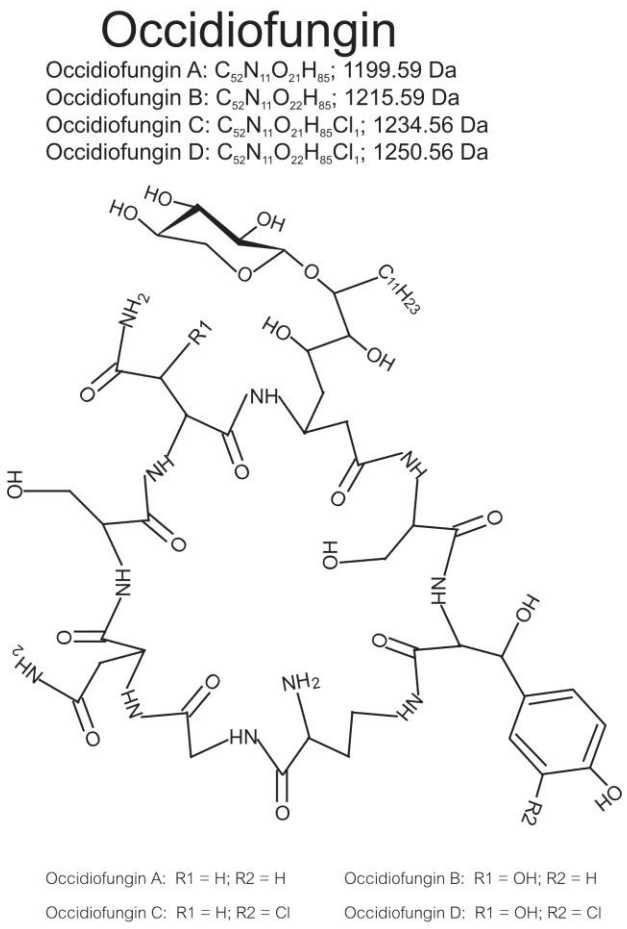

Figure 1.2 Structure of occidiofungins

Monoisotopic masses for occidiofungin variant A to D are listed and the location of each variant designated by R1 and R2 (8).

References

1. **Anagnostakis, S. L.** 1982. Biological control of chestnut blight. Science **215**:466-471.

2. **Berriatua, S., P. Vanamme, and J. R. Goven**. 2001. Outbreak of sublinical mastitis in a flock of dairy sheep associated with *Burkholderia cepacia* complex infection. J. Clin. Microbiol **39**:990-994.

3. **Borel, J. F.** 1986. Cyclosporin-A and its future. Progr. Allergy **38**:9-18.

4. **Campbell, J. A., G. J. Davies, V. Bulone, and B. Henrissat**. 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. J. Biochem. **326**:929-939.

5. **Cook, R. J.** 2000. Advances in plant health management in the twentieth century. Annu. Rev. Phytopathol. **38**:95-116.

6. **Coutinho, P. M., E. Deleury, G. J. Davies, and B. Henrissat**. 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. **328**:307-317.

7. **de Wit, P. J. G. M.** 1987. Specificity of active resistance mechanisms in plant-fungus interactions, p. 1-24. *In* Fungal infection of plants. University Press, Cambridge, UK.

8. **Gu, G., L. Smith, A. Liu, and S. E. Lu**. 2011. A genetic and biochemical map for the biosynthesis of occidiofungin, an antifungal produced by *Burkholderia contaminans* strain MS14. J. Appl. Environ. Microbio. **77**:6189-6198.

9. **Gu, G., N. Wang, N. Chaney, L. Smith, and S. E. Lu**. 2009. AmbR1 is a key transcriptional regulator for production of antifungal activity of *Burkholderia contaminans* strain MS14. FEMS Microbiol. Lett. **297**:54-60.

10. **Haggag, W. M. and H. A. A. Mohamed**. 2007. Biotechnological aspects of microorganisms used in plant biological control. Am. -Eurasian J Sustain. Agric. **1**:7-12.

11. **Herrera-Estrella, A. and I. Chet**. 1989. Biocontrol of bacteria and

phytopathogenic fungi., p. 263-282. *In* Agricultural biotechnology. Marcel Dekker Inc., New York.

12. **Hillocks, R. J.** 1986. Cross protection between strains of *Fusarium oxysporum* f. sp. *vasinfectum* and its effect on vascular resistance mechanisms. J. Phytopathol. **117**:216-225.

13. **Hofte, M., J. Boelens, and W. Verstraete**. 1992. Survival and root colonization of mutants of plant growth-promoting pseudomonads affected in siderophore biosynthesis or regulation of siderophore production. J. Plant Nutr. **15**:2253-2262.

14. **Jasniewski, J., C. Cailliez-Grimal, E. Gelhaye, and A. M. Revol-Junelles**. 2008. Optimization of the production and purification processes of carnobacteriocins Cbn BM1 and Cbn B2 from *Carnobacterium maltaromaticum* CP5 by heterologous expression in *Escherichia coli*. J. Microbiol. Methods **73**:41-48.

15. **Loper, J. E. and M. D. Henkels**. 1997. Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. Appl. Environ. Microbiol. **63**:99-105.

16. **Lu, S. E., J. Novak, F. W. Austin, G. Gu, D. Ellis, M. Kirk, S. Wilson-Stanford, M. Tonelli, and L. Smith**. 2009. Occidiofungin, a unique antifungal glycopeptide produced by a strain of *Burkholderia contaminans*. Biochemistry **48**:8312-8321.

17. **Lu, S.-E., S. Woolfolk, and J. Caceres**. 2005. Isolation and identification and genetic analysis of rhizobacteria antagonistic to plant soilborne fungal pathogens. Phytopathology **95**:62-63.

18. **Mahenthiralingam, E., A. Baldwin, P. Drevinek, E. Vanlaere, P. Vandamme, J. J. LiPuma, and C. G. Dowson**. 2006. Multilocus sequence typing breathes life into a microbial metagenome. PloS One 1 **e17**.

19. **Mizumoto, S., M. Hirai, and M. Shoda**. 2006. Production of lipopeptide antibiotic iturin A using soybean curd residue cultivated with *Bacillus subtilis* in solid-state fermentation. Appl. Microbiol Biotechmol. **72**:869-875.

20. **Mizumoto, S. and M. Shoda**. 2007. Medium optimization of antifungal lipopeptide, iturin A, production by *Bacillus subtilis* in solid-state fermentation by response surface methodology. Appl. Microbiol. Biotechnol. **76**:101-108.

21. **Moore, L. W. and G. Warren**. 1979. *Agrobacterium radiobacter* strain 84 and biological control of grown gall. Ann. Rev. Phytopathol. **17**:163-179.

22. **National Academy of Sciences**. 1987. Components of biological control, p. 3-12. *In* Research briefing panel on biological control in managed ecosystems. National

Academy Press, Washington, D.C.

23. **Parke, J. L. and D. Gurian-Sherman**. 2001. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Annu. Rev. Phytopathol. **39**:225-258.

24. **Stabb, E., L. M. Jacobson, and J. Handelsman**. 1994. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl Environ Microbiol. **60**:4404-4412.

25. **Survase, S. A., N. S. Shaligram, R. C. Pansuriya, U. S. Annapure, and R. S. Singhal**. 2009. A novel medium for the enhanced production of Cyclosporin A by *Tolypocladium inflatum* MTCC 557 using solid state fermentation. J. of Microbiol. and Biotechnol. **19**:462-467.

26. **Vanlaere, E., A. Baldwin, D. Gevers, D. Henry, E. De Brandt, J. J. Lipuma, E. Mahenthiralingam, D. P. Speert, C. Dowson, and P. Vandamme**. 2009. Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp nov and *Burkholderia lata* sp nov. Int. J. of Syst. and Evol. Microbiol. **59**:102-111.

27. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane**. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**:697-703.

28. **Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa**. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36**:1251-1275.

CHAPTER II

FUNCTIONAL CHARACTERIZATION OF THE *OCFC* GENE IN OCCIDIOFUNGIN PRODUCTION BY *BURKHOLDERIA CONTAMINANS* STRAIN MS14

Abstract

Burkholderia contaminans strain MS14 shows a significant level of antifungal activity. In previous studies, a 56-kb *ocf* gene cluster was determined to be essential for occidiofungin production. In this study the *ocfC* gene, which is located downstream of *ocfD* and upstream of *ocfB* gene in the *ocf* gene cluster, was examined. The antifungal activity of the *ocfC* gene mutant MS14KC1 was significantly reduced against the indicator fungus *Geotrichum candidum* compared with the wild type strain, which indicates a functional *ocfC* gene was important for occidiofungin. Furthermore, the analyses of the protein sequence and the chemical structure of occidiofungin produced by MS14KC1 reveals that the *ocfC* gene encodes a xylosyltransferase. Possible functions of the *ocfC* gene in occidiofungin production were discussed.

Introduction

Members of the bacteria *Burkholderia* exist naturally in environments such as water, soil, and the rhizosphere of crop plants (17). Some *Burkholderia* bacteria show striking efficacy in controlling fungal diseases of crops as biological control agents for plant disease management (4). However, the application of *Burkholderia* bacteria was prohibited because of difficulty differentiating taxonomically these beneficial strains from the *Burkholderia* strains that are opportunistic pathogens associated with the human disease cystic fibrosis (17). Understanding the molecular mechanisms of antifungal activities of the *Burkholderia* strains will provide important clues for the development of biologically based fungicides while eliminating potential health risks.

Genetic analysis revealed that a 56-kb *ocf* gene cluster is required for production of antifungal activity by *Burkholderia contaminans* strain MS14. Strain MS14 showed a broad range of antifungal activity to plant and human fungal pathogens (14). A glycopeptide, occidiofungin, produced by strain MS14 is responsible for its antifungal activity (9,13). Sixteen genes have been predicted in the *ocf* gene cluster, including the genes encoding nonribosomal peptide synthetases (*ocfD, ocfE, ocfF, ocfH, and ocfJ*), the bacterial LuxR regulatory proteins (*ambR1* and *ambR2*), and an ATP-binding cassette (*ocfA*) (9). Mutagenesis and sequence analysis revealed that these genes are associated with occidiofungin production by strain MS14. However, functions of the remaining genes including *ocfC* had yet to be studied regarding their contribution to occidiofungin production.

