# CLONING AND HETEROLOGOUS EXPRESSION OF CHITINASE GENE FROM AN ENDOPHYTIC ACTINOMYCETE FOR ANTIFUNGAL ACTIVITY IMPROVEMENT

THONGCHAI TAECHOWISAN

DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

GRADUATE SCHOOL
CHIANG MAI UNIVERSITY
SEPTEMBER 2003

ขอมอบแด่ "โครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย"

"Biodiversity Research and Training Program"

ขอแสดงความนับถือ

นายธงชัย เตชะวิศาล.

1054/46

RECEIVED
BY - 8 S.A. 2546
DATE

โครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย
c/o ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
อาคารสำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
73/1 ถนนพระรามที่ 6 เขตราชเทวี
กรุงเทพฯ 10400

# CLONING AND HETEROLOGOUS EXPRESSION OF CHITINASE GENE FROM AN ENDOPHYTIC ACTINOMYCETE FOR ANTIFUNGAL ACTIVITY IMPROVEMENT

THONGCHAI TAECHOWISAN

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

GRADUATE SCHOOL
CHIANG MAI UNIVERSITY
SEPTEMBER 2003

# CLONING AND HETEROLOGOUS EXPRESSION OF CHITINASE GENE FROM AN ENDOPHYTIC ACTINOMYCETE FOR ANTIFUNGAL ACTIVITY IMPROVEMENT

THONGCHAI TAECHOWISAN

THIS THESIS HAS BEEN APPROVED

TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

EXAMINING COMMITTEE

..... CHAIRPERSON

Assoc.Prof.Dr.Saisamorn Lumyong

..... MEMBER

Prof.Dr.John F Peberdy

..... MEMBER

Dr.Rungrach Wangspa

..... MEMBER

Assoc.Prof.Dr.Pairote Wiriyacharee

..... MEMBER

Asst.Prof.Dr.Sittisin Bovonsombut

30 September 2003

## ACKNOWLEDGEMENTS

I am extremely grateful to my advisor Assoc.Prof.Dr.Saisamorn Lumyong. Her great enthusiasm, knowledge and endless supply of ideas have been a constant source of inspiration, ever since I first met her. It was a great pleasure to me to conduct this thesis under her supervision. My deep gratitude to my co-advisor Prof.Dr.John F Peberdy, whose insightful hints, stimulating suggestions and encouragement have helped me on numerous occasions throughout the writing period. I am also indebted to the other committee members, Dr.Rungrach Wangspa, Assoc.Prof.Dr. Pairote Wiriyacharee and Asst.Prof.Dr.Sittisin Bovonsombut for their worth while advice and comments.

I would like to acknowledge the Biodiversity Research and Training Program of Thailand for the graduated student research grant (BRT T_646003) and the graduate school of Chiang Mai University which partially enabled me to carry out this study successfully.

I wish to thank all the laboratory persons of the Department of Biology, Faculty of Science, Chiang Mai University for their friendship and support.

I am thankful to my friends, who sharing the knowledge, fun and happiness together.

Finally, much appreciation in specially expressed to my parents for their loves, understanding, encouragement throughout my study.

Mr.Thongchai Taechowisan

| | |
|---|---|
| **Thesis Title** | Cloning and Heterologous Expression of Chitinase Gene from An Endophytic Actinomycete for Antifungal Activity Improvement |
| **Author** | Mr.Thongchai Taechowisan |
| **Degree** | Doctor of Philosophy (Biotechnology) |
| **Thesis Advisory Committee** | Assoc.Prof.Dr.Saisamorn Lumyong Chairperson |
| | Dr.Uraporn Sardsud Member |
| | Dr.Chartchai Kanongnuch Member |
| | Prof.Dr.John F Peberdy Member |


## ABSTRACT

The isolation of endophytic actinomycetes from surface sterilised tissues of 36 plant species was carried out using Humic acid – Vitamin (HV) agar as a selection medium. Of the 330 isolates recovered, 212 were from roots, 97 from leaves and 21 isolates from stems with a revelance of 3.93%, 1.79% and 0.39% respectively. Identification of the isolates was based on their morphology and the amino acid composition of the whole-cell extract. Most were classified as *Streptomyces* sp. (n=277), with the remainder belonging to *Microbispora* sp. (n=14), *Nocardia* sp. (n=8) and *Micromonospora* sp. (n=4). Four isolates were unclassified. *Zingiber officinale* was the most significant host for the *Streptomyces* sp. isolates with 6.44% of the tissue samples giving rise to cultures. Scanning electron microscopic investigations of this plant revealed that 7.5% of the root and 5% of the leaf samples contained endophytes. Three of the *Streptomyces* sp. isolates strongly inhibited

*Colletotrichum musae*, five were very active against *Fusarium oxysporum* and two strongly inhibited growth of both test fungi.

More than 300 isolates were screened for their potential for chitinase production. The strain identified as *Streptomyces aureofaciens* CMUAc130 was the most effective amongst those investigated. Production of the chitinase from *S. aureofaciens* CMUAc130 was optimal with 1% colloidal chitin, at 30-40°C, pH 6.5-7.0 and 100-150 rev $min^{-1}$ shaking. *N*-acetylglucosamine was a good inducer and the enzyme complex was repressed by several mono- and disaccharides including lactose, mannose, glucose, cellobiose, arabinose, raffinose, sucrose, xylose and fructose. Addition of pectin, starch or carboxymethyl cellulose to the colloidal chitin-containing medium, increased chitinase production. The enzyme tolerated a wide range of temperature (30-50°C) and pH (5.5-8). Among various divalent cations $Hg^{2+}$ $Cd^{2+}$ and $Ni^{2+}$ completely inhibited the purified enzyme while $Mg^{2+}$ stimulated its activity. Crude and purified enzyme had potential for cell wall lysis of many phytopathogenic fungi tested.

The gene *chi40*, which codes for chitinase, was cloned from the endophytic *Streptomyces aureofaciens* CMUAc130. Its complete sequence was determined, and the deduced amino acid sequence of the enzyme designated Chi40_Sau yielded an open reading frame coding for 413 amino acids of a 40-KDa precursor protein with a putative leader peptide at its N-terminus. The nucleotide and polypeptide sequences of Chi40_Sau showed 95 and 87% identity with the corresponding gene and enzyme, Chia of *Streptomyces thermoviolaceus*, respectively. *Escherichia coli* JM109 carrying the *S. aureofaciens* CMUAc130 *chi40* gene produced a secreted Chi40_Sau. The

antifungal activity of the chitinase was demonstrated *in vitro* by inhibition of hyphal extension and spore germination in *Fusarium oxysporum*.

**ชื่อเรื่องวิทยานิพนธ์** การโคลนและการแสดงออกแบบเฮเทอโรโลกัสของยีนไคติเนสจากแอคติโนมัยซีสในพืชเพื่อเพิ่มฤทธิ์ต้านเชื้อรา

**ผู้เขียน** นายธงชัย เตโชวิศาล

**ปริญญา** วิทยาศาสตรดุษฎีบัณฑิต (เทคโนโลยีชีวภาพ)

**คณะกรรมการที่ปรึกษาวิทยานิพนธ์**

| | |
|---|---|
| รศ.ดร.สายสมร ลำยอง | ประธานกรรมการ |
| อ.ดร.อุราภรณ์ สอาดสุด | กรรมการ |
| อ.ดร.ชาติชาย โชนงนุช | กรรมการ |
| Prof.Dr.John F Peberdy | กรรมการ |


**บทคัดย่อ**

แยกเชื้อแอคติโนมัยซีสในพืชจากส่วนต่างๆ ของพืช 36 ชนิด ด้วยวิธีฆ่าเชื้อที่ผิวตัวอย่างและเลี้ยงบนอาหาร Humic acid – Vitamine agar ได้เชื้อแอคติโนมัยซีสในพืช 330 สายพันธุ์ (จากใบ 97 สายพันธุ์ จากลำต้น 21 สายพันธุ์ และจากราก 212 สายพันธุ์) โดยความชุกในการแยกเชื้อจากใบ 1.79% จากลำต้น 0.39% และจากราก 3.93% จำแนกเชื้อแอคติโนมัยซีสในพืชที่แยกได้ด้วยลักษณะสัณฐานและองค์ประกอบของกรดอะมิโนของสารสกัดจากเซลล์ พบว่าเป็น *Streptomyces* sp. 277 สายพันธุ์ *Microbispora* sp. 14 สายพันธุ์ *Nocardia* sp. 8 สายพันธุ์ *Micromonospora* sp. 4 สายพันธุ์ และไม่สามารถจำแนกได้ 4 สายพันธุ์ ความชุกในการแยกเชื้อแอคติโนมัยซีสในพืชสูงที่สุด คือ 6.44% จากขิง (*Zingiber officinale*) จากการศึกษาภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าเส้นใยเชื้อแอคติโนมัยซีสในพืชแทรกตัวอยู่ภายในเนื้อเยื่อของพืช ตรวจพบได้ 5.0% ของตัวอย่างใบ และ 7.5% ของตัวอย่างรากของขิง แต่ไม่พบในตัวอย่างลำต้น เชื้อแอคติโน

มัยซีสในพืชที่แยกได้ 3 สายพันธุ์ให้ผลยับยั้งการเจริญเชื้อรา *Colletotrichum musae* ได้มากกว่า 2 เซนติเมตร 5 สายพันธุ์ที่ให้ผลยับยั้งการเจริญเชื้อรา *Fusarium oxysporum* ได้มากกว่า 2 เซนติเมตร และ 2 สายพันธุ์ที่ให้ผลยับยั้งการเจริญเชื้อรา *C. musae* และ *F. oxysporum* ได้มากกว่า 2 เซนติเมตร ในอาหารแข็ง