Glycosyltransferases (GTs) (EC 2.4.1.-) are the enzymes that catalyze the transfer of a monosaccharide unit from an activated nucleotide sugar, also known as the "glycosyl donor", to a glycosyl acceptor molecule such as alcohols or oligopeptides (3). Glycosyltransferases have high donor and acceptor substrate specificities and form one glycosidic linkage (2). Classification of glycosyltransferase is based on the amino acid sequence similarities except families 19, 22, 24, 25 and 26 (3). In 1997, 26 glycosyltransferase families were found based on this classification (3). However, due to the sequencing of genomes for eukaryotic organisms, the growth of glycobiology and the extension of integrated glycosyltransferase, the number of known sequences encoding glycosyltransferase have increased enormously (5). According to the carbohydrate- active enzymes (CAZy) database (http://www.cazy.org/), there are 94 families for glycosyltransferases and many glycosyltransferases have not been well studied.

The *Burkholderia contaminans* MS14 glycosyltransferase OcfC is predicted to enable the addition of xylose on the occidiofungin and belongs to the GT25 family, which is a family involved in lipooligosaccharide (LOS) biosynthesis, such as Lgt2 of *Moraxella catarrhalis* and Lic2A of *Haemorphilus influenza* for beta- (1,4) galactosyltransferase (8,15). According to the CAZy database, the Arad_3667 protein of *Agrobacterium radiobacter* K84 (21), the EcolC_3376 protein of *Escherichia coli* ATCC 8379, the MCA1423 protein of *Methylcoccus capsulatus* str. Bath(25), the Xcc-b100_0220 protein of *Xanthomonas campestris* pv, *campestris* (24), and the PXO_04234 protein of *Xanthomonas oryzae* pv. *oryzae* PXO99 (20) also belong to the GT25 family.

Occidiofungin has showed great potential as a biofungicide and medical drug (13). Understanding the genetics and biosynthesis of this antifungal compound will

provide deep insights to optimizing the production by the host bacteria. A few genes of the *ocf* gene cluster, such as *ocfC*, remain to be investigated. There is a xylose in the occidiofungin molecule and preliminary sequencing analysis indicated the *ocfC* gene codes for the putative glycosyltransferase (9). We hypothesized that the *ocfC* gene codes for an enzyme to catalyze addition of xylose to the backbone peptide of occidiofungin. In this study, the *ocfC* gene was disrupted with a nonpolar mutation and effects of the mutation on occidofugin production were evaluated. Possible functions of the *ocfC* gene are discussed.

Materials and methods

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* JM109 (Promega, Madison, WI) was used in cloning and was cultured at 37℃ on Luria-Bertani (LB) agar. Nutrient broth-yeast extract (NBY) agar media (23) were used to culture *Burkholderia contaminans* strain MS14 at 28℃. Potato dextrose agar (PDA) (Difco, Detroit, MI) and *Burkholderia* minimum media (BM) (26) were used for antifungal activity assays. Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media at the following concentrations: ampicillin (100 μg/mL), kanamycin (100 μg/mL for *E. coli* and 300 μg/mL for the MS14 mutants) and trimethoprim (50 μg/mL).

DNA isolation and manipulation

The cetyl trimethyl ammonium bromide protocol (7) or Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI) was used for extraction of bacterial genomic DNA. Primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA) and Eurofins MWG Operon (Huntsville, AL). Plasmid extraction was done using the QIAprep® Spin Miniprep kit (QIAGEN Inc., Valencia, CA). Wizard® SV Gel and PCR Clean-Up System kit (Promega) was used to recover DNA fragments for cloning. Sequencing was sent to Eurofins MWG Operon. Phylogenetic analysis was conducted using MEGA4 (22).

Mutagenesis of the *ocfC* gene

Primers 6471R1649 (5'- GCCTACCTGCGCGTCTATCA) and 6471F137 (5'- CCATGGCGGCGATTTGCTTTGA) were designed in order to amplify the *ocfC* gene by polymerase chain reaction (PCR). The final concentrations of PCR reagents in the 50 µl reaction were: $MgCl_2$, 2 mM; dNTPs, 0.4mM; primers, 0.6mM each; Taq DNA polymerase, 0.75 units. The PCR cycling conditions were 4 min at 95˚**C**, then 50 s at 95˚**C**, 50 s at 56˚**C** and 2 min at 72˚**C** for 30 cycles, followed by 8 min at 72˚**C**. The PCR amplicon containing the *ocfC* gene with the flanking regions was cloned into the vector pGEM-T Easy (Promega) to generate the plasmid pKC1. Plasmid pBSL15 was partially digested with *Eco*RI and then self-ligated to remove the restriction endonuclease *Eco*RI digestion site as described previously (11). A 1.3-kb *Pst*I fragment of the plasmid pBSL15 lacking *Eco*RI site and carrying the non-polar kanamycin cassette (the *nptII* gene fragment) was cloned into the *ocfC* gene of the plasmid pKC1 using a *Pst*I partial

digestion strategy. The resulting plasmid pGEM-T Easy-*ocfC*::*nptII* was named pKC2. The *ocfC*::*nptII* DNA fragment was introduced to the *Eco*RI site of the vector pBR325 (18) to generate plasmid pKC3. Plasmid pKC3 was electroporated into cells of the wild type MS14 for marker exchange mutagenesis (C=25μ, 200Ω, v=1.8KV, and cuvette 1mm). NBY media containing kanamycin (300 μg/mL) were used for selection of the mutants. PCR amplification and sequencing were used for confirmation of double crossover mutagenesis. Plate bioassays were used to evaluate the production and biological activity of occidiofungin as described (11).

Results

Sequence analysis of the *ocfC* gene

The *ocfC* gene is located downstream of the *ocfD* gene and upstream of the *ocfB* gene in the *ocf* gene cluster (9-11). No significant promoter region or terminator was identified from the 5' and 3' termini of the *ocfC* gene. The 657 base-paired *ocfC* gene was predicted to code for a 218-residue putative protein which was predicted to be the glycosyltransferase that presumably catalyzes the transfer of a xylose to the C-7 site of occidiofungin (9). Sequence analysis showed this putative protein encoded by the *ocfC* gene shared 94.0 % identity with the putative glycosyltransferase (Bamb_6471) of *Burkholderia ambifaria* AMMD (GenBank accession number: NC_008392). There is one conserved domain from amino acid 13 to 89 on the *ocfC* gene. According to the phylogenetic tree (Fig 2.1), the OcfC putative protein of *Burkholderia contaminans* MS14 is clustered with Bamb_6471 of *Burkholderia ambifaria* AMMD with a bootstrap

value of 100 and both were predicted to be members of the glycosyltransferase family 25. The Xcc-b100_0220 protein for *Xanthomonas campestris* pv. *campestris* B1 and the PXO_04234 protein of *Xanthomonas oryzae* pv. *oryzae* PXO99A shared 55% of similarity to OcfC.

Site-directed mutagenesis of the *ocfC* gene

A 1.5-kb PCR product was amplified using the primers 6471R1649 and 6471F137 (Fig. 2.2) and confirmed by sequencing to be the *ocfC* gene with its flanking regions. The plasmid pKC1, which is the vector pGEM-T Easy carrying the *ocfC* gene, was partially digested by restriction enzyme *Pst*I to confirm the insertion of the *ocfC* fragment (Fig. 2.3). Sequencing further confirmed its identity.

A nonpolar mutation was obtained by insertion of an *nptII* cassette, resulting in plasmid pKC2. The plasmid pKC2 was digested by *Eco*RI and *Pst*I, which confirmed the insertion of *nptII* in the plasmid (Fig. 2.4). The plasmid pKC3, which is the suicide vector pBR325 carrying the *nptII*- disrupted *ocfC* gene, was confirmed by the *Eco*RI and *Hind*III digestion (Fig. 2.5). Introduction of pKC3 into cells of strain MS14 resulted in generations of the bacterial colonies with kanamycin resistance (300 µg/mL), which were candidates of the *ocfC* mutants. The mutant generated via a double-crossover, named as MS14KC1, was confirmed by PCR technique (Fig. 2.6) and sequencing.

Effect of mutation in *ocfC* on antifungal activity

Mutant MS14KC1 with nonpolar insertion of the *nptII* gene was evaluated for occidiofungin production by inhibitory activities against the indicator fungus *G.*

candidum (Fig. 2.7). MS14KC1 remained the ability to inhibit the growth of the indicator fungus *G. candidum* with a reduced inhibitory zone (0.817 ± 0.022 cm). In contrast, the antifungal activity for wild-type strain MS14 was 1.417 ± 0.056 cm in radius. The disruption of *ocf*C gene caused a decrease of 42% of the antifungal activity against *G. candidum* (Fig. 2.8). Based on the Fisher's least significant difference (LSD) test, this was a significant difference ($\alpha < 0.05$). This result revealed the importance of *ocfC* gene for the production of the antifungal activity of strain MS14.

Discussion

The *ocfC* gene encodes a member of the GT25 family of glycosyltransferases. Glycosyltransferases are identified from various organisms and classified based on amino acid sequence similarity (3,11). The GT25 family includes the known activities of beta-1,4-galactosyltransferase and lipopolysaccharide biosynthesis protein (5). For *Haemophilus influenza*, glycosyltransferase LpsA is responsible for the addition of a hexose which can be either glucose or galactose (6). In this study, we hypothesized that the *ocfC* gene codes for glycosyltransferase, which was predicted to add xylose to oligopeptide to form occidiofungin. This is proved by the sequence analysis showing that this putative protein encoded by *ocfC* gene shared 94.0 % identity to glycosyltransferase of *B. ambifaria* AMMD in the phylogenetic tree generated. The known activities of the GT25 family include beta-1, 4- galactosyltransferase, beta-1, 3- glucosyltransferase, beta-1, 2 – glucosyltransferase and beta-1, 2- galactosyltransferase (CAZy database). This study is the first evidence that xylosyltransferase is found in GT25 family. This novel glycosyltransferase has expanded the function categories of this family. Based on the

evidence of this study, glycosyltransferase encoded by the *ocfC* gene should be named xylosyltransferase.