เชื้อแอคติโนมัยซีสในพืชที่แยกได้ทั้งหมดเมื่อนำมาตรวจกรองความสามารถในการสร้างเอ็นไซม์ไคติเนส พบว่าเชื้อแอคติโนมัยซีส CMUAc130 ที่ศึกษาเทียบเคียงโดยอาศัยสัณฐานวิทยาองค์ประกอบของผนังเซลล์ และลำดับกรดนิวคลีอิกของยีน 16SrRNA ว่าเป็น *Streptomyces aureofaciens* CMUAc130 สามารถสร้างเอ็นไซม์ไคติเนสสูงสุด จึงนำ *S. aureofaciens* CMUAc130 มาศึกษาการสร้างเอ็นไซม์ไคติเนสในสภาวะต่างๆ และการย่อยสลายผนังเซลล์ของเชื้อราบางชนิด *S. aureofaciens* CMUAc130 สามารถสร้างเอ็นไซม์ไคติเนสได้ดีที่สุดในอาหารเลี้ยงเชื้อที่ใช้ทดสอบ โดยมีคอลลอยดอลไคติน 1% pH ระหว่าง 6.5-7.0 บ่มที่อุณหภูมิ 30-40°C เขย่าด้วยความเร็ว 100-150 รอบต่อนาที สร้างเอ็นไซม์ไคติเนสได้มากที่สุดในช่วง วันที่ 7-10 ของการบ่มเลี้ยงเชื้อ การเติม *N*-acetylglucosamine 0.5% ในอาหารเลี้ยงเชื้อที่มีคอลลอยดอลไคติน 1% เร่งการสร้างเอ็นไซม์ไคติเนส ขณะที่การเติมน้ำตาลชนิดอื่นๆ เช่น lactose, mannose, glucose, cellobiose, arabinose, raffinose, sucrose, xylose และ fructose ในความเข้มข้น 0.5% ในอาหารเลี้ยงเชื้อมีผลยับยั้งการสร้างเอ็นไซม์ไคติเนส การเติม pectin, starch และ carboxymethyl cellulose 0.3% ในอาหารเลี้ยงเชื้อที่มีคอลลอยดอลไคติน 1% ทำให้การสร้างเอ็นไซม์ไคติเนสมากขึ้น เอ็นไซม์ไคติเนสมีความทนทานในการย่อยสลายคอลลอยดอลไคติน ในช่วงอุณหภูมิ 30-50°C และ pH 5.5-8 $Hg^{2+}$, $Cd^{2+}$ และ $Ni^{2+}$ ยับยั้งการทำงานของเอ็นไซม์ไคติเนส ขณะที่ $Mg^{2+}$ และ β-mercaptoethanol

ส่งเสริมการทำงานของเอ็นไซม์ไคติเนสให้ดีขึ้น เอ็นไซม์ไคติเนสทั้งหยาบและบริสุทธิ์มีความคุณสมบัติย่อยสลายผนังเซลล์ของเชื้อราก่อโรคพืชหลายชนิด

ยีนควบคุมการสร้างเอ็นไซม์ไคติเนสชนิด *Chi40* ถูกโคลนจาก *S. aureofaciens* CMUAc130 โดยวิธี PCR cloning และทำการวิเคราะห์ยีนที่โคลนได้โดยแปลงเป็นลำดับกรดอะมิโน แล้วให้ชื่อว่า Chi40_Sau ซึ่งประกอบด้วยกรดอะมิโน 413 หน่วย น้ำหนักโมเลกุลประมาณ 40-KDa จากการวิเคราะห์ลำดับกรดนิวคลีอิก และลำดับกรดอะมิโนของยีนนี้ พบว่ามีความคล้ายกับยีน *ChiA* จาก *Streptomyces thermoviolaceus* ถึง 95% และ 87% ตามลำดับ ยีนที่โคลนได้นี้ถูกชักนำให้แสดงออกใน *E. coli* JM109 และสามารถตรวจสอบการแสดงออกโดยวิธี Western Blot เอ็นไซม์ไคติเนสที่สร้างจาก *E. coli* JM109/pChi40_Sau มีคุณสมบัติต้านเชื้อรา *Fusarium oxysporum* โดยยับยั้งการเจริญของเส้นใย ยับยั้งการงอกของสปอร์ และย่อยสลายผนังเซลล์ของ *F. oxysporum*

# LIST OF CONTENTS

## LIST OF CONTENTS (CONTINUED)

## LIST OF CONTENTS (CONTINUED)

## LIST OF CONTENTS (CONTINUED)

## LIST OF CONTENTS (CONTINUED)

## LIST OF CONTENTS (CONTINUED)

## LIST OF CONTENTS (CONTINUED)

# LIST OF TABLES

# LIST OF TABLES (CONTINUED)

# LIST OF ILLUSTRATIONS

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

## LIST OF ILLUSTRATIONS (CONTINUED)

# ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| A | absorbance |
| ATP | adenosine triphosphate |
| °C | degree celcius |
| ca | capacity |
| g | gram |
| h | hour |
| IPTG | isopropyl thio-β-galactoside |
| KDa | kilo Dalton |
| l | liter |
| M | Molar |
| mg | milligram |
| min | minute |
| ml | milliliter |
| MW | molecular weight |
| µg | microgram |
| µl | microliter |
| OD | optical density |
| rev | revolution |
| s | second |
| SD | standard deviation |
| U | unit |
| w/v | weight by volume |

# CHAPTER I

# GENERAL INTRODUCTION

Antagonistic microorganisms, by their interactions with various plant pathogens, play a major role in the microbial equilibrium serving as powerful agents for biological disease control which may involve antibiosis, competition, or exploitation. Exploitation processes are subdivided into predation and direct parasitism. The latter relies on lytic enzymes for the degradation of cell walls of pathogenic fungi. Cell walls of Basidiomycete and Ascomycete fungi contain chitin and many bacteria and fungi have been shown to produce hydrolytic enzymes after induction by the appropriate substrates. Several reports have demonstrated the biological control of *Fusarium* spp. and *Pythium* sp. by bacteria that degrade the cell wall of these plant pathogens (Hadar *et al.* 1983, Campbell 1983). Thus, biocontrol of plant pathogens provides an alternative means of reducing the incidence of plant disease without the negative aspects of chemical control. Chemical fungicides are costly, can cause environmental pollution, and may induce pathogen resistance. Additionally, they can cause developmental effects with young seedlings (Jones 1985).

Actinomycetes, especially *Streptomyces* spp., isolated from the rhizosphere have been a focus as excellent biocontrol agents to soil-borne plant diseases (Yuan and Crawford 1995). Such effective activity is largely dependent on secondary metabolites produced by these organisms. *Streptomyces* spp. are capable of producing a remarkably wide spectrum of antibiotics as secondary metabolites and the discovery

of new molecules still continues. Actinomycetes have been reported as biocontrol candidates against fungal plant pathogens for example several reports refer to the influence of metabolic products from actinomycetes on plant growth and physiology (Drautz and Zahner 1986, Schipper *et al.* 1987). Okazaki (1995) reported that the distribution of actinomycete genera in healthy plant leaves was different compared to soils and they show different physiological characteristics and HPLC profiles of culture broths. These facts mean that plant leaves might be one of the important sources for taxonomically rare actinomycetes as well as proving to be a reliable screening source for new metabolites. Furthermore, some strains of actinomycetes can produce chitinase (William and Robison 1981) and during the last decade, this enzyme has received increased attention due to its wider range of biotechnological applications, for example: in the biocontrol of fungal phytopathogens (Lim *et al.* 1991) in the possible new biopesticides (Shapairo *et al.* 1987) and through gene manipulation (Oppenheim and Chet 1992). Many bacterial chitinase genes from species including *Aeromonas hydrophila* (Chen *et al.* 1991), *Alteromonas* sp. O-7 (Tsujibo *et al.* 1993a), *Bacillus circulans* WL-12 (Watanabe *et al.* 1992), *Cellvibrio mixtus* (Wynne and Pemberton 1986), *Streptomyces liquefaciens* (Joshi *et al.* 1988), *Serratia marcescens* (Sundheim *et al.*, 1988), *Streptomyces lividans* 66 (Matashita *et al.* 1991), *S. plicatus* (Robbins *et al.* 1992), *S. olivaceoviridis* (Blaak *et al.* 1993), *S. thermoviolaceus* OPC-520 (Tsujibo *et al.* 1993b), *Vibrio harveyi* (Jannatipour *et al.* 1987), and *V. vulnificus* (Wortman *et al.* 1986) have been successfully cloned. Reports describing the characteristics of many chitinase genes are now available. Recently, Tsujibo (Tsujibo *et al.* 2000) reported the cloning and sequencing of chitinase gene (*chi35*) of *S. thermoviolaceus* OPC-520 and the characterization of its

translated product. They also showed the role of the *chi35* product in the hydrolysis of insoluble chitin and antifungal activity.

As to the ability of some strains of endophytic actinomycetes which can produce chitinase, these microorganisms might protect against phytopathogenic fungi in plant tissue by this property. Thus the role of chitinase of endophytic actinomycetes in antifungal activity should be studied.

**The objectives of this research**

1. To isolate and screen endophytic actinomycetes from plants for the production of chitinase and the secondary metabolites and assess their significance as biocontrol agents.
2. To isolate a chitinase gene from an actinomycete with proven biocontrol function and analyse its nucleotide sequence.
3. To construct and heterologously express a recombinant plasmid containing chitinase gene in *E. coli* JM109.
4. To evaluate the recombinant chitinase in antifungal activity.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Biology of actinomycetes

The order Actinomycetales includes bacteria that are characterized by the formation of branching filaments. In the more evolved forms of the order, this property results in a somewhat fungal appearance. Abundantly distributed in nature, actinomycetes may be separated into two large but unequal subgroups: the oxidative forms, which are very numerous and are basically soil inhabitants, and the fermentative types, which are primarily found in the natural cavities of man and animals (Lechevalier and Lechevalier 1967). In general, actinomycetes are Gram positive, but part of their thallus may be Gram negative. Certain filamentous, branching, Gram-negative bacteria belonging to the genus *Mycoplana* may be classified with the actinomycetes as a matter of convenience, although they are not phylogenetically related to the other members of the order. Other Gram-negative bacteria that reproduce by budding, such as members of the genus *Hyphomicrobium*, from branching filaments but have never been considered members of the *Actinomycetales*. Closely related to the actinomycetes are the corynebacteria and their relatives, the latter often described as "coryneforms". The mycobacteria, which are related to the corynebacteria by many properties, are usually more rod shaped than filamentous and are currently included in the order *Actinomycetales*. The separation between corynebacteria, mycobacteria, and some of the other pleomorphic bacteria is not easy. The same strain, depending on the observer, may be classified in one of the

genera *Corynebacterium*, *Arthrobacter*, *Mycobacterium*, or *Nocardia* (Gordon 1966). In general, actinomycetes will grow on ordinary laboratory media, but their growth is usually slower than that of ordinary bacteria. A division cycle in actinomycetes may take 2-3 h as compared with 20 min for *Escherichia coli*. Some actinomycetes grow even more slowly. *Mycobacterium tuberculosis*, for example, has a generation time of about 15 h under optimal conditions. Other actinomycetes, such as *Mycobacterium leprae*, have never been grown on laboratory media. Some endophytes of plants, placed in the genus *Frankia*, grow on present-day laboratory media only with the greatest difficulty. Actinomycetes are very important from a medical point of view as the agents of tuberculosis, nocardiosis, mycetomas, streptothricosis, allergic pneumonias, bovine farcy, paratuberculosis, actinomycosis, and various abscesses. They also cause a few plant diseases, including potato scab, a rot of sweet potato, and a disease of blueberry. Their main ecological role is in the decomposition of organic matter in soil which is their main habitat (Waksman 1959, 1961). They may be a nuisance, as when they decompose rubber products or grow in aviation fuel, they produce odorous substances that pollute water supplies, or grow in sewage-treatment plants where they form thick clogging foams (Lechevalier and Lechevalier 1967). In contrast, actinomycetes are the producers of most of the antibiotics those are useful not only in human and veterinary medicine but also in agriculture and in biochemistry (as metabolic poisons) (Lechevalier 1968). At first, actinomycetes were thought of only as pathogens. Ferdinand Cohn probably published the first description of an actinomycete in 1875 when he had observed a filamentous organism in a concretion from a human lacrimal duct and called it *Streptothrix foersteri* (Waksman 1959). Two years later, C.O. Harz gave the name *Actinomyces bovis* to an organism that he

observed in a case of bovine lumpy jaw (Waksman 1959). The name "actinomyces", which is of Greek derivation, means "ray-fungus", and actinomycetes are still referred to as "ray-fungi", particularly by German and Russian authors. The importance of actinomycetes in soil was largely realized through the work of Beijerinck, Krainsky, Conn, and Waksman during the first two decades of the last century (Waksman 1959). With these early studies, there began a controversy about the nature of actinomycetes. Some considered them to be filamentous bacteria, other minute fungi. The advent of electron microscopy and the development of our knowledge of the fundamental properties of microorganisms have resolved the controversy in favor of the bacteria (Lechevalier and Lechevalier 1967). There are, however, some minute fungi such as *Fusarium* spp. That can be mistaken for actinomycetes (Lechevalier *et al.* 1977). There is so much variation in the morphology of actinomycetes. Generally, one may say that on solid media most actinomycetes form a mycelium (substrate or primary) that grows on and into the agar. In addition, there may be a mycelium (aerial or secondary) growing away from the medium. In some cases, the primary mycelium is short-lived and soon breaks up into bacillary or coccoid elements that may be flagellated. In some species, the mycelium may be so transient as to escape notice, or be nonexistent. These atypical organisms are considered members of the Actinomycetales because they share other properties with more orthodox members of the order. Thus, actinomycetes form a morphological spectrum ranging from diphtheroid bacilli to filamentous forms with intricate modes of sporulation. The actinomycetes are separated into groups on the basis of physiology (fermentative vs. oxidative metabolism), morphology (type and stability of mycelium, types, number, and disposition of spores, formation of sclerotia, sporangia, or synnemata, formation

of flagellate elements), physical qualities (heat resistance), and chemistry (cell wall and whole cell composition, types of lipids, isoprenoid quinones). The fermentative actinomycetes are morphologically simple organisms. They form neither aerial mycelia nor spores. Their primary mycelium may be quite well developed or rudimentary. When assessing the morphology of an actinomycete, it is important not to distort or destroy the arrangement of hyphae and spores. Actinomycetes should be studied microscopically by the methods that are used for the study of molds. The *in situ* examination of cultures growing on agar plates is helped by the use of long-working distance condensers and objectives. Particular attention should be paid to the formation and location of the hyphae, the formation and arrangements of the conidia, the presence of sporangia (called spore vesicles by Cross and Attwell 1975), the release of motile elements, and the occurrence of special structures such as a sclerotia and spore-bearing synnemata. Some thermophilic actinomycete is distinguished by the presence of endospores having high heat resistance. In general, the G+C content of the DNA of actinomycetes is high, the mycobacteria and nocardiae being at the low end of this high spectrum (60-70 mol%). And the streptomycetes on the high side (70-75 mol%). Some thermophilic actinomycetes have DNAs with low G+C percentages (44-54 mol%) (Lechevalier *et al.* 1971). From a chemical point of view, cell wall composition has been found to be especially useful (Lechevalier and Lechevalier 1967). Most oxidative actinomycetes can be separated into four groups on the basis of their cell wall composition (Table 2.1), which is of the Gram-positive type. The most common cell wall types (I to IV) have peptidoglycans containing diaminopimelic acid. This amino acid, which occurs in three isomeric forms, is easy to detect in hydrolysates of whole cells. Two of the isomers, the *meso* and L forms,

are readily separable by paper chromatography. The D isomer is not readily separated from the *meso* form and is of unknown taxonomic significance.

**Table 2.1** Major constituents of cell walls of actinomycetes. (Modified from Mortineer *et al.* 1981)

| Cell wall type | Major constituents[a] | Genera | |
|---|---|---|---|
| | | Example | Total no.[b] |
| I | L-DAP[c], glycine | *Streptomyces* | 11 |
| II | Meso-DAP, glycine | *Micromonospora* | 5 |
| III | Meso-DAP | *Actinomodura* | 13 |
| IV | Meso-DAP, arabinose, galactose | *Nocardia* | 8 |
| V | Lysine, ornithine | *Actinomyces* | 1 |
| VI | Lysine (aspartic acid; galactose)[d] | *Oerskovia* | 4 |
| VII | DAB[e] glycine (lysine) | *Agromyces* | 1 |
| VIII | Ornithine | *Bifidobacterium* | 2 |
| IX | Meso-DAP, numerous amino acids | *Mycoplana* | 1 |

[a] All cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine and muramic acid.
[b] Total number of actinomycete genera known to have this cell wall type.
[c] DAP = 2,6-diaminopimelic acid.
[d] Bracketed constituents are variable.
[e] DAB = 2,4-diaminobutyric acid.

In most cases, it is also possible to recognize cell wall types II, III, and IV without having to isolate the cell walls. This is done by the detection of certain sugars that are found in whole-cell hydrolysates (Table 2.2) (Lechevalier 1968). In addition, some of the organisms with type IV cell walls produce α–branched, β-hydroxylated fatty acids called mycolic acids. These lipids fall into three broad groups on the basis of molecular weight: the largest are typical of the genus *Mycobacterium*, the smallest are produced by some members of the genus *Corynebacterium*, and those of intermediate molecular weight are found in species of *Nocardia* (Lechevalier 1965). Phospholipid patterns have recently been shown to cast considerable light on the interrelationships of various aerobic actinomycete genera (Lechevalier and Lechevalier 1967).

**Table 2.2** Cell wall types and whole cell sugar patterns of aerobic actinomycetes containing *meso*-diaminopimelic acid.[a] (Modified from Mortineer *et al.* 1981)

| Cell wall | | Whole cell sugar pattern | |
|---|---|---|---|
| Type | Distinguishing major constituents[b] | Type | Diagnostic sugars |
| II | Glycine | D | Xylose, arabinose |
| III | None | B | Madurose[c] |
| | | C | None |
| IV | Arabinose, galactose | A | Arabinose, galactose |

[a] No differentiation is made between *meso*-DAP and D-DAP.
[b] All cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine and muramic acid.
[c] Madurose = 3-*O*-methyl-D.galactose.

Five groups (Table 2.3) are recognizable on the basis of their content of nitrogenous phospholipids (Mortineer *et al.* 1981). More detailed data are given at the end of this Introduction. The genera of actinomycetes whose members have a type I cell wall are listed in Table 2.4. The most important genus of the group is *Streptomyces*. Streptomycetes are abundantly distributed in soil and are the source of most of the antibiotics in current have been described probably exceeds a thousand, although it is felt by many investigators that several different names have probably been given to the same species (Mortineer *et al.* 1981).

**Table 2.3** Phospholipid patterns of aerobic actinomycetes (Modified from Mortineer *et al.* 1981).

| Phospholipid pattern | Characteristic phospholipids[a] | | | | |
|---|---|---|---|---|---|
| | PE | PME | PC | GluNU | PG |
| PI | -[b] | - | - | - | V[c] |
| PII | +[d] | - | - | - | - |
| PIII | V | V | + | - | V |
| PIV | V | V | - | + | - |
| PV | - | - | - | + | + |

[a] PE, phosphatidyl ethanolamine; PME, phosphatidyl methylethanolamine; PC, phosphatidyl choline; GluNU, Phospholipids of unknown structure containing glucosamine; PG, Phosphatidyl glycerol. Other phospholipids of no taxonomic value may be present.
[b] Not present.
[c] Variably present.
[d] Present.

**Table 2.4** Genera of Actinomycetales with a type I cell wall (Modified from Mortineer *et al.* 1981).

| Generic name | Morphological characteristics |
|---|---|
| *Streptomyces* | Aerial mycelium with chains (usually long) of nonmotile conidia. |
| *Streptoveticillium* | Same as *Streptomyces*, but the aerial mycelium bears verticils consisting of at least three side branches, which may be chains of conidia or hold sporulating terminal umbels. |
| *Nocardioides* | Both substrate and aerial mycelium fragment into rod- and coccus-shaped elements. |
| *Chainia* | Same as *Streptomyces*, but sclerotia are also formed. |
| *Actinopycnidium* | Same as *Strep tomyces*, but pycnidia-like structures are also formed. |
| *Actinosporangium* | Same as *Streptomyces*, but spores actcumulate in drops. |
| *Elytrosporangium* | Same as *Streptomyces*, but merosporagia are also formed. |
| *Microellobosporia* | No chains of conidia; merosporangia with nonmotile spores are formed. |
| *Sporichthya* | No substrate mycelium is formed; aerial chains of motile, flagellated conidia are held to the surface of the substratum by holdfasts. |
| *Intrasporangium* | No aerial mycelium; substrate mycelium forms terminal and subterminal vesicles. |
| *Arachnia* | No aerial mycelium; substrate mycelium is branched and may fragment. |

The genera of actinomycetes with a type II cell wall are listed in Table 2.5. The genera *Micromonospora* and *Actinoplanes* are the two most frequently encountered members of this group. Micromonosporae favor water-logged soils and actinoplanetes are found on organic matter decomposing at the edge of water bodies and in soils rich in organic matter.

**Table 2.5** Genera of Actinomycetales with a type II cell wall (Modified from Mortineer *et al.* 1981).

| Generic name | Morphological characteristics |
|---|---|
| *Micromonospora* | Aerial mycelium absent. Conidia single. |
| *Actinoplanes* | Globose to lageniform sporangia; globose spores with one polar tuft of flagella. |
| *Amorphosporangium* | Same as Actinoplanes, but the sporangia are often very irregular; sporangiospores are usually nonmotile. |
| *Ampullariella* | Lageniform to globose sporangia; rod shaped spores with one polar tuft of flagella. |
| *Dactylosporangium* | Claviform sporangia, each with one chain of spores with one polar tuft of flagella. |

Actinomycetes with a type III cell wall (Table 2.6) composed of morphologically physiologically, and ecologically very different organisms. From a chemical point of view, one can recognize two kinds: those with and those without

madurose (3-*O*-methyl-D-galactose). This compound, though not a cell wall constituent, appears to be constantly present in the organisms, that produce it. The madurose-positive group includes the pathogens *Actinomadura madurae* and *Dermatophilus congolensis*. The madurose negative thermoactionomycetes form true heat-resistant bacterial endospores, and may cause serious allergic reactions. Actinomycetes whose members have cell walls of type IV and include the important genera *Mycobacterium* and *Nocardia* are listed in Table 2.7. The separation of actionomycetes into families is not satisfactory. Some of the actinomycete families and genera are delineated in the key presented in Table 2.8.