The *ocfC* gene is essential for the production of occidiofungin. The *ocfC* mutant strain MS14KC1 has approximately 42% less inhibitory activity to *G. candidum* than the wild-type strain. It was hypothesized that a derivative of occidiofungin produced by the *ocfC* mutant MS14KC1 lacks xylose and this hypothesis was confirmed by matrix-assisted laser desorption ionization (MALDI)-MS analysis (L. Smith, unpublished data).

The effect of a lack of xylose on the production of occidiofungins remains to be investigated. In this study, a mutation of the *ocfC* gene results in significant reduction of occidiofungin production based on the standard plate bioassays. However, the purified xylose-free occidiofungin, which is produced by the mutant MS14KC1, showed higher antifungal activity than the original occidiofungin (L. Smith, personal communication). Therefore, we hypothesize that xylose in occidiofungin may be associated with efficient secretion of occidiofungin by bacterial cells. In a previous work, Yethon et al. found that mutation of the *waaG* gene, a glycosyltransferase gene for liposaccharide (LPS), destabilized the outer membrane of *Escherichia coli*(27). It was shown that this glycosyltransferase gene, *waaG*, played an important role in stabilization of the lipopolysaccharide core protein of the outer cell membrane. Likewise, the *ocfC* gene plays an important role in occidiofungin production based on the results of bioassay. In a previous study the biosynthesis pathway of occidiofungin was proposed based on genetic information and chemical structure of occidiofungin (9). According to this pathway, the addition of xylose is one of the last two steps so it might be needed for translocation of the antifungal compound across cytoplasmic membrane. However, it seems that

antifungal activity may not require the presence of xylose. Further studies are needed to verify these hypotheses and the resulting findings will be informative for development of pharmaceutical drugs and biofungicides based on occidiofungin.

In conclusion, this study demonstrated that the *ocfC* gene encoding glycosyltransferase is important for the production of occidiofungin. The genetic study of the *ocf* gene cluster could help us understand the mechanism of the antifungal compound. Furthermore, it was found that xylose-free occidiofungin showed higher antifungal activity than the naturally-occuring occidiofungin. This discovery may provide us some clues for engineering new chemical variants to have high antifungal activity and minimize the potential toxicity to plants and animals. This might also be the key to higher- efficiency production of occidiofungin in the pharmaceutical and agricultural industry. Functions of a few genes in the *ocf* gene cluster in occidiofungin production remain unknown and further genetic analysis of these genes will be future work to understand biosynthesis pathway of occidiofungin.

Table 2.1 Bacterial strains and plasmids

Strains or plasmids	Relevant Characteristics*	Sources or reference
Strains		
E. coli JM109	*recA1*, *endA1*, *gyr*A96, *thi,*hsdR17, *sup*E44, *rel*A1, Δ(lac-proAB)/F'[traD26, rpoAB+, *laclq*, lacZΔM15]	Promega
B. contaminans		
MS14	Wild type strain	(10)
MS14KC1	*occF*::*nptII* derivative of MS14; km^r	This study
Plasmids		
pBR325	Cloning vector; Cm^r, Tc^r, Ap^r	(18)
pMLS7	Expression vector of *Burkholderia*; Tp^r	(12)
pGEM-T Easy	Cloning vector; Ap^r	Promega
pBSL15	Kanamycin resistance gene cassette; Km^r	(1)
pKC1	pGEM-T Easy carrying 1.5-kb PCR product containing the intact *occF* gene; Ap^r	This study
pKC2	pGEM-T Easy containing 2.8-kb *occF* and *nptII*; Km^r	This study
pKC3	pBR325 carrying 2.8-kb *Eco*RI fragment containing the intact *occF* gene; Km^r, Cm^r, Tc^r, Ap^r	This study

*Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Tp^r, trimethoprim resistance; Cm^r, chloramphenicol resistance

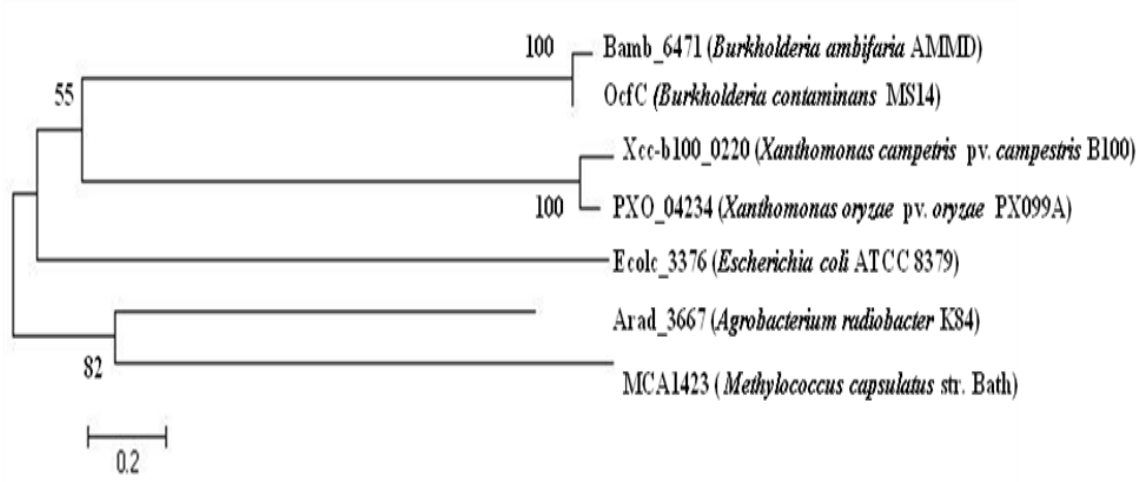

Figure 2.1 Evolutionary relationships of glycosyltransferase family 25

The evolutionary history was inferred using the Minimum Evolution method (19). The optimal tree with the sum of branch length = 6.91717329 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (28) and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (16) at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 218 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (22). (Bamb_6471 for *Burkholderia ambifaria* AMMD; OcfC for *Burkholderia contaminans* MS14; Xcc-b100_0220 for *Xanthomonas campetris* pv. *campestris* B100; PXO_04234 for *Xanthomonas oryzae* pv. *oryzae* PX099A; Ecolc_3376 for *Escherichia coli* ATCC 8379; Arad_3667 for *Agrobacterium radiobacter* K84; MCA1423 for *Methylococcus capsulatus* str. Bath)

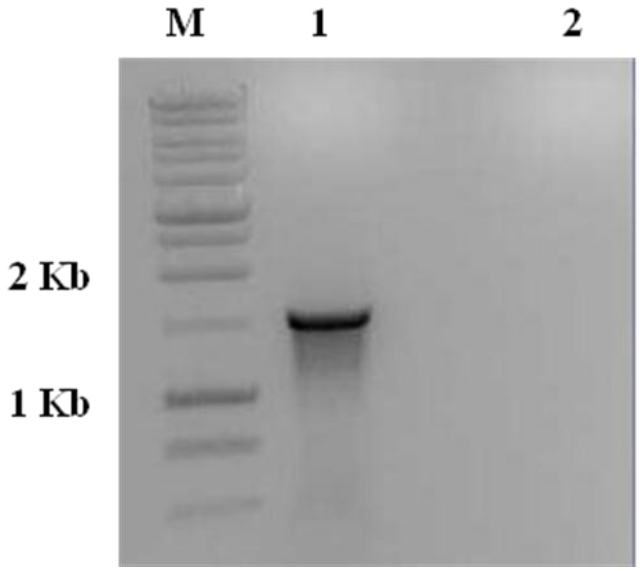

Figure 2.2 PCR amplification of the *ocfC* gene with its flanking regions

DNA marker (BenchTop 1-kb DNA ladder, Promega) is in lane M. 1.5-kb *ocfC* gene with its flanking region is in lane 1, and negative control is in lane 2.

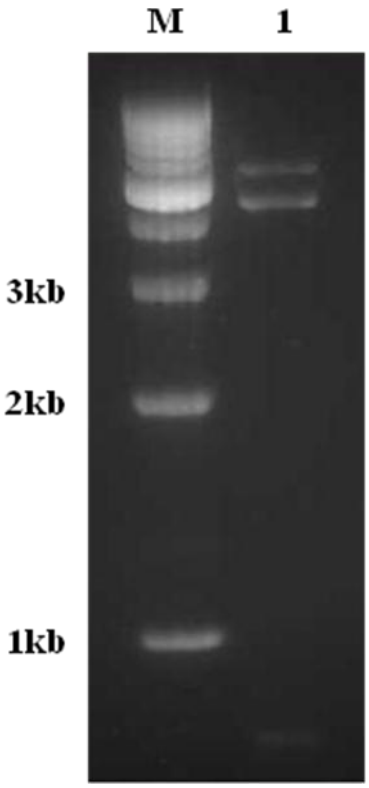

Figure 2.3 Partial digestion of plasmid pKC1

DNA marker (BenchTop 1-kb DNA ladder, Promega) is in lane M. Partial digestion with *Pst*I of pKC1 is in lane 1(pGEM T-Easy::*ocfC* is 4.5-kb, partial digestion of pGEM T-Easy::*ocfC* is 3.9-kb, and there is a small and weak band for 0.7-kb).