**Table 2.6** Genera of Actinomycetales with a type III cell wall (Modified from Mortineer *et al.* 1981).

| Generic name | Morphological characteristics |
|---|---|
| *Thermoactinomyces* | Single spores are formed on the aerial and substrate mycelia; the spores are heat-resistant endospores. |
| *Thermomonospora* | Single spores are formed on the aerial mycelium or on both the aerial and substrate mycelia; the spores are not heat-resistant endospores. |
| | Longitudinal pairs of conidia on the aerial mycelium. |
| *Microbispora* | Short chains of conidia on the aerial mycelium. |
| *Actinomadura* | Same as Actinomadura; number of spores in chain not exceeding 6; |
| *Microtetraspora* | usually 4. |
| | Very long chains of conidia on the aerial mycelium. |
| *Nocardiopsis* | Single and short chains of conidia on both the aerial and the substrate |
| *Excellospora* | mycelia. |
| | Cylindrical sporangia, each containing one motile spore with one polar |
| *Planomonospora* | tuft of flagella. |
| | Cylindrical sporangia, each containing two motile spore with |
| *Planobispora* | peritrichous flagella. |
| | Globose sporangia containing nonmotile spores. |
| *Streptosporangium* | Globose sporangia with rod-shaped spores, each with a subpolar tuft of |
| *Spirillospora* | fragella. |
| | Formation of synnemata bearing chains of motile conidia. |
| *Actinosynnema* | Hyphae dividing in all planes, forming packets of cocci motile by |
| *Dermatophilus* | means of a tuft of flagella; pathogenic to animals. |
| | Similar to Dermatophilus; nonpathogenic; found in soil. |
| *Geodermatophilus* | Sporangia with nonmotile spores. Grow in symbiotic association with |
| *Frankia* | roots of higher plants. Fix nitrogen. Culture in vitro with difficulty. |

**Table 2.7** Genera of Actinomycetales with a type IV cell wall (Modified from Mortineer *et al.* 1981).

| Generic name | Morphological characteristics |
|---|---|
| *Mycobacterium* | Filamentation is usually limited, and aerial mycelium is usually not formed; filaments fall easily apart into rods and cocci. |
| *Nocardia* | Filamentation is abundant, and aerial mycelium is often formed; chains of conidia may be formed. |
| *Micropolyspora* | Short chains of globose conidia are formed on both the aerial and the substrate mycelia. |
| *Pseudonocardia* | Long, cylindrical conidia in chains on the aerial mycelium. |
| *Saccharomonospora* | Single spores, mainly on the aerial mycelium. |
| *Saccharopolyspora* | Morphology similar to that of *Nocardiopsis*. |
| *Bacterrionema* | No aerial mycelium; substrate mycelium is branched and fragments. Filaments often terminate in a bacillus-like body. |
| *Actinopolyspora* | Long chains of conidia on the aerial mycelium; substrate mycelium may fragment. Halophile. |

**Table 2.8** Key to some families and genera of the Actinomycetales.[a](Modified from Mortineer *et al.* 1981).

| | |
|---|---|
| Fermentative, nonmotile organisms, CW I, IV, V, VI, or VII | *Actinomycetaceae* |
| Facultatively fermentative; hyphae breaking into motile elements. CW VI; Phos P V. | *Oerskovia* |
| Oxidative metabolism. | |
| Hyphae dividing in more than one plane to form masses of motile cocci. CW III; WCS B or C; Phos P I or Phos P II. | *Dermatophilaceae* |
| Symbionts in plant nodules; hyphae dividing in more than one plane; sporangia present, nonmotile spores formed. CW III. | *Frankia* |
| Hyphae dividing only perpendicularly to their long axis. | |
| Gram-negative; rudimentary branching; breaking into motile elements. Cell wall with *meso*-diaminopimelic acid. Phos P III | *Mycoplana* |
| Gram-positive. | |
| Mycelium usually rudimentary or absent; often acid-fast. CW IV; WCS A; Phos P II. | *Mycobacterium* |
| Little or no aerial mycelium; no spores. Non motile. Vegetative hyphae may fragment. | |
| CW IV; WCS A; Phos P II. Nocardomycolates present. | *Nocardia, Rhodococcus* |
| CW VI; Phos P V. Nocardomycolates absent. | *Promicromonospora* |
| CW I; Phos P IV. Vegetative hyphae bear terminal and subterminal vesicles. | *Intrasporangium* |
| Aerial hyphae sparse to abundant; may fragment. No spores. | |
| CW IV; WCS A; Phos P II. Nocardomycolates present. | *Nocardia* |
| CW I; Phos P I. Nocardomycolates absent. | *Nocardioides* |
| Single spores produced. | |
| Heat-sensitive spores on primary mycelium only; no to sparse aerial mycelium. Usually mesophilic. CW II; WCS D; Phos P II. | *Micromonospora* |
| Heat-sensitive spores on aerial and vegetative mycelium. Usually thermophilic. CW III; WCS B or C. | *Thermomonospora* |
| Heat-sensitive spores on aerial mycelium only. Vegetative hyphae may fragment. Occasional pairs of spores may occur. Thermophilic and mesophilic. CW IV; WCS A; Phos P II. | *Saccharomonospora* |
| Heat-resistant endospores on aerial and vegetative mycelium. Usually thermophilic. CW III; WCS B or C. | *Thermoactinomyces* |
| Pairs of spores produced longitudinally. | |
| Spores on the aerial mycelium only. CW III; WCS B; Phos P IV. | *Microbispora* |

| | |
|---|---|
| Spores on the primary and aerial mycelium. CW IV; WCS A; Phos P III. No nocardomycolates. | *Micropolyspora* |
| Spore chains produced on aerial mycelium only, except as noted. | |
| Chains of 1-6 spores (average 4). CW III; WCS B; Phos P I or P IV. | *Microtetraspora* |
| Chains of 2-35 spores. | |
| CW III; WCS B; Phos P I or P IV. | *Actinomadura* |
| CW III; WCS B; P? | *Excellospora* |
| Chains of 4 to more than 100 spores. Chains may be straight, flexuous, spiral or verticillate. | |
| CW I; Phos P II. | *Streptomyces*<br>*Streptoverticillium* |
| CW III; WCS C; Phos P III. | *Nocardiopsis* |
| CW IV; WCS A; Phos P II. Nocardomycolates present. | *Nocardia* |
| CW IV; WCS A; Phos unknown. Nocardomycolates absent. | *Saccharopolyspora* |
| CW IV; WCS A; Phos P III. Mycolates unknown. Halophilic. | *Actinopolyspora* |
| Chains of long cylindrical spores formed by budding, redividing to form shorter spores. CW IV; WCS A; Phos P III. No Nocardomycolates. | *Pseudonocardia* |
| Chains of spores that aggregate to sticky masses. CW I. | *Streptomyces* |
| Chains of spores on both aerial and primary mycelia. CW I; Phos P II. | *Streptomyces* |
| Chains of about 2-15 spores on primary and aerial mycelia and at agar surface. | |
| Nocardomycolates present. CW IV; WCS A; Phos P II. | *Micropolyspora* |
| Nocardomycolates absent. CW IV; WCS A; Phos P III. | *Micropolyspora* |
| Chains of spores on the aerial mycelium; podlike structures on the primary mycelium. CW I. | *Elytrosporangium* |
| Sporangia present containing; | |
| Single motile spores. Aerial hyphae abundant. CW III; WCS B; Phos P IV. | *Planomonospora* |
| Pairs of motile spores. Aerial hyphae abundant. CW III; WCS B; Phos P IV. | *Planobispora* |
| Single row (3-6) of motile spores. Aerial hyphae scant. CW II; WCS D; Phos P II. | *Dactylosporangium* |
| Single row (2-6) of nonmotile spores. Aerial hyphae abundant. CW I; Phos P II. | *Microellobosporia* |
| Coils or rows of motile, round spores. Aerial hyphae scant. CW II, VI or VIII; WCS D; Phos P II. | *Actinoplanes* |
| Rows of motile, rod-shaped spores. Aerial hyphae scant. CW II WCS D; Phos P II. | *Ampullariella* |
| Coils of motile, rod-shaped spores. Aerial hyphae scant. CW II; WCS D; Phos P II. | *Spirillospora* |
| Coils of nonmotile spores. Aerial hyphae scant. CW II; WCS D; Phos P II. | *Amorphosporangium* |
| Coils of nonmotile round spores. Aerial hyphae abundant, CW III; WCS B; Phos P IV. | *Streptosporangium* |
| Sclerotia present; long chains of spores also formed by most strains. CW I; Phos P II. | *Chainia* |
| Pycnidia-like structures formed. Long chains of spores on aerial mycelium. CW I. | *Actinopysnidium* |
| Synnemata present bearing chains of motile rod-shaped spores. CW III; WCS C; Phos P II. | *Actinosynnema* |

[a] CW, cell wall composition; WCS, whole cell sugar pattern; Phos, phospholipid pattern.

## 2.2 Taxonomy of *Streptomyces*

A whole array of taxonomic tools has been used to define genera and suprageneric groups of actinomycetes (Goodfellow 1989, Embley and Stackebrandt 1994), but partial sequence analysis of 16S ribosomal RNA is the most significant. An abridged tree base on this analysis is shown in Figure 2.1 to give a flavor of the diversity of the actinomycete families and the place of the genus *Streptomyces* in the group.

### The genus *Streptomyces*

The genus is defined by both chemotaxonomic and phenotypic ("phenetic") characters. The major emphasis is now on 16S rRNA homologies, in addition to cell wall analysis and fatty acid and lipid patterns (Williams *et al.* 1989, Wellington *et al.* 1992). One of the quickest methods for preliminary identification to genus level was the presence of the LL isomer of diaminopimelic acid (LL – DAP) as the diamino acid in the peptidoglycan. This feature, when combined with the characteristic substrate and aerial mycelium, was diagnostic for *Streptomyces*. However, it turned out that some strains, proviously classified as *Kitasatosporis* may contain major amounts of DL – DAP in the vegetative mycelium only (Wellington *et al.* 1992) species from this former genus were found by Wellington *et al.* (1992) to lie within the 16S rRNA clade for *Streptomyces*, but this was recently contradicted when more strains were studied (Zhand *et al.* 1997).