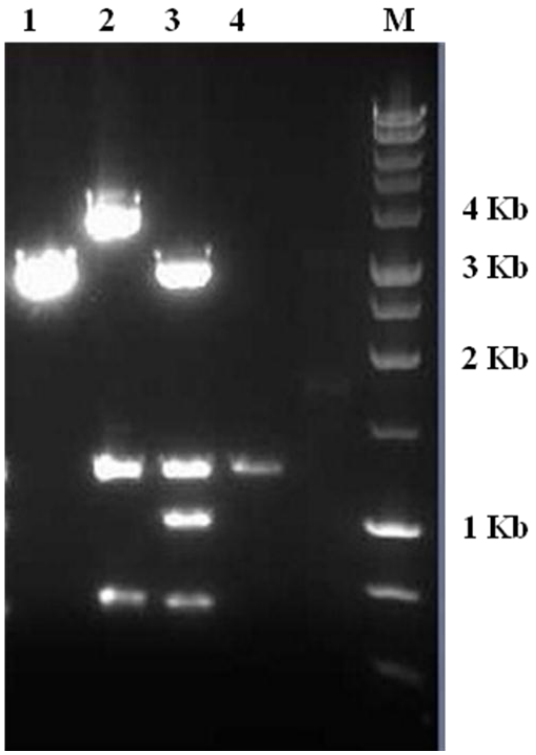

Figure 2.4 Restriction enzyme digestion to confirm plasmid pKC2

DNA marker (BenchTop 1-kb DNA ladder, Promega) is in lane M. pKC2 digested with *Eco*RI is in lane 1 (pGEM-T Easy vector is 3-kb, and *ocfC* ::*nptII* is 2.8-kb). pKC2 digested with *Pst*I is in lane 2 (pGEM-T Easy and part of *ocfC* ::*nptII* is 3.8-kb). The *nptII* is 1.3-kb. Part of *ocfC*::*nptII* is 0.7-kb). pKC2 digested with *Eco*RI and *Pst*I is in lane 3 (pGEM-T Easy is 3-kb. The *nptII* is 1.3-kb. Part of pGEM-T Easy and *ocfC*::*nptII* is 1-kb. Part of *ocfC*::*nptII* is 0.7-kb). The *nptII* gene is in lane 4.

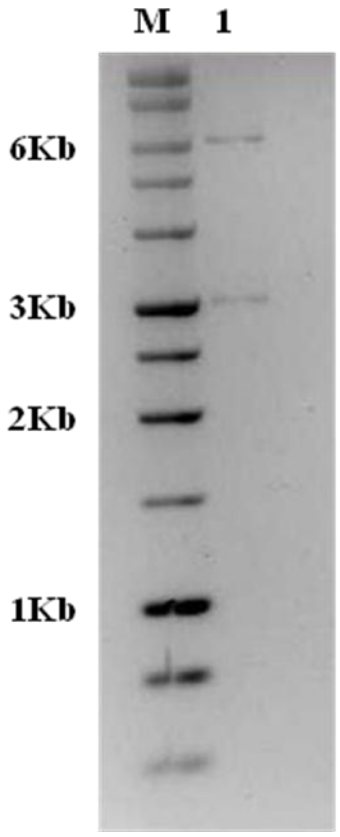

Figure 2.5 Restriction enzyme digestion to confirm plasmid pKC3

DNA marker (BenchTop 1-kb DNA ladder, Promega) is in lane M; pKC3 digested with *Eco*RI and *Hind*III is in lane 1. The size of pBR325 is 6-kb and *ocfC*::*nptII* is 2.8-kb.

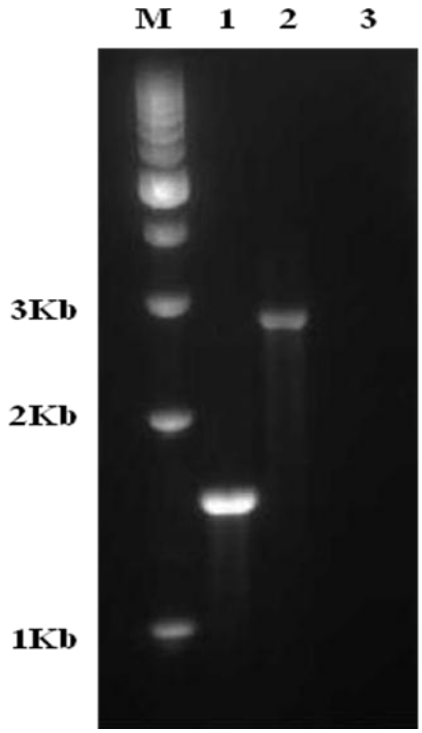

Figure 2.6 PCR analysis to confirm the identity of the *ocfC* mutant

DNA marker (1-kb DNA step ladder, Promega) is in lane M, 1.5-kb *ocfC* gene is in lane 1, 2.8-kb *ocfC*::*nptII* gene is in lane 2, and negative control is in lane 3.

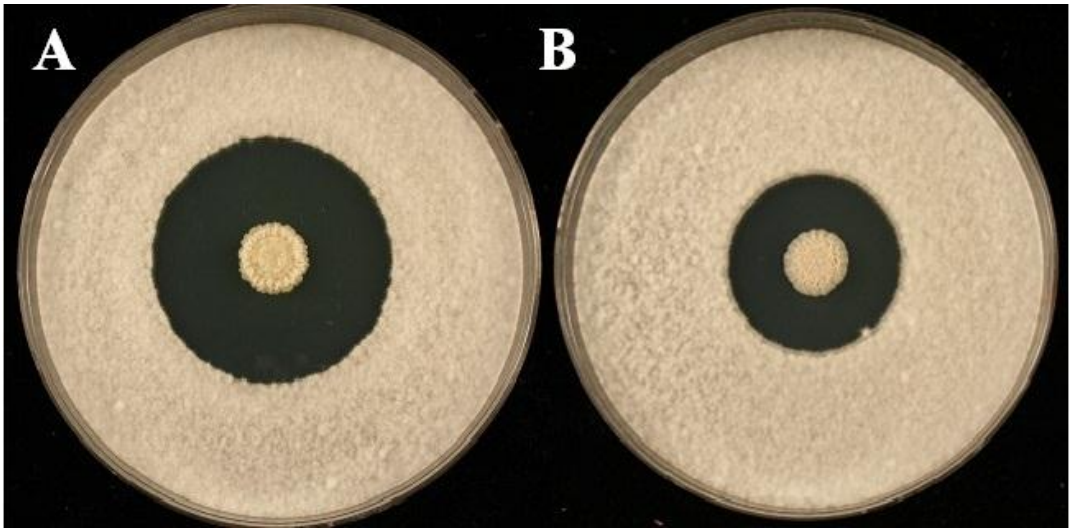

Figure 2.7 Plates bioassays for antifungal activities of *Burkholderia contaminans* against indicator fungus *Geotrichum candidum*

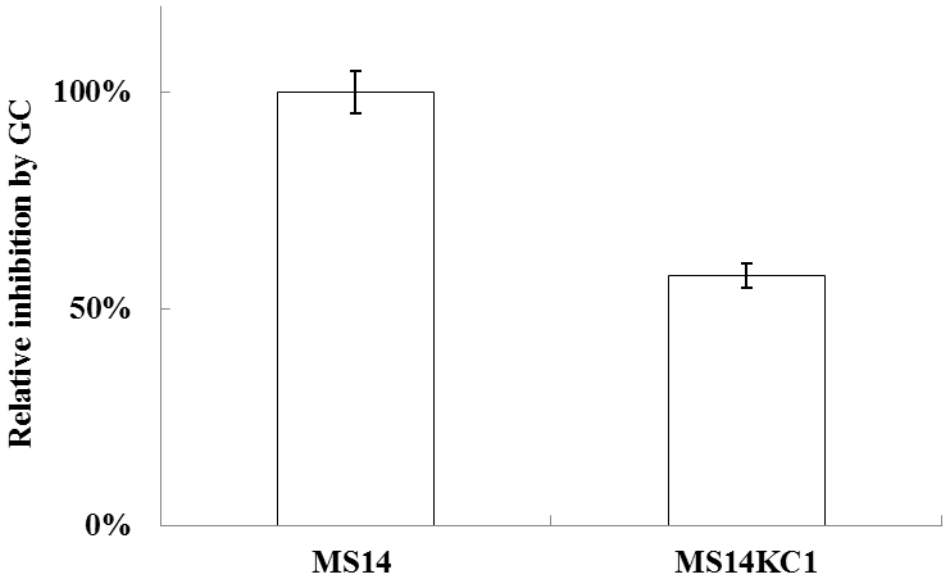

Figure 2.8 Relative inhibition of production of occidiofungin by *Burkholderia contaminans* strain MS14 and its mutant MS14KC1

The strains were inoculated on potato dextrose agar, incubated at 28˚C for 4 days and then over-sprayed with *Geotrichum candidum*. Difference between treatments was tested by Fisher's least significant difference (LSD) test. Vertical bars were standard error of mean.

References

1. **Alexeyev, M.** 1995. Three kanamycin resistance cassettes with different polylinkers. Biotechniques **18**:52-56.

2. **Amado, M., R. Almeida, T. Schwientek, and H. Clausen**. 1999. Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions. Biochem Biophys Acta. **1473**:35-53.

3. **Campbell, J. A., G. J. Davies, V. Bulone, and B. Henrissat**. 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. J. Biochem. **326**:929-939.

4. **Chiarini, L., A. Bevivino, C. Dalmastri, S. Tabacchioni, and P. Visca**. 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends Microbiol. **14**:277-286.

5. **Coutinho, P. M., E. Deleury, G. J. Davies, and B. Henrissat**. 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. **328**:307-317.

6. **Deadman, M. E., S. L. Lundstrom, E. K. H. Schweda, E. R. Moxon, and D. W. Hood**. 2006. Specific amino acids of the glycosyltransferase LpsA direct the additon of glucose or galactoseto the terminal inner core heptose of *Haemophilus influenzae* lipopolysacchraide via alterative linkages. J. Biol. Chem. **281**:29455-29467.

7. **Del Sal, G., G. Manfioletti, and C. Schneider**. 1989. The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. Biotechniques. **7**:514-520.

8. **Faglin, I., J. C. Wilson, J. Tiralongo, and I. R. Peak**. 2010. *Moraxella catarrhali*s Lgt2, a galactosyltransferase with broad acceptor substrate specificity. Carbohyrdrate research **345**:2151-2156.

9. **Gu, G., L. Smith, A. Liu, and S. E. Lu**. 2011. A genetic and biochemical map for the biosynthesis of occidiofungin, an antifungal produced by *Burkholderia contaminans* strain MS14. J. Appl. Environ. Microbio. **77**:6189-6198.

10. **Gu, G., L. Smith, N. Wang, H. Wang, and S.-E. Lu**. 2009. Biosynthesis of an antifungal oligopeptide in *Burkholderia contaminans* strain MS14. Biochem. Biophys. Res. Commun. **380**:328-332.

11. **Gu, G., N. Wang, N. Chaney, L. Smith, and S. E. Lu**. 2009. AmbR1 is a key transcriptional regulator for production of antifungal activity of *Burkholderia contaminans* strain MS14. FEMS Microbiol. Lett. **297**:54-60.

12. **Lefebre, M. D. and M. A. Valvano**. 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. Appl. Environ. Microbiol. **68**:5956-5964.

13. **Lu, S. E., J. Novak, F. W. Austin, G. Gu, D. Ellis, M. Kirk, S. Wilson-Stanford, M. Tonelli, and L. Smith**. 2009. Occidiofungin, a unique antifungal glycopeptide produced by a strain of *Burkholderia contaminans*. Biochemistry **48**:8312-8321.

14. **Lu, S.-E., S. Woolfolk, and J. Caceres**. 2005. Isolation and identification and genetic analysis of rhizobacteria antagonistic to plant soilborne fungal pathogens. Phytopathology **95**:62-63.

15. **Martí-Lliteras, P., A. López-Gómez, S. Mauro, D. W. Hood, C. Viadas, L. Calatayud, P. Morey, A. Servin, J. Liñares, A. Oliver, J. A. Bengoechea, and J. Garmendia**. 2011. Nontypable *Haemophilus influenzae* displays a prevalent surface structure molecular pattern in clinical isolates. Plos One **6**: e21133.

16. **Nei, M. and S. Kumar**. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

17. **Parke, J. L. and D. Gurian-Sherman**. 2001. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Annu. Rev. Phytopathol. **39**:225-258.

18. **Prentki, P., F. Karch, S. Iida, and J. Meyer**. 1981. The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. Gene **14**:289-299.

19. **Rzhetsky, A. and M. Nei**. 1992. A simple method for estimating and testing minimum evolution trees. Mol. Biol. Evol. **9**:945-967.

20. **Salzberg, S. L., D. D. Sommer, M. C. Schatz, A. M. Phillippy, P. D. Rabinowicz, S. Tsuge, A. Furutani, H. Ochiai, A. L. Delcher, D. Kelley, R. Madupu, D. Puiu, D. Radune, M. Shumway, C. Trapnell, G. Aparna, G. Jha, A. Pandey, P. B. Patil, H. Ishihara, D. F. Meyer, B. Szurek, V. Verdier, R. Koebnik, J. M. Dow, R. Ryan, H. Hirata, S. Tsuyumu, S. Won Lee, Y. S. Seo, M. Sriariyanum, P. C. Ronald, R. V. Sonti, M. A. Van Sluys, J. E. Leach, F. F. White, and A. J. Bogdanove**. 2008. Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99A. BMC Genomics **9**:204.

21. **Slater, S. C., B. S. Goldman, B. Goodner, J. C. Setubal, S. K. Farrand, E. W. Nester, T. J. Burr, L. Banta, A. W. Dickerman, I. Paulsen, L. Otten, G. Suen, R. Welch, N. F. Almeida, F. Arnold, O. T. Burton, Z. Du, A. Ewing, E. Godsy, S. Heisel, K. L. Houmiel, J. Jhaveri , J. Lu, N. M. Miller, S. Norton, Q. Chen, W. Phoolcharoen, V. Ohlin, D. Ondrusek, N. Pride, S. L. Stricklin, J. Sun, C. Wheeler, L. Wilson, H. Zhu, and D. W. Wood**. 2009. Genome sequences of three *Agrobacterium* biovars help elucidate the evolution of multichromosome genomes in bacteria. J Bacteriol. **191**:2501-2511.

22. **Tamura, K., J. Dudley, M. Nei, and S. Kumar**. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24**:1596-1599.

23. **Vidaver, A. K.** 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: Effect of the carbon source. Appl. Microbiol. **15**:1523-1524.

24. **Vorhölter, F. J., S. Schneiker, A. Goesmann, L. Krause, T. Bekel, O. Kaiser, B. Linke, T. Patschkowski, C. Rückert, J. Schmid, V. K. Sidhu, V. Sieber, A. Tauch, S. A. Watt , B. Weisshaar, A. Becker, K. Niehaus, and A. Pühler**. 2008. The genome of *Xanthomonas campestris* pv. *campestris* B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. J Biotechnol. **134**:33-45.

25. **Ward, N., Ø. Larsen, J. Sakwa, L. Bruseth, H. Khouri, A. S. Durkin, G. Dimitrov, L. Jiang, D. Scanlan, K. H. Kang, M. Lewis, K. E. Nelson, B. Methé, M. Wu, J. F. Heidelberg, I. T. Paulsen, D. Fouts, J. Ravel, H. Tettelin, Q. Ren, T. Read, R. T. DeBoy, R. Seshadri, S. L. Salzberg, H. B. Jensen, N. K. Birkeland, W. C. Nelson, R. J. Dodson, S. H. Grindhaug, I. Holt, I. Eidhammer, I. Eidhammer, I. Jonasen, S. Vanaken, T. Utterback, T. V. Feldblyum, C. M. Fraser, J. R. Lillehaug, and J. A. Eisen**. 2004. Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). PLoS Biol. **2**:e303.

26. **West, T. P.** 1989. Isolation and characterization of thymidylate synthetase mutants of *Santhomonas maltophilia*. Arch Microbiol **151**:220-222.

27. **Yethon, J. K., E. Vinogradov, M. B. Perry, and C. Whitfield**. 2000. Mutation of the lipopolysaccharide core glycosyltransferase encoded by waaG destabilizes the outer membrane of *Echerichia coli* by interfering with core phosphorylation. J. Bacterol. **182**:5620-5623.

28. **Zuckerkandi, E. and L. Pauling**. 1965. Evolutionary divergence and convergence in proteins, p. 97-166. *In* Evolving Genes and Proteins. Academic Press, New York.

CHAPTER III

OPTIMIZATION OF MEDIUM COMPONENTS AND CULTURE CONDITIONS FOR OCCIDIOFUNGIN PRODUCTION BY *BURKHOLDERIA CONTAMINANS* STRAIN MS14

Abstract

Occidiofungin produced by *Burkholderia contaminans* MS14 is an antifungal compound with potential for future bio-control applications. In order to obtain high yields of this antifungal compound it is necessary to have suitable cultural conditions and medium components. This study presents the effects on occidiofungin production of different medium components such as amino acids, carbon sources, metal ions and nitrogen sources as well as pH values in solid medium. The results of optimization of cultural conditions indicate increased inoculation cell density (20% culture volume) produces the highest amount of occidiofungin. Addition of casamino acid enhanced the antifungal activity by 32%. Supplementation of xylose as a carbon source produced the best antifungal activity. Urea was the best nitrogen source and zinc promoted production of occidiofungin. In addition, an acidic environment (pH5) was better for the antifungal compound production. These preliminary data provide a foundation for further optimization of occidiofungin production.

Introduction

Occidiofungin, a glycolipopeptide, shows a broad range of antifungal activities against plant and animal pathogens (7). It is produced by *Burkholderia contaminans* strain MS14 that was isolated from disease-suppressive soil (8). A 56-kb genomic DNA fragment, named the *ocf* gene cluster, harboring 16 open reading frames (ORFs) was identified (4). Of the 16 ORFs, the genes encoding biosynthetases and LuxR regulators (ambR1 and ambR2) have been characterized (5, 6). The backbone of occidiofungins is composed of eight amino acids with a xylose and it has four variants: variants A, B, C, and D. Variants A and B were both composed by eight amino acids (4). The only difference between A and B variants is an addition of oxygen on asparagine to form occidiofungin B (4).Variant C has an addition of oxygen on asparagine to form occidiofungin D. The variant C contains beta- hydroxy modification of tyrosine; however, there is a 3-chloro addition to beta- hydroxytyrosine (4).

Production of bacterial secondary metabolites can be promoted by optimization of culture conditions and culture medium compositions. Lantibiotic mutacin production was increased more than 100-fold on an optimized culture medium (1). Cyclosporin A produced by *Tolypocladium inflatum* was optimized from 792 mg/kg to 6,480 mg/kg by using solid state fermentation (10). Optimization of media components and culture condition for the production of occidiofungin has not been studied yet. Current data showed that the yield of occidiofungin is about 100-500 µg/L. This study focuses on optimization of occidiofungin produced by *Burkholderia contaminans* strain MS14 through changing the components of *Burkholderia* minimal media (14). Different

components such as amino acids, carbon sources, nitrogen sources, inorganic salts, and pH values were investigated in this study in order to increase yield of occidiofungin.

Materials and methods

Bacterial strains and media

B. contaminans MS14 was routinely grown was on nutrient broth-yeast extract (NBY) agar medium (12) at 28℃. Potato dextrose agar (PDA) (Difco, Detroit, MI) and *Burkholderia* minimum media (BM) (14) were used for bioassay of *B. contaminans* MS14 for antifungal activities.

Liquid bioassay

Spore suspension of *Geotrichum candidum* F-260 (3) was prepared to 0.3 $O.D_{420}$. The culture extract was serially diluted using the BM medium. *B. contaminans* strain MS14 was streaked out from glycerol stock on a NBY plate. Single colonies were used to inoculate into 100 mL BM and cultured at 28℃ for 4 days without shaking. Bacterial cells were collected by centrifugation for 4 min at 5000 *g*. The supernatant of BM media was used for evaluation of occidiofungin activity. The culture extract was mixed with the *G. candidum* spore suspension to 0.3 OD_{420}. The mixture was incubated at 28^{o}C overnight. Snowflake- like *G. candidum* hyphae were visible macroscopically if there was no antifungal activity (6).