The genus *Streptomyces* has been subject to numerous systematic studies during the past 30 years but it is still difficult to identify unknown isolates. Many type species have been described, but there has been much over-speciation resulting from antibiotic patents and the consequent need to assign a name to the producing

organism. Since the International *Streptomyces* Project in 1964, an attempt was made to produce valid species descriptions with at least a minimal number of standard phenotypic criteria. However, the criteria turned out to be too minimal and the proliferation of species continued without any real attempt to compare species thoroughly with each other. The first study to do this relied on numerical phenetic techniques to define clusters of strains or species based on comparison of many on numerical phenetic techniques to define clusters of strains or species based on comparison of many phenotypic traits (Williams *et al.* 1983). This established 23 major clusters and some 20 minor groups; (Williams *et al.* 1989). Because the Williams *et al.* (1983) study included only one representative for each species, very many species were reduced to synonyms. However their specific epithets are still in constant use; for example *S. griseus* is synonymous with *S. anulatus*, *S. lividans* with *S. violaceoruber*, and *S. hygroscopicus* with *S. violaceoniger*. The major species groups (Williams *et al.* 1983), are given in Figure 2.2, with some minor groups of interest also included. The names are those of the oldest extant type species within each cluster.

A second numerical phenetic analysis was made by Kampfer *et at.* (1991); it differed from the earlier study in including many more species, and more than one strain of each species when available. Many of the clusters defined by Williams *et al.* (1983) were recognized; an example is the *albidoflavus/anulatus/griseus/halstedii* group, appearing as cluster 1 in both studies, in which 28 of the *S. griseus* strains were grouped. Despite the problems associated with phenotypic characterization, most of the strains sharing the same specific epithet were grouped together, indicating previously reliable identification. But there were some notable exceptions; for

example *S. hygroscopicus* strains were recovered in cluster 1 subclusters 6, 8, 9, 10, 13, 24, 25, 35, 53, 54, 55, 56, 57 and 85. This may indicate either problems in the identification of the group or considerable phenotypic variation.

Several studies have attempted to use sequence data from variable regions of 16S rRNA to establish taxonomic structure within the genus, but the variation was regarded as too limited to help resolve problems of species differentiation (Stackebrandt *et al.* 1991). For example, species with a phenotype characteristic of the *Streptoverticillia* grouped as a clade, but were not distinct from other species in the genus, in contrast to the results of the phenotypic analysis but were not distinct from other species in the genus, in contrast to the results of the phenotypic analysis. (clusters 55, 56, 58 and 59). The close phenotypic relationship between *S. lavendulae* and the *Streptoverticillial* species (Figure 2.2) was also confirmed by 16S rRNA sequence comparisons (Stackedbrandt *et al.* 1990). The type species of the genus, *S. albus*, retained a distinct position in the phylogenetic trees and had unique sequences in the variable $\alpha$ and $\beta$ regions of the gene (Stackebrandt *et al.* 1991).

Total DNA homology studies (Labeda 1992) have indicated genetic heterogeneity within some of the large phenotypic species groups defined by numerical taxonomy. The *S. cyaneus* cluster 18 was studied in detail: strains showed DNA relatedness of 20-85% with the majority of values around 50% (Labeda and Lyons 1991). Selected species within the cluster were reduced to synonymy with others if the comparisons gave homology values > 70%.

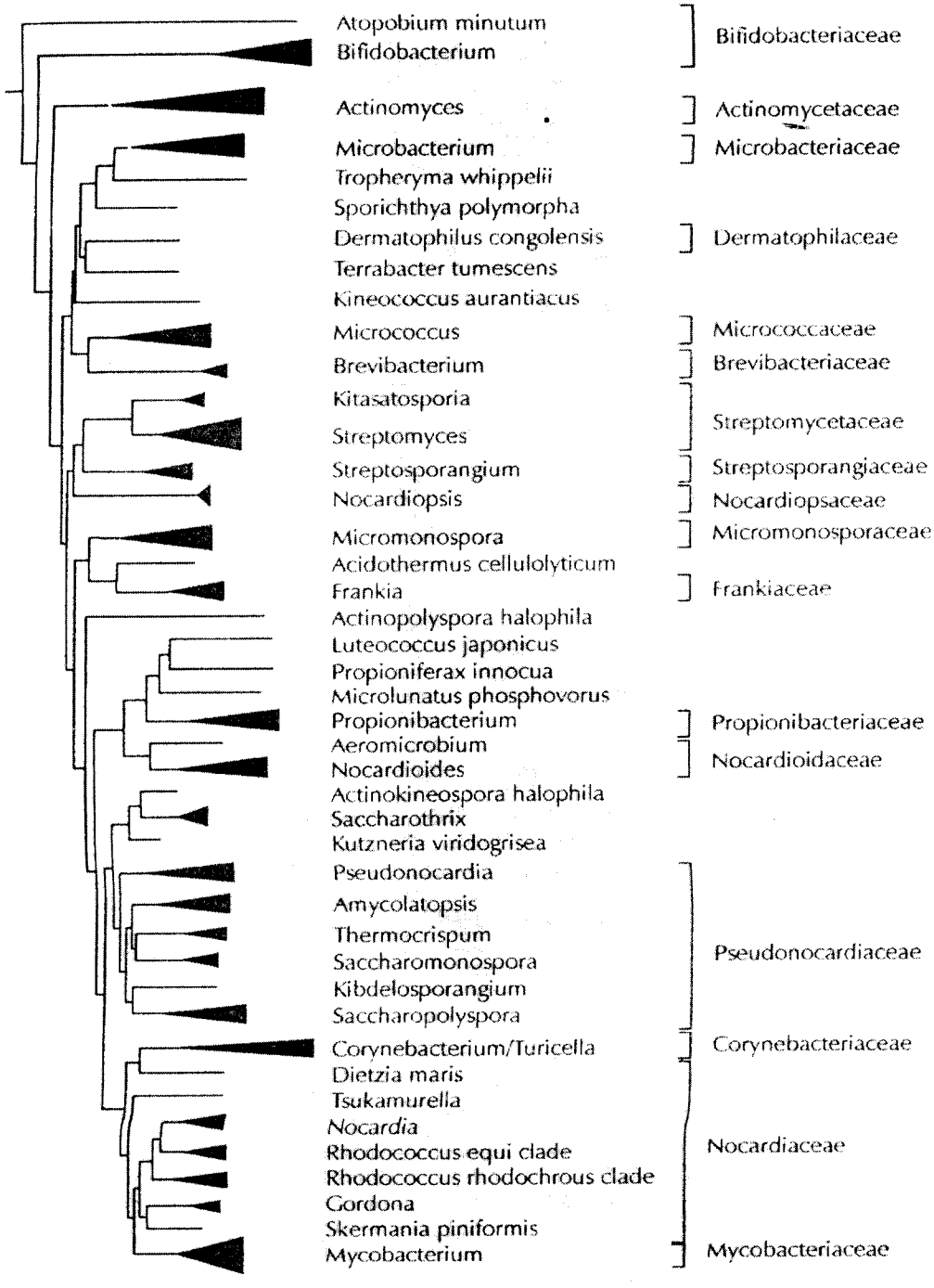


**Figure 2.1** Abridged phylogenetic tree of actinomycetes based on almost full 16S ribosomal RNA sequences. The tree was constructed by using the neighbour joining method with *Bacillus subtilis* as the outgroup. There are now over 100 balidly described actinomycete genera, but not all have been assigned to families. Data were based on Williams *et al.* (1983).

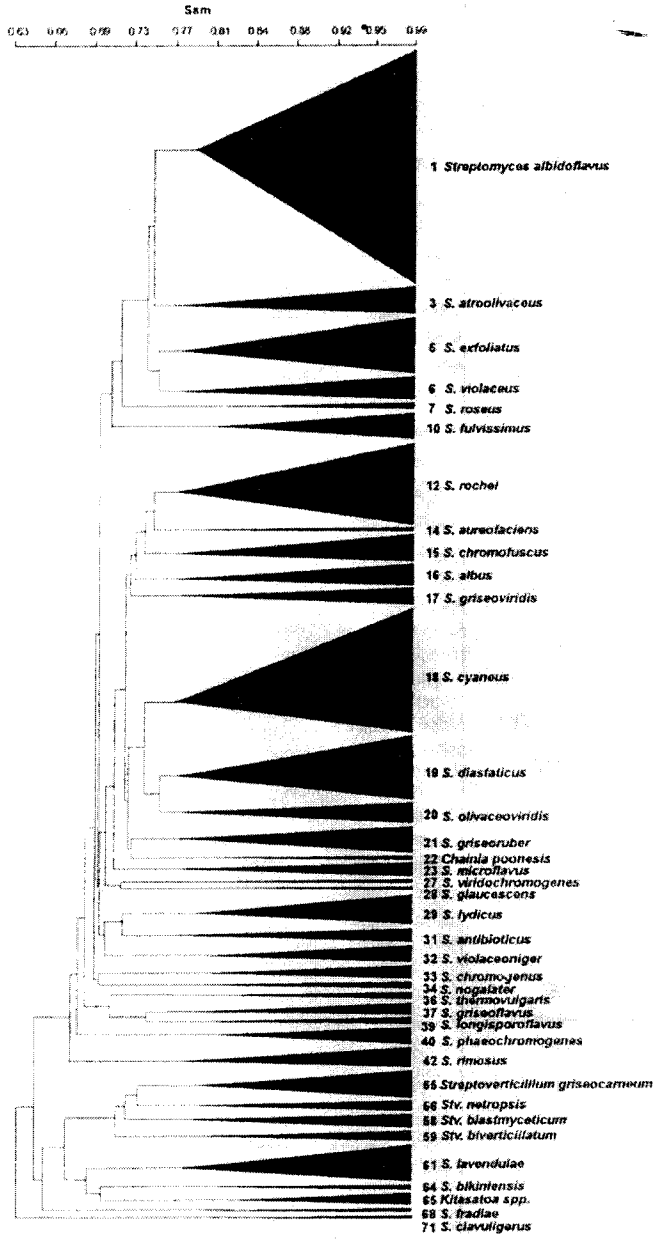


**Figure 2.2** Dendrogram derived using phenotypic characters for selected major clusters derived using simple matching coefficient (Ssm) and clustering by the unweighted pair group method based on arithmetic averages (UPGMA). Data were based on Williams *et al.* (1983).

## 2.3 Ecology of *Streptomyces*

Streptomycetes are ubiquitous in nature. Their ability to colonize the soil is greatly facilitated by growth as a vegetative hyphal mass which can differentiate into spores that assist in spread and persistence. The spores are a semi-dormant stage in the life cycle and can survive in soil for long periods (Ensign 1978): viable *Streptomyces* cultures were recovered from 70 year old soil samples (Morita 1985). The spores impart resistance to low nutrient and water availability, whereas the mycelial stage is sensitive to drought (Karagouni *et al.* 1993). The relatively high numbers of Streptomycetes in soil exist largely as inactive spores for most of the time. When laboratory-grown spores were added to non-sterile soil, they exhibited very low germination efficiencies, probably because of competition with indigenous microorganisms, but pre-germinated spores grew for a short time and then re-sporulated (Lloyd 1969). Germination can be partially density-dependent, but the interaction did not cross species boundaries (Triger *et al.* 1991), suggesting special signaling factors between spores of the same strain, causing inhibition of germination above a certain concentration. The advantage would be to limit the number of germinating propagules in accordance with as available resources. Spore germination requires exogenous nutrients, water and $Ca^{2+}$ (Ensign 1978). And nutrient status of the germination site limits the extent of hyphal growth and the time to differentiation into aerial hyphae and eventually spores (Wellington *et al.* 1992).

Actinomycetes produce many extracellular enzymes in soil. By decomposing complex mixtures of polymers in dead plant, animal and fungal material (McCarthy and Williams 1992, Wang *et at.* 1989), they are important in soil biodegradation and the recycling of nutrients associated with recalcitrant polymers (McCarthy and Williams, 1992). Addition of chitin to acidic soil led to chitinolytic activity of

acidophilic Streptomycetes, with subsequent ammonification of the soil and eventual colonisation by neutrophiles (Williams and Robinson, 1981). The enzymes required to catabolise such a common material as straw are very complex and recently much effort has been devoted to characterizing the cellulases, xylanases, amylases, maltases, etc, involved (McCarthy and Williams 1992).

In addition to their ability to conlonise bulk soil, many Streptomycetes successfully colonize the rhizosphere (Watson and Williams 1974). This ability may be partly due to the antagonistic characteristics of Streptomycetes in competition with other rhizosphere bacteria such as pseudomonads and bacilli. An added advantage over Gram-negative soil bacteria is their ability to spread through relatively dry soil via hyphal growth; in wetter soil, motile bacteria such as *Pseudomonas fluorescens* show more extensive colonization of rhizospheres than Streptomycetes (Karagouni *et al.* 1993).

## 2.4 Chitinase and Chitinolytic enzyme

Chitin, an insoluble linear β-1,4-linked polymer of *N*-acetyl-(D)-glucosamine (GlcNAc or NAG), is the second abundant polysaccharides in nature, highly resistant to organic solvents and requires strong mineral acids for solubilization (Jeuniaux 1966, Flach *et al.* 1992). Chitin could be served as major carbon and nitrogen sources (Flach *et al.* 1992, Clarke 1956) for microorganisms similar to cellulose. In general, growth on these carbohydrate polymers is associated with the secretion of a number of "endo" and "exo" enzymes that act at random internal sites or the non-reducing ends of polymer chains. These enzymes have differing enzymatic properties, but are probably induced coordinately. As in the case of cellulose degradation, the major

product of extracellular hydrolysis of chitin is a disaccharide. Other postulated steps in chitin utilizations are transport of the disaccharide into the cell, hydrolysis of the disaccharide to acetylglucosanime, phosphorylation, deacetylation and deamination of acetylglucosamine. It will be interesting to determine whether these steps are regulated in a coordinate fashion with the synthesis and secretion of the extracellular chitinase. There is little information concerning the regulation of any of these steps (Vorgias *et al.* 1992).

Chitin is present in diatoms, yeast, fungi, protozoans, arachnids, insects, crustaceans, nematodes, some tunicates and other invertebrates (Jeuniaux 1966, Fuchs *et al.* 1986, Flach *et al.* 1992). These chitin containing organisms produce chitinases. Some organisms which do not contain chitin also produce chitinases, which catalyse the hydrolysis of chitin for example plants and bacteria (Flach *et al.* 1992, Trudel and Asselin 1989). Because of the abundance of this compound in nature and the possibility of its conversion to useful products, increased attention has been focused on chitinolytic microorganisms.

Microbial conversion of chitin to NAG requires the sequential action of two hydrolytic enzymes: (i) chitinase (chitin glucohydrolase) and (ii) chitobiase (acetylaminodexoglucohydrolase). Some bacterial species produce both enzymes (chitinolastic) while others exhibit activity of either enzyme (Jeuniaux 1966). The enzymes appear to be coordinately controlled and in different organisms can be induced by chitosan, chitobiose, *N*-acetylglucosamine, or glucosamine. Chitinolytic enzymes are found in bacteria, fungi, plants, and invertebrates (Jeuniaux 1966, Flach *et al.* 1992), for example, bacteria from the genera *Streptomyces*, *Aeromonas* and *Serratia*, and fungi *Gliocladium* and *Trichoderma* (Chen *et al.* 1991, Clarke 1956).

Better knowledge of chitinolytic enzymes is useful for several applications: they have been implicated in the biological control of plant pathogenic fungi (Oppenheim and Chet 1992, Romaguera *et al.* 1992) by degrading the newly synthesized chitin at the hyphal tips (Beyer and Diekmann 1985). They may be useful in degradation of chitin-containing waste, e.g., crab shells, and they may be used for chemical modification of chitin and chitosan (Chernin *et al.* 1995).

Chitin application leads to increase populations of chitinolytic bacteria, especially actinomycetes, and fungi. These increases are correlated with reductions of pathogenic fungi and nematodes and more importantly, with the reduction of infectivity and hence crop damage. Although the evidence for the role of chitinase in fungi and nemathodes control is indirect, the correlation is strong and suggestive (Fuchs *et al.* 1986). There are a number of ways by which chitinases could be used to protect growing plants against pathogenic fungi. The free enzyme could be introduced into the irrigation water or incorporated into a coating around the seed to protect germinating seedlings. However, the activity of free enzyme in the soil is probably rather short (Clarke 1956, Gopalakrishnan *et al.* 1995). The isolation and cloning of chitinase determinants from naturally occuring organisms can provide an efficient source of such enzymes, such as, Actinomycetes (*S. plicatus, S. lividans*), bacteria (*Serratia marcescens, S. liquefaciens, B. circulans, A. hydrophila, Ps. maltophilia*), fungi (*Trichoderma harzianum, Vibro vulnificus*), and yeast (*Saccharomyces cerevisiae, Aphanocladium album*) (Tsujibo *et al.* 1993, Fuchs *et al.* 1986, Wortman *et al.* 1986, Felse and Panda 1999).

**Determination and Nomenclature**

Enzymes that hydrolyze chitin are categorized as hydrolytic enzyme systems. Chitinases are designated as endo- or exoenzymes according to where hydrolytic cuts are adminstered within chitin chains and activity is logged according to their reactions with structurally defined isomers of chitin aggregates (Bade and Hickey 1988). Chitinases are defined as enzymes cleaving a band between the C1 and C4 of two consecutives *N*-acetylglucosamines of chitin (Flach *et al.* 1992). In general, two chitinolytic enzymes, chitinase and *N*-acetyl-β-glucosaminidase, are recognized chitinolastic activity (Jeuniaux 1966, Flach *et al.* 1992, Chernin *et al.* 1995). In addition, the term chitinase may be used to refer to any enzyme with chitinolytic activity, or may refer only to endochitinases. Chitobiase is considered to be synonymous with *N*-acetyl-β-D-glucosaminidase or *N*-acetyl-β-glucosaminidase or β-*N*-acetylglucosaminidase (Chernin *et al.* 1995, Beyer and Diekmann, 1985). The degree of activity for chitobiase and chitinase was not necessarily directly correlated (O'Brien and Colwell 1987). Chitinases randomly cleave chitin polymers (polymers of *N*-acetyl-D-glucosaminidase hydrolyses chitobiose (the dimer of *N*-acetyl-D-glucosamine) and chitotriose (Jeuniaux 1966). Thus, chitobiase hydrolyzes non reducing *N*-acetyl-β-glucosamine residues from terminal non-reducing ends of chitobiose and higher analogues (Chernin *et al.* 1995).

Endochitinases, exochitinases, *N*-acetyl-β-D-glucosaminidase and chitobiases have been characterized (Flach *et al.* 1992, Oppenheim and Chet 1992).

Exochitinase activity can be defined by its progressive action starting at the non-reducing end of the chitin chains, and release of diacetylchitobiose units (Flach *et al.* 1992, Oppenheim and Chet 1992) from the chitin chain as the sole product (Bade and Hickey 1988), including the activity of enzymes that release monomeric units

from chitin. The term chitobiosidase (Chernin *et al.* 1995) or chitibiosidase (Romaguera *et al.* 1992), is the exochitinase, it requires at least the trimer for activity (Chernin *et al.* 1995). Supportly, during the enzymatic degradation of chitin, disaccharide, presumedly chitobiose was found. *N*-acetyl-glucosamine occurred only in small amounts indicating exo-splitting activity in such a way that dimeric units were cleaved from the end of the chitin molecule. Commercial chitobiose and the dimeric product were not split by the exochitinase but rapidly split by *N*-acetyl-β-D-glucosaminidase, which had no activity against chitin (Beyer and Diekmann 1985).

Endochitinase is defined as an enzyme catalyzing the random hydrolysis of 1,4-β-linkages of NAG at internal sites over the entire length of chitin microfibrils (Oppenheim and Chet 1992, Romaguera *et al.* 1992, Bade and Hickey 1988). It requires at least the tetramer for activity (Chernin *et al.* 1995).

A different approach for classification of endo and exo chitinases based on relative activities toward fibrous and collapsed chitin has been proposed by Bade and Hickey (Bade and Hickey 1988). In the presence of fibrous particles, or in a mixture of fibrous and collapsed chitin, reduction in particle size through exochitinase attack will be slow compared to the effect of endochitinase on the same particles. Pure exochitinase will digest the fibrous elements while leaving collapsed particles visible even after lengthy digestion. Whereas the ultimate outcome of endochitinase attack is that no residue remains that is visible to the naked eye or microscopically, since endochitinases hydrolyse bonds randomly in the interior of chitin strands, both collapsed and fibrous chitins.

*N*-acetyl-β-glucosaminidase (*N*-acetyl-β-1,4-D-glucosaminidase or *N*-acetylglucosaminidase or β-*N*-acetylglucosaminidase) is a chitinolytic enzyme which

also acts in exo-splitting mode on dimer and higher analogues of chitin (Oppenheim and Chet 1992, Romaguera *et al.* 1992), releasing *N*-acetylglucosamine monomers from chitin (Flach *et al.* 1992, Oppenheim and Chet 1992, Romaguera *et al.* 1992), same as chitobiase hydrolyses chitobiose (Flach *et al.* 1992, Chernin *et al.* 1995). *N*-acetyl-β-glucosaminidase requires at least the dimer of chitin for activity (Chernin *et al.* 1995), is also active against *N*-acetylhexosaminidase (Beyer and Diekmann 1985).