Plate bioassay

Strain MS14 was cultured overnight in NBY liquid medium and bacterial cells were harvested by centrifuge. The cell suspension was adjusted to 0.3 $O.D_{420}$. MS14 suspension (5µl) was dropped onto the center of BM media and the plates were incubated at 28˚**C** for 4 days. Spore suspension of *G. candidum* was adjusted into 0.3 OD_{420} and was oversprayed onto the media. Sprayed plates were incubated at 28˚**C** overnight (6). The radius of the inhibitory zone was used for evaluating antifungal activities.

Effect of inoculation cell density

Initial inoculations of 1, 5, 10, and 20 ml of MS14 suspension at 0.3 OD_{420} were placed in 80 ml liquid BM medium. Samples (1 ml for each) were taken at a 12-hour interval after two days to evaluate antifungal activity using the liquid bioassay method described previously. Bacterial cells were collected by centrifugation for 4 min at 5000 *g*. The supernatant of BM medium was used for evaluation of occidiofungin activity. The culture extract was serially diluted in to final concentrations of 1/2, 1/4, 1/8, 1/16 and 1/32 of original culture extract. Spore suspension of *G. candidum* was prepared to 0.3 $O.D_{420}$. The diluted culture mix and spore suspension were combined and placed at 28˚**C** overnight without shaking. Three replicates for the liquid bioassay were performed independently.

Effects of amino acids

Effects of amino acids on antifungal compound production were evaluated. Asparagine (Asn), glycine (Gly), lysine (Lys), serine (Ser), tryptophan (Typ), and

tyrosine (Tyr) were individually added to BM media, respectively, to a final concentration of 20 mM for each amino acid. Furthermore, casamino acid was also added to the BM at a final concentration at 4g/L. Three replicates for the plate bioassay were performed independently.

Effects of carbon sources

Effects of glucose, galactose, lactose, mannose and xylose on the production of occidiofungin were examined. The final concentrations of these carbon sources in the BM media were 22.2 mM. These carbon sources were sterilized separately and aseptically added to the sterilized BM medium. Three replicates for the plate bioassay were performed independently.

Effects of metal ions

Effects of different metal ions on occidiofungin production were investigated using $MgSO_4$, $ZnCl_2$, $MnSO_4$, $MnCl_2$, $CaCl_2$, and $FeCl_3$. These were added in BM to a final concentration of 10 mM. Three replicates for the plate bioassay were conducted independently.

Effects of nitrogen sources

Effects of ammonium chloride, ammonium sulfate, sodium nitrate and urea on production of occidiofungin were tested. These nitrogen sources were added to BM medium to a final concentration of 20 mM. Three replicates for the plate bioassay were performed independently.

Effects of pH values

Effects of pH values of media on occidiofungin activity were tested with different pH values as follows: pH 4, 5, 6, 7, 8, 9 and 10. The pH values of BM were adjusted with 1N sodium hydroxide (NaOH) or 1N hydrogen chloride (HCl). Three replicates for the plate bioassay were performed independently.

Results and Discussion

Effect of optimal inoculation cell density

The fermentation solution of MS14 was collected every twelve hours from day 2 to 6. As expected, greater initial inoculum resulted in earlier production of the antifungal compound (Fig. 3.1). When the amount of initial inoculum was higher, the metabolites produced by MS14 were higher in the limited nutrient conditions. No significant differences were found for initial 1 and 5ml inoculum levels.

Effects of optimal amino acids

Twenty amino acids could be tested for the effect of occidiofungin production. However, the previously determined structure of occidiofungin indicates that only Asn, Gly, Lys, Ser, Typ, and Tyr are present in this compound (7). These six amino acids and casamino acid, a mixture of amino acids, were tested in this study. There was no difference in the production of antifungal compound between the six individual amino acids (Fig. 3.2). Based on previous study, additional casamino acid could enhance the

production of the syringomycin (3). Interestingly, according to Fisher's least significant difference (LSD) test, adding casamino acid in the BM media could enhance the production of antifungal compound. For the production of occidiofungin, all six amino acids are needed. This might explain why single amino acids caused relatively lower production of the antifungal compound than the production when casamino acid was added. Therefore, casamino acid resulted in a greater amount of the occidiofungin production.

Effects of optimal carbon sources

In the BM medium, 0.02M glucose is used as carbon source. Two experiments were conducted to see if the glucose concentration and glucose are the optimal carbon source for the antifungal compound production. First, different concentrations of glucose were tested (Fig. 3.3). Based on the LSD t-test statistical analysis, 1 g/L concentration of glucose produced relatively high yields of antifungal compound. Second, some monosaccharide and disaccharide sugars were examined in order to find the optimal carbon sources. Glucose, galactose, lactose, mannose and xylose were tested at concentration of 0.02M. PDA was used as a control. Of all the carbon sources tested, xylose increased the production of antifungal compound compared with glucose, galactose, lactose and mannose (Fig. 3.4). In fact, the bacteria *Burkholderia* spp. can utilize the xylose as a sole carbon source (11). More importantly, xylose is required for biosynthesis of occidiofungin (7).

Effects of optimal metal ions

In this study, 10mM $CaCl_2$, $FeCl_3$, $MgSO_4$, $MnCl_2$, $MnSO_4$, and $ZnCl_2$ were examined (Fig. 3.5). Fisher's LSD test was used to do data analysis. Calcium was no better than magnesium and manganese. In a previous study, the yield of bacitracin produced by *Bacillus licheniformis* was increased by adding magnesium, and manganese (2). Magnesium ions are essential in various kinds of enzyme reactions in the production of bacterial metabolites (1). However, in the current study the addition of magnesium did not result in as great a production of the antifungal agent as did some other metal ions. The addition of manganese also produced no differences compared to the other metal ions tested. The addition of ferric ions resulted in a lack of growth of the indicator fungus. It might result in inhibition of the growth of the fungus at the tested concentration of ferric ion. The addition of zinc ions resulted in the greatest antifungal production among the metal ions tested. Zinc is important for the production of some antibiotics such as lantibiotics (1), so zinc may be important for the enzymatic modification of occidiofungin. However, further studies are needed to understand the role of zinc in occidiofungin.

Effects of optimal nitrogen sources

The nitrogen source in BM media is ammonium sulfate. Several nitrogen sources were tested at 0.02M such as ammonium chloride, sodium nitrate and urea. Ammonium nitrogen present in liquid manure resulted in a significantly increased production of polyamino acids produced by *Bacillus* strain (9). For cyclosporine produced by *Tolypocladium inflatum*, additional ammonium sulfate resulted in the maximum

production. For the *Myrothecium verrucaria*, the chitinase production was enhanced in the presence of urea (13). In this study, nitrogen sources such as ammonium sulfate, ammonium chloride, and sodium nitrate produced low antifungal compound production. Therefore, the production of antifungal compound was significantly enhanced only by addition of urea base on Fisher's LSD analysis (Fig. 3.6).

Effects of optimal pH values

The optimal pH is 5 for production of lactocin, a bacteriocin produced by *Lactobacillus rhamnosus* (1). In this study, pH 4, 5, 6, 7, 8, 9, and 10 were tested. *B. contaminans* MS14 did not grow on the media having pH 4 and 10. In general, the yield of antifungal compound is higher in acidic and neutral (pH 5, 6 and 7) than in basic (pH 8 and 9) environments (Fig.3.7) based on Fisher's LSD test.

Effect of casamino acid at pH 5 and 6

For all of the tested factors, the addition of casamino acid significantly increased occidiofungin production as compared to the other amino acids tested. For pH values, the acidic condition resulted in higher occidiofungin production. These two factors were investigated together. Casamino acid in pH 5 and 6 were examined with 2.5, 5, 10, and 20 g/L of casamino acid tested at each pH (Fig.3.8 and 3.9, respectively). BM medium at pH 7 with no casamino acid was the control. At pH 5 there is no difference between each concentration of casamino acid. However, additional casamino acid in BM media in pH 6 produced relatively higher yields of occidiofungin.

Conclusions

In this study, production of occidiofungin by strain MS14 was optimized in liquid BM medium by inoculation cell density and in plate bioassay with regulated amino acids, carbon sources, metal ions, nitrogen sources and pH values. Our data show that optimized individual conditions for the production of occidiofungin are higher inoculation cell density (20% culture volume), additional casamino acid (32% increase), xylose (22%), urea (13%), zinc ions (18%), and pH 5 (10%). For further study, two or more factors should be combined for optimization of occidiofungin production.

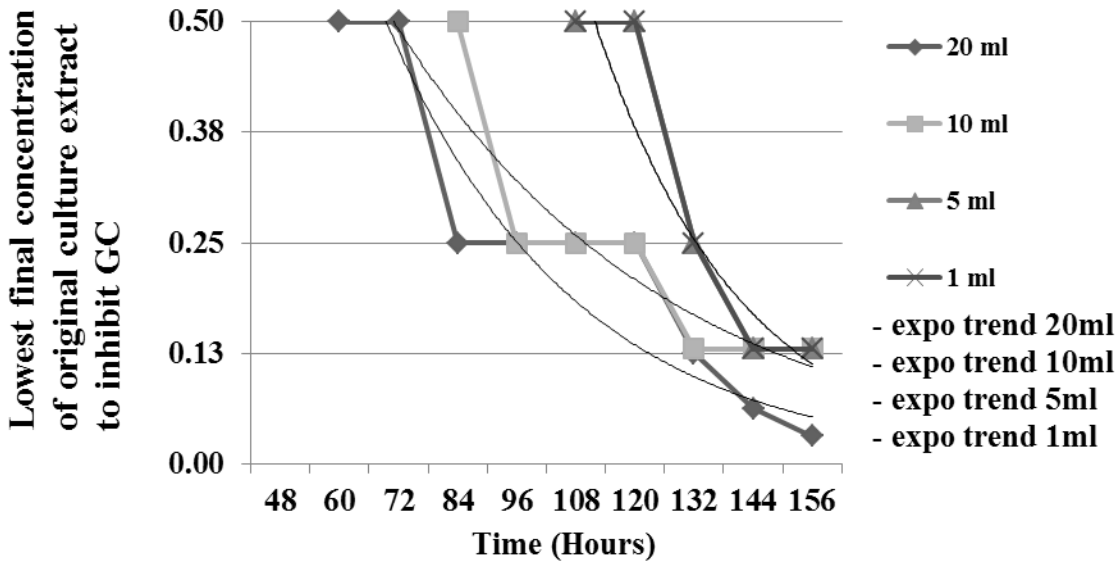

Figure 3.1 Effect of inoculation cell density on antifungal activity

1, 5, 10, 20 ml of MS14 solution were used for initial inoculation. The culture extract was serially diluted in the final concentration of 1/2, 1/4, 1/8, 1/16 and 1/32 of original culture extract for every 12 hour. Spore suspension of *Geotrichum candidum* (GC) was prepared to 0.3 O.D420. Culture extract and spore suspension were mixed overnight without shaking to observe the antifungal activity. Expo trend means exponential trend line for that treatment.

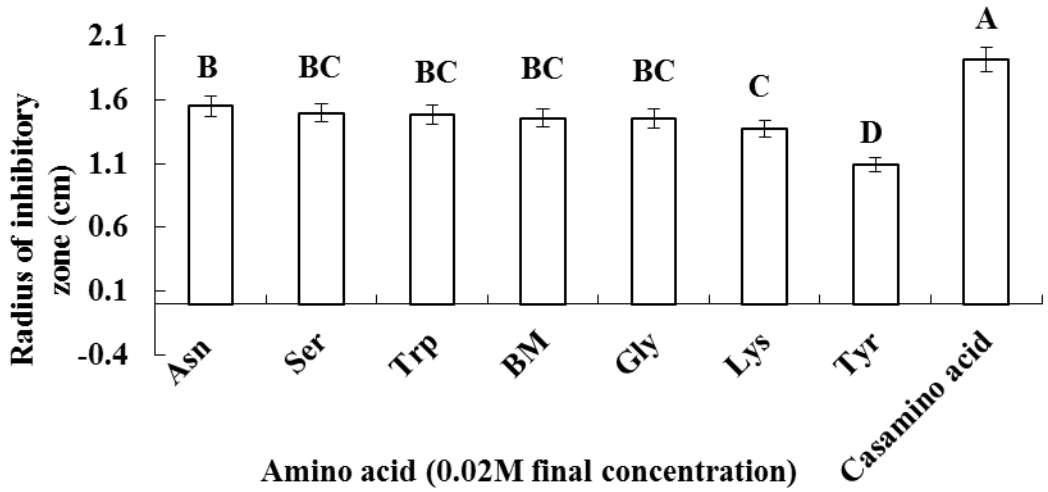

Figure 3.2 Optimization of occidiofungin production using amino acids

Concentration of each amino acid is 0.02M. Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars represent the standard errors of the mean.

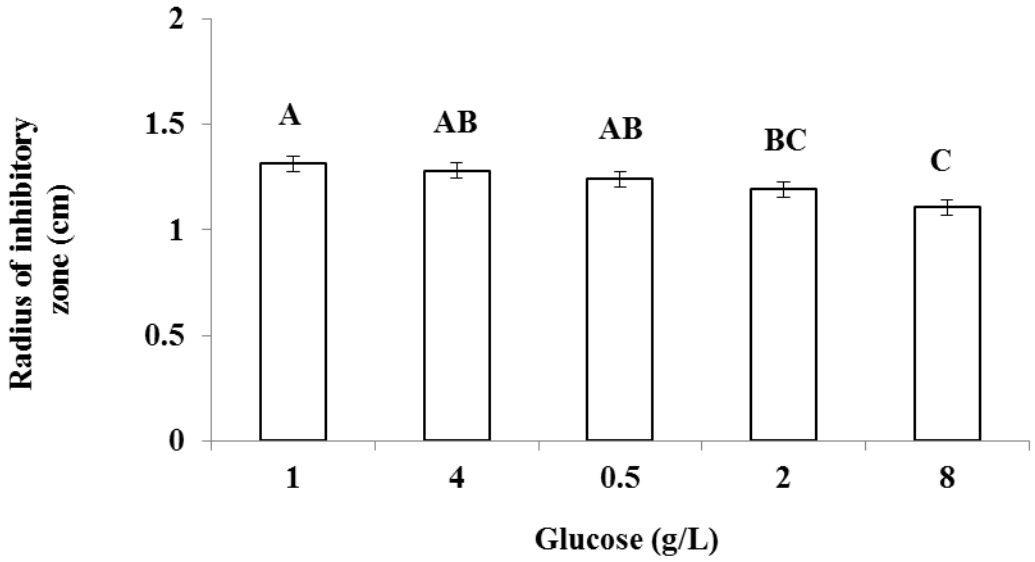

Figure 3.3 Optimization of occidiofungin production using different concentrations of glucose

Glucose concentrations are ranging from 0.5 to 8 g/L. Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars indicate the standard errors of the mean.

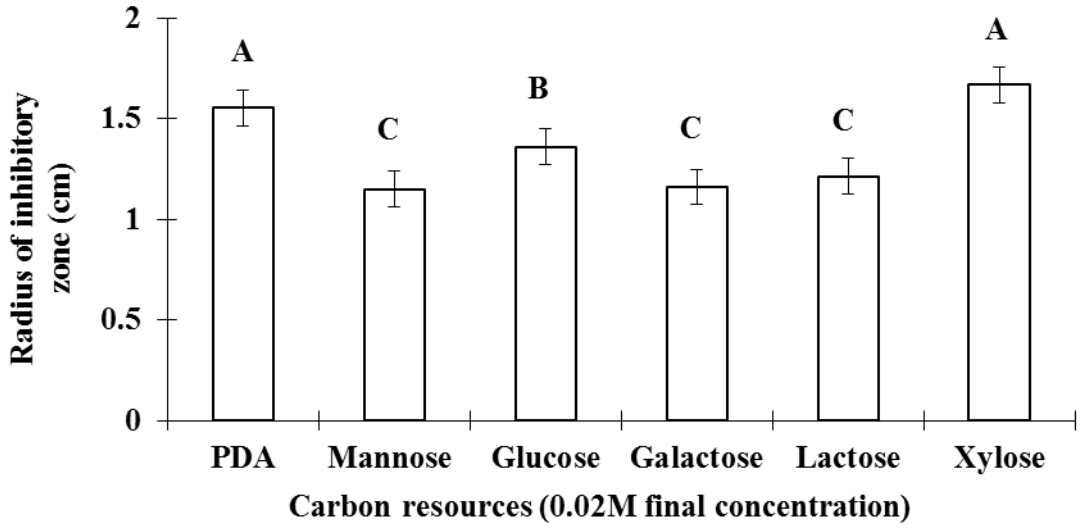

Figure 3.4 Optimization of occidiofungin production for carbon sources

Concentration of each carbon source is 0.02M. Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars indicate the standard errors of the mean.

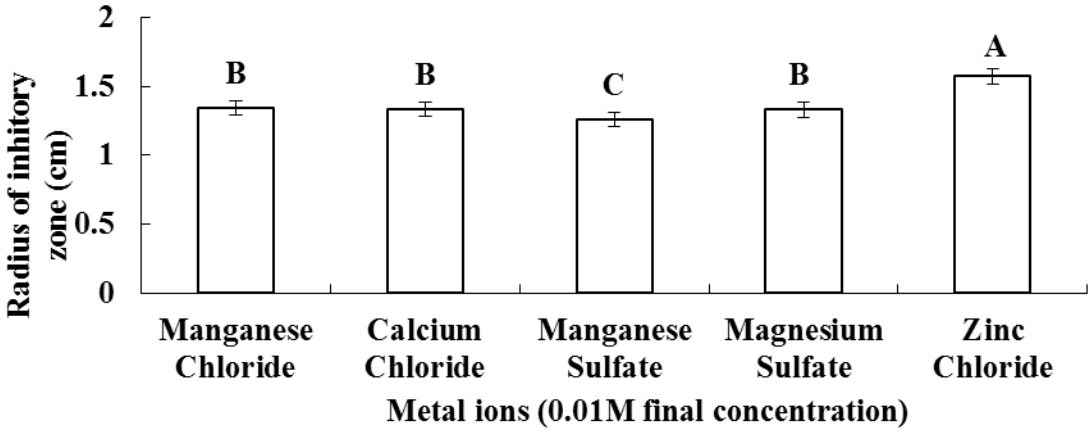

Figure 3.5 Optimization of occidiofungin production using metal ions

Concentration of each metal ion is 0.01M. Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars indicate the standard errors of the mean.

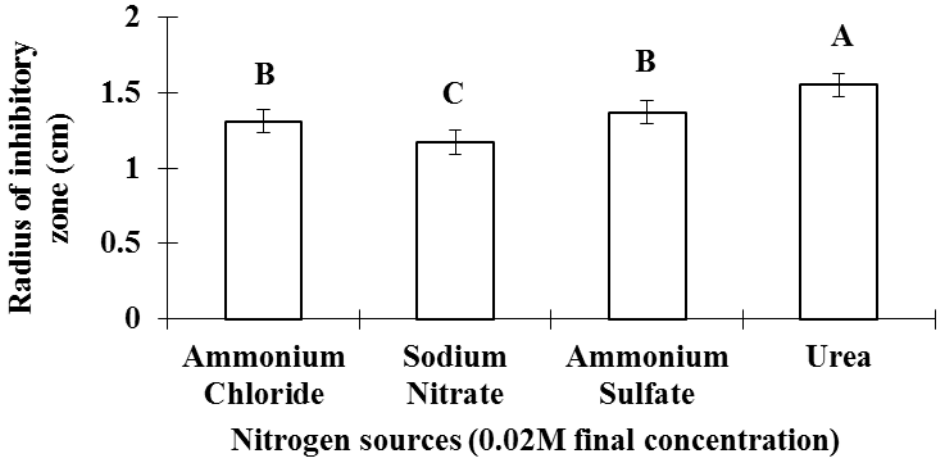

Figure 3.6 Optimization of occidiofungin production using various nitrogen sources

Concentration of each nitrogen source is 0.02M. Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars indicate the standard errors of the mean.