Some chitinases also display a more or less pronouced lysozyme activity corresponding to the cleavage of a glycosidic bond between the C1 of *N*-acetylmuramic acid (Mur-NAc) and the C4 of *N*-acetylglucosamine in the bacterial peptidoglycan. Transglycosidase activities associated with exochitinase activities have also been detected.

Chitinolastic activity is demonstrated by a wide variety of microorganisms (Jeuniaux 1966, Wortman *et al.* 1986, Flach *et al.* 1992), plants and animals (Flach *et al.* 1992). The enzymes involved in chitin degradation are quite stable, withstanding overnight incubation in toluene, even in the absence of substrate (Jeuniaux 1966, Wortman *et al.* 1986, Trudel and Asselin 1989). Extracts of toluene-killed cells show persistent and measurable acetylglucosamine production from chitin for more than 10 days after treatment (Trudel and Asselin 1989).

Furthermore, endochitinase and chitobiase appear to be coordinately controlled and can be induced by chitosan, chitobiose, *N*-acetylglucosamine, or glucosamine (Jeuniaux 1966, Wortman *et al.* 1986).

**Occurance of chitinases**

1. Bacterial chitinases

Chitin decomposition is known to be initiated by a large number of chitinase producing bacteria isolated from marine environments, soil and fresh water (Wortman *et al.* 1986, Trudel and Asselin 1989). A number of cultures of bacteria of medical provenance as well as soil and plant pathogenic organisms were screened for chitinase.

2. Plant chitinases

Chitinases may be produce constitutively and are found at low levels in healthy plant, and their expression is increased along with the induction of other components. These include other antimicrobial compounds, eg protease inhibitors and a β-1,3-glucanase, which form a plant defense system during attack by pathogens (Jeuniaux 1966, Flach *et al.* 1992).

Purified plant enzymes have been shown to hydrolyse isolated fungal cell walls, inhibit the growth of pathogenic fungi in culture, and inhibit the induction of the chitinase promoter associated with the plant response to infection (Flach *et al.* 1992).

Three classes of chitinases have been characterized. It appears that all three can be present in the same plant and their occurrence is not correlated with phylogenetic classification, but the major chitinase produced in different materials does not always belong to the same class.

Class I chitinases are found in the basic chitinases of tobacco, potato, *Arabidopsis* and *Populus* (*chiX*). Class I chitinases possess a leucine rich or valine rich signal peptide. They usually have a basic isoelectric point and are located in the vacuole. However Win6 and Win8 from poplar and PR4 from bean are acidic. RP4 is

extracellular. Class I chitinase was divided into two subclasses: class I a for the basic chitinases and class I b for the acidic ones (Flach *et al.* 1992).

Class II chitinases are similar to class I enzymes, but the cysteine-rich domain and the proline-rich small region, that follows, are missing. Class II chitinases are acidic proteins, such as PR-P and PR-Q proteins from tobacco (Flach *et al.* 1992).

Class III chitinases have no sequence similarities with classes I and II chitinases. Class III chitinases were described in *Rubus*, *Parthenoocissus*, *Vigna*, *Cucumis* and *Hevea*. They can be acidic (*Cucumis*) or basic proteins (Flach *et al.* 1992).

Although all types of chitinases can be induced by infection or by elicitor treatment, ethylene treatment induces class I chitinases only. Some chitinases have too high molecular weight to a known class. It is possible that they may be assigned to a fourth class.

3. Fungal chitinases

Fungal wall-degrading enzymes could be involved in the growth of the fungus itself. Chitinases may be sequestered in lysosomal vacuoles, be membrane-bound or wall-bound. All types of chitin-degrading activities were found in fungi. For example, an endochitinase and a β-*N*-acetylglucosaminidase were found in *Aspergillus nidulans*, and an exochitinase was found in *Mucor rouxii*. In *Nomuraea rileyi*, high levels of both endo-and exochitinase activities were detected in virulent isolates but not in an avirulent one. The greatest difference in chitinase activity occurred at germination (Flach *et al.* 1992).

4. Animal chitinases

Chitinases are synthesized by some protozoans (Jeuniaux 1966, Huber *et al.* 1991, Walters *et al.* 1993), and by different glandular tissues of the digestive system of many coelenterates, nematodes, polychaetes, oligochaetes, mollusks, and arthropods. Chitinases are also secreted by the epidermis of nematodes during the hatching process and by the epidermis of arthropods at the time of molting. In vertebrates, chitinases are secreted by the pancreas and the gastric mucosa of insectivorous fishes, amphibians, and reptiles, as well as by the gastric mucosa of some insectivorous birds and mammals (Jeuniaux 1966).

**2.5 Chitinase system in *Streptomyces* sp.**

The chitinases, whose encoding genes have been cloned and sequenced, are classified into two families, family 18 and 19 (Henrissat and Bairoch 1993). Family 18 and 19 chitinases differ from each other not only in their primary and higher structures (Davies and Henrissat 1995) but also in their catalytic mechanisms of hydrolyzing chitin. Acid-catalyzed glycoside hydrolysis may proceed to yield a hydrolyzed product with either retention or inversion of the anomeric configuration (at Cl') relative to the starting conformation. Family 18 chitinases yield β-anomers as a hydrolyzed product (a retaining mechanism) (Armand *et al.* 1994), whereas family 19 chitinases yield the α-anomers (an inverting mechanism) (Iseli *et al.* 1996). Family 18 and 19 chitinases are considered to be derivatives from different ancestral proteins.

Family 18 chitinase genes were cloned from many kind of organisms including bacteria, and the bacterial family 18 chitinases can be further classified into three groups A, B, and C, depending on the amino acid sequences of their catalytic domains (Watanabe *et al.*, 1993). Family 19 chitinase genes, on the other hand, have

been cloned exclusively from higher plants. Mitsutomi *et al.* (1995) found a unique chitinase in the culture of *S. griseus*, which was different from microbial family 18 chitinases in the specificity for β-1,4-*N*-acetylglucosamine versus β-1,4-glucosamine linkages in the hydrolysis of partially acetylated chitosan (Mitsutomi *et al.* 1995). Ohno *et al.* (1996) cloned a gene for the chitinase from *S. griseus* and showed this belong to family 19 (Ohno *et al.* 1996). *S. griseus* is the first organism which was found to produce family 19 chitinase other than plants.

**High-multiplicity of chitinase genes in *Streptomyces***

Chitinases have been purified from various *Streptomyces* species (Skujins *et al.* 1970, Tominaga and Tsujisaka 1976). Romaguera *et al.* (1992) purified four chitinases from *S. olivaceoviridis*, and thus showed the multiplicity of chitinases in *Streptomyces* (Romaguera *et al.* 1992). However, it was not known whether the multiplicity was the results of multiple genes for chitinases or of processing (proteolytic cleavage) of a single chitinase molecule. Three distinct genes for chitinase were cloned from *S. lividans* (*chiA, chiB*, and *chiC*), and thus the multiplicity of chitinase genes in the bacterium was confirmed (Miyashita and Fujii 1993, Miyashita *et al.* 1997). The presence of additional chitinase genes in *S. lividans* was inferred from southern-blot hybridization studies (Miyashita *et al.* 1997). The discovery of the family 19 chitinase gene in *S. griseus* (Ohno *et al.* 1996) also raised the question whether family 19 chitinase genes are present in other *Streptomyces* species. The set of ordered cosmid library representing the 8 Mb chromosome of *S. coelicolor* A3(2), a species very closely related to *S. lividans* (Leblond *et al.* 1993, Kataoka *et al.* 1997), was constructed by Redenbach and his co-workers in 1996 (Redenbach *et al.* 1996). This library is useful for identifying and locating the genes

from the bacterium or sequences similar to the genes of other *Streptomyces* species. Using this library, six chitinase genes were cloned and their localization on the chromosome was determined (Saito *et al.* 1999). Seven chitinase genes have been characterized and located on the chromosome of *S. coelicolor* A3(2). Five of them belong to family 18 chitinase genes (*chiA, chiB, chiC, chiD*, and *chiE*) and two belong to family 19 (*chiF* and *chiG*) (Figure 2.3). Four out of the five family 18 chitinase genes reside on the AseI-B or -P fragments while the remaining one (*chiD*) is on the AseI-G fragment of the chromosome (Figure 2.4). As for the family 19 chitinase genes, *chiF* and *chiG* maps on the AseI-A. and the AseI-F fragment, respectively. Family 19 chitinase genes are thus close to both ends of the linear chromosome of *S. coelicolor* A3(2). This was the first report confirming the coexistence of family 18 and 19 chitinase genes in organisms other than higher plants (Saito *et al.* 1999).

The multiplicity of chitinase genes in *S. coelicolor* A3(2) is remarkably high as compared with other chitin-hydrolyzing bacteria from which only two or three family 18 chitinase genes have been cloned. To determine whether such high-multiplicity of chitinase genes is confined to *S. coelicolor* A3(2), Southern-blot analysis was performed using various *Streptomyces* species. It was found that the sequences homologous to the three family 18 chitinase genes of *S. lividans*, i.e., *chiA*, *chiB*, and *chiC*, are present in many *Streptomyces* species (Miyashita *et al.* 1997). The sequences homologous to *chiD* and *chiE* gene from *S. coelicolor* A3 (2) and that to the family 19 chitinase gene of *S. griseus* (*chiC*) are also present in many *Streptomyces* species (Saito *et al.* 1999). These results suggest that the high-multiplicity of family 18 and 19 chitinase genes is a characteristics of the genus *Streptomyces*.

In addition to such multiplicity of chitinase genes, proteolytic cleavage of primary gene products has been shown to contribute to the multiplicity of chitinolytic molecules in *Streptomyces*. The proteolytic derivatives of ChiA and ChiC were found in the culture supernatant of *S.lividans* grown in liquid medium containing chitin (Miyashita and Fujii, 1993). These observations indicate that the presence of multiple chitinase genes and the processing of chitinases cause the multiplicity of chitinases in *Streptomyces*.