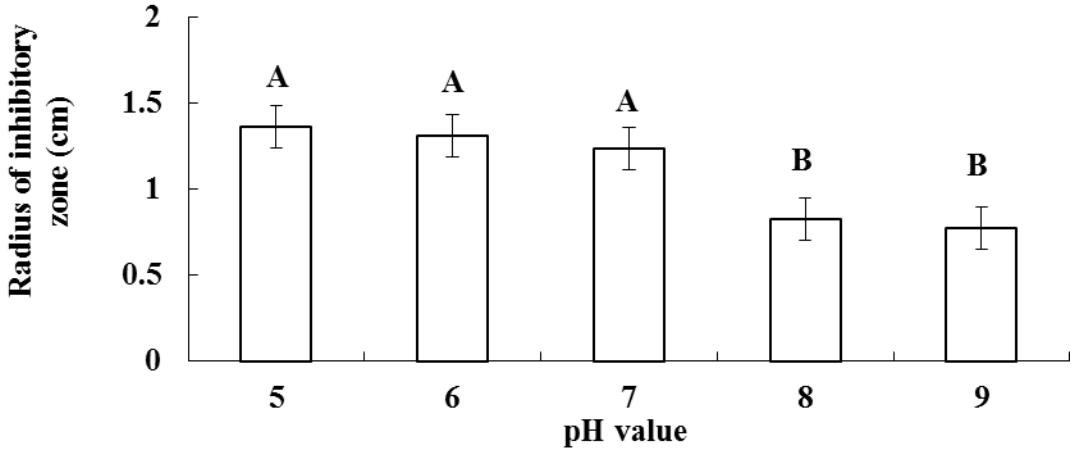

Figure 3.7 Optimization of occidiofungin production for pH ranging from pH 5 to 9

Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars indicate the standard errors of the mean.

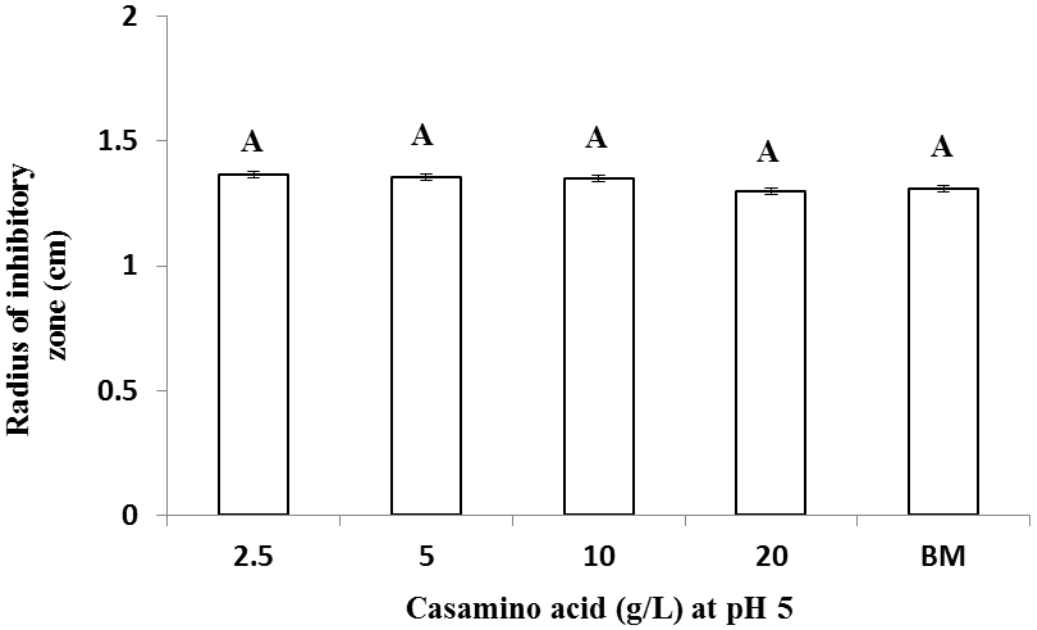

Figure 3.8 Optimization of occidiofungin production for casamino acid concentration ranging from 2.5 to 20g/L at pH 5

Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given and error bars represent the standard errors of the mean.

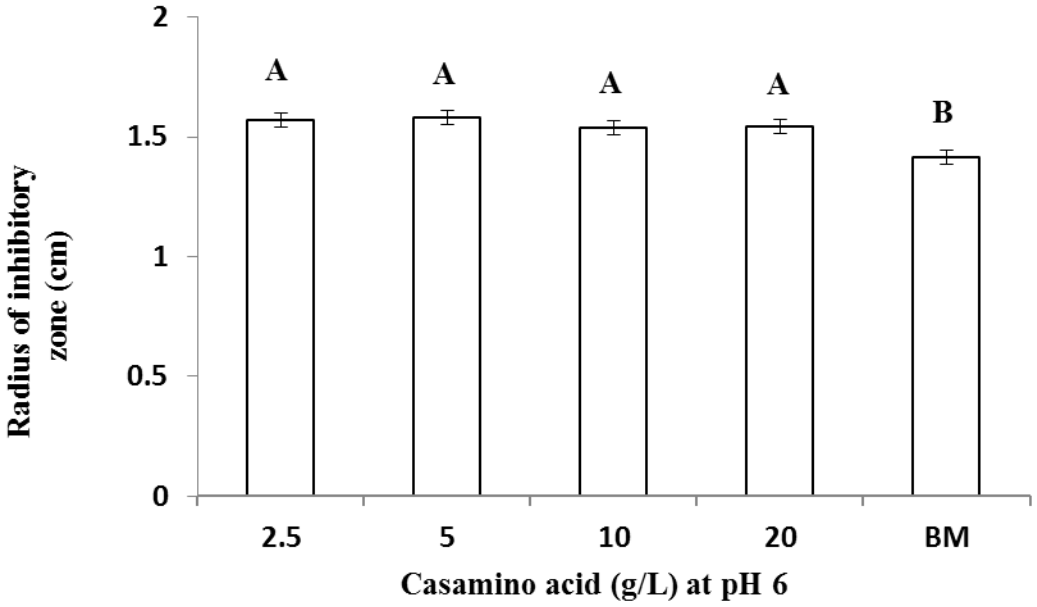

Figure 3.9 Optimization of occidiofungin production for casamino acid concentration ranging from 2.5 to 20g/L at pH 6

Differences between treatments are determined by Fisher's least significant difference (LSD) test. Different letters for each treatment indicate statistically significant difference. Mean values for three biological replicates are given, and error bars are the standard errors of the mean.

References

1. **Dahal, N., N. Chaney, D. Ellis, S. E. Lu, and L. Smith**. 2010. Optimization of the production of the lantibiotic mutacin 1140 in minimal media. Process Biochem. **45**:1187-1191.

2. **Frøyshov, O. and A. Mathiesen**. 1979. Tryptic cleavage of enzyme A in bacitracin synthetase. FEBS Lett. **106**:275-278.

3. **Gross, D. C. and J. E. DeVay**. 1977. Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae*. Physiol. Plant Pathol. **11**:13-28.

4. **Gu, G., L. Smith, A. Liu, and S. E. Lu**. 2011. A genetic and biochemical map for the biosynthesis of occidiofungin, an antifungal produced by *Burkholderia contaminans* strain MS14. J. Appl. Environ. Microbio. **77**:6189-6198.

5. **Gu, G., L. Smith, N. Wang, H. Wang, and S.-E. Lu**. 2009. Biosynthesis of an antifungal oligopeptide in *Burkholderia contaminans* strain MS14. Biochem. Biophys. Res. Commun. **380**:328-332.

6. **Gu, G., N. Wang, N. Chaney, L. Smith, and S. E. Lu**. 2009. AmbR1 is a key transcriptional regulator for production of antifungal activity of *Burkholderia contaminans* strain MS14. FEMS Microbiol. Lett. **297**:54-60.

7. **Lu, S. E., J. Novak, F. W. Austin, G. Gu, D. Ellis, M. Kirk, S. Wilson-Stanford, M. Tonelli, and L. Smith**. 2009. Occidiofungin, a unique antifungal glycopeptide produced by a strain of *Burkholderia contaminans*. Biochemistry **48**:8312-8321.

8. **Lu, S.-E., S. Woolfolk, and J. Caceres**. 2005. Isolation and identification and genetic analysis of rhizobacteria antagonistic to plant soilborne fungal pathogens. Phytopathology **95**:62-63.

9. **Pötter, M., F. B. Oppermann-Sanio, and A. Steinbüchel**. 2001. Cultivation of bacteria producing polyamino acids with liquid manure as carbon and nitrogen source. Appl Environ Microbiol. **67**:617-622.

10. **Survase, S. A., N. S. Shaligram, R. C. Pansuriya, U. S. Annapure, and R. S.**

Singhal. 2009. A novel medium for the enhanced production of Cyclosporin A by *Tolypocladium inflatum* MTCC 557 using solid state fermentation. J. of Microbiol. and Biotechnol. **19**:462-467.

11. **Vanlaere, E., A. Baldwin, D. Gevers, D. Henry, E. De Brandt, J. J. Lipuma, E. Mahenthiralingam, D. P. Speert, C. Dowson, and P. Vandamme**. 2009. Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp nov and *Burkholderia lata* sp nov. Int. J. of Syst. and Evol. Microbiol. **59**:102-111.

12. **Vidaver, A. K.** 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: Effect of the carbon source. Appl. Microbiol. **15**:1523-1524.

13. **Vyas, P. and M. V. Desphpande**. 1989. Chitinase production by Myrothecium verrucaria and its significance for fungal mycelia degradation. J Gen Appl Microbiol. **35**:343-350.

14. **West, T. P.** 2000. Role of cytosine deaminase and beta-alanine-pyruvate transaminase in pyrimidine base catabolism by *Burkholderia cepacia*. Antonie Van Leeuwenhoek **77**:1-5.