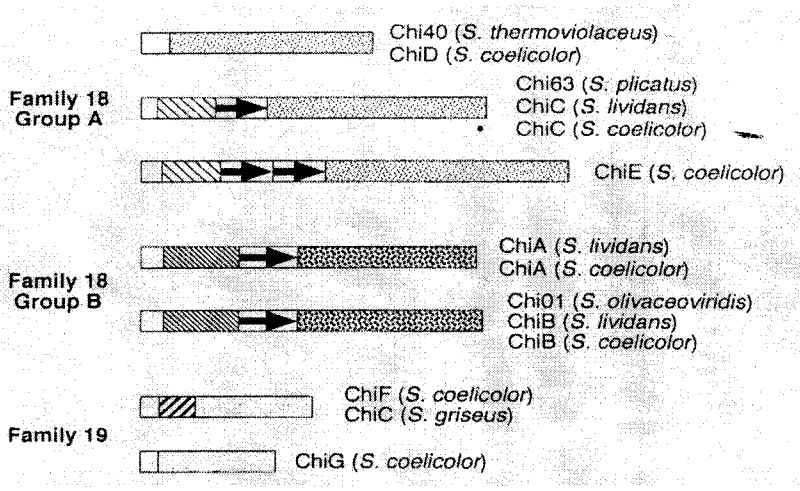


**Figure 2.3** Multiple domain structure and classification of chitinase of *Streptomyces*. Open boxes, striped boxes with arrows, and dotted boxes indicate signal domain, substrate-binding domain, fibronectin type III-like domain, and catalytic domain, respectively (From Saito *et al.* 1999).

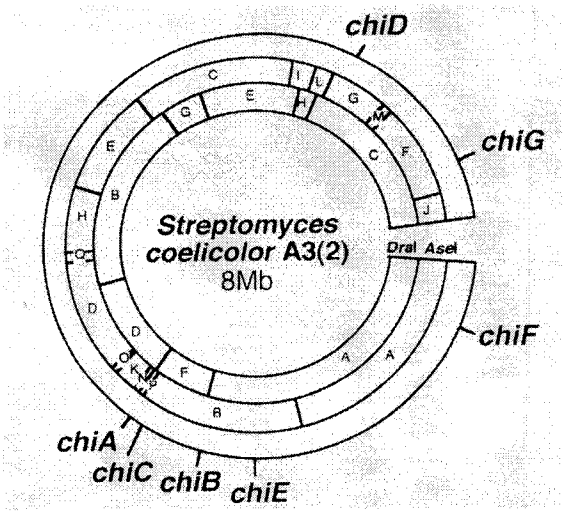


**Figure 2.4** Location of the chitinase genes on the chromosome of *S. coelicolor* A3(2). The inner circle indicates the alignment of *Dra*I restriction fragments. The middle open circle represents the orientation of the *Ase*I macro restriction map. The position of each chitinase gene is shown outside (From Saito *et al.* 1999).

**Multiple domain structures of Chitinases**

So far, a total of fourteen chitinase genes have been cloned from five species of *Streptomyces* (Ohno *et al.*, 1996, Miyashita *et al.*, 1997, Blaak *et al.*, 1993). According to their deduced amino acid sequences, these chitinase genes are classified into groups A and B of family 18 chitinases, and family 19 chitinases. Most of the chitinases encoded by these genes have multiple domain structures. Members of group B of family 18 chitinases are composed of four domains; a signal domain, a substrate binding domain, a fibronectin type Ill-like domain, and a catalytic domain. The chitin-binding activity of the chitin-binding domain was confirmed in Chi0l of *S. olivaceoviridis* (Blaak and Schremph, 1995). A matured 54-KDa chitinase, Chi0l, is proteolytically processed to a 47-KDa truncated chitinase lacking the chitin-binding domain during cultivation with chitin. This truncated chitinase has no affinity to crystalline chitin, whereas the 54-KDa chitinase binds to the substrate very strongly. The 47-KDa chitinase hydrolyses crystalline chitin considerably less efficiently than the 54-KDa enzyme, whereas colloidal chitin and low-molecular-mass substrates are quite equally degraded by both enzymes at identical optimal conditions. The sequences of chitin-binding domains of family 18 group B chitinases do not share significant similarity with any sequences in the database.

The chitinases belonging to family 18 group A show some diversity in domain structure. Chi40 of *S. olivaceoviridis* (Tsujibo *et al.*, 1993) and ChiD of *S.coelicolor* A3(2)(Saito *et al.*, 1999), do not have a multiple domain structure and are composed only of a signal sequence and a catalytic domain. The other chitinases in group A have additional domains; substrate-binding domains and one or two copies of the fibronectin type Ill-like domains. The substrate-binding domains of these

chitinases show similarity to the cellulose-binding domains of cellulases (Fujii and Miyashita 1993). Hence the origin of the substrate-binding domain of the *Streptomyces* group A chitinases seems to be different from that of group B chitinases.

The fibronectin type III-like domains are homologous to the original fibronectin type III domains that are very common constituents of animal proteins (Bork and Doolittle 1992). Since the domains have been found only in the animal proteins and in the glycosyl hydrolases of bacteria, the fibronectin type III-like domains are considered to have been transferred from animals (Little *et al.* 1994). Although the function of the fibronectin type III-like domains in the hydrolysis of chitin is not clear, the co-existence of the domain with substrate-binding domain might suggest that the fibronectin type III-like domain is involved in the binding of the enzyme to the substrate or has been involved in the shuffling of the functional domains.

The family 19 chitinases, ChiC of *S. griseus* and ChiF of *S. coelicolor* A3 (2) consist of a signal domain, a catalytic domain, and a putative substrate-binding domain which is lacking in ChiG *of S. coelicolor* A3 (Ohno *et al.* 1996).

*Streptomyces*, thus, has multiple genes for chitinases that are diverse both in the amino acid sequences of their catalytic domains and in their domain structures. Multiple domain structures found in chitinases of *Streptomyces* may suggest that these domains have been shuffled independently during the evolution of these enzymes.

**Genes for chitin-binding proteins and *N*-acetylglucosaminidases in *Streptomyces***

Chi01, a 20-KDa protein, purified from culture supernatant of *S. olivaceoviridis* lacks the catalytic ability to hydrolyze chitin (Schnellmann *et al.*, 1994), but binds to $\alpha$-chitin. Binding of the protein seems to be very specific since it does not bind to $\beta$ -chitin, chitosan or cellulose. The protein is produced in the presence of chitin (Schnellmann *et al.*, 1994). The genes for chitin-binding proteins were also cloned from *S. reticuli* (chb2) (Kolbe *et al.*, 1998) and *Serratia marcescens* (cbp21) (Suzuki *et al.*, 1998). An ORF with a deduced amino acid sequence similar to those of the chitin-binding proteins was found in *S. coelicolor* A3(2) (Saito *et al.*, 1999) . The prevalence of genes for chitin-binding proteins in chitinolytic bacteria might suggest that these proteins play a role in the utilization of chitin by the bacteria.

*N*-acetylglucosaminidase is an enzyme which releases *N*-acetylglucosamine from chitin or chitin-oligomers. One and two genes for *N*-acetyl-glucosaminidases were cloned from *S. plicatus* (Robbins *et al.* 1984) and *S. thermoviolaceus* (Tsujibo *et al.* 1998), respectively. *N*-acetylglucosaminidase H *of S. plicatus* belongs to the family 18 (Henrissat 1991), NagB of *S. thermoviolaceus* belongs to the family 20, and NagA of *S. thermoviolaceus* belongings to the family 3. Adding to these enzymes, the gene for *N*-acetylhexosaminidase, which removes *N*-acetylglucosamine or *N*-acetylgalactosamine residues from the nonreducing end of oligosaccharides and their conjugates, was cloned from *S. plicatus* and the protein encoded by the gene belongs to the family 20 (Mark *et al.*, 1998). Neugebauer *et al.* (1991) suggested that the production of extracellular *N*-acetyl-glucosaminidase was independent of the presence of chitin in *S. lividans* (Neugebauer *et al.* 1991).

**Direct repeat sequences in the promoter regions of chitinase genes**

In *S. lividans*, chitinase production is induced in the presence of chitin (Neugebauer *et al.*, 1991), and is repressed in the co-existence of glucose with chitin (Miyashita and Fujii, 1991). The induction and repression of chitinase production are regulated at the transcriptional level (Miyashita and Fujii, 1993, Robbins *et al.*, 1992).

In the promoter regions of *Chi63* from *S. plicatus*, *ChiC* from *S. lividans*, and *ChiC* from *S. coelicolor* A3(2), a pair of 12 bp direct repeat sequences is found to overlap the putative RNA polymerase binding sites (Fujii and Miyashita, 1993, Robbins *et al.*, 1992). Similar direct repeat-like sequences are found in the promoter regions of most of the genes for other chitinases and chitin-binding proteins from *Streptomyces* (Miyashita *et al.*, 1997). A single-base change within the direct repeat causes the constitutive and glucose-insensitive expression of *Chi63* of *S. plicatus* and *ChiA* of *S. lividans* promoter in vivo (Miyashita and Fujii, 1993, Delic *et al.*, 1992). Ni and Westpheling (1997) further made a detailed analysis of the promoter region of *Chi63* by integrating single-base mutagenesis (Ni and Westpheling, 1997). Single base substitution in the putative RNA polymerase binding site resulted in the loss of transcriptional activity from the promoter. Substitution of some of the bases within the direct repeat sequences resulted in the constitutive transcription from the promoter, and others resulted in the loss of glucose repression of the transcription while retaining the induction by chitin. These results indicated that the direct repeat sequences are involved in both the induction and repression of the transcription from *Chi63* promoter. Considering that the direct repeat-like sequences are present in most of the chitinase genes from *Streptomyces*, common factors or mechanisms might direct the regulation of the transcriptions of the chitinase genes in *Streptomyces*.

## 2.6 Regulation of Gene Expression

The expression of genetic information present in DNA requires a complex set of reactions. Initially, information is transferred from DNA by transcription to several major classes of RNA, including transfer RNA (tRNA), ribosomal RNA (rRNA), and messenger RNA (mRNA). The sequence of bases in mRNA is translated into the amino acid sequence of proteins, the catalysts that carry out the vital functions of the cell. The mature tRNA and rRNA, which are derived from larger precursor RNA molecules by RNA processing enzymes, play essential roles in this translation process. Thus, all transcripational products play a major part in gene expression (Doi 1991).

The major component of the enzymatic machinery that carries out transcription is the enzyme DNA-dependent RNA polymerase, which called RNA polymerase. The RNA polymerase core enzyme interacts with a sigma ($\sigma$) factor which confers promoter specificity to the holoenzyme (core enzyme + $\sigma$ factor = holoenzyme). This led to the identification of promoters, which are DNA sequences located at the 5' end of genes and are the RNA polymerase binding site.

The promoter region contains the promoter, which covers approximately 40-50 bp, and other sequences that regulate the binding of the RNA polymerase to promoter. The $\sigma$ factor directs the RNA polymerase to specific promoters. These promoters can be distinguished by the conserved sequences in the –10 (TATAAT) and –35 (TTGACA) hexamer region of the promoter (+1 is the first base of the transcript; -10 indicates the position of a base that is 10 bases to the 5' end of +1; +10 indicates the position of the $10^{th}$ base of the transcript). Mutation in the –10 and –35

hexamer regions can drastically reduce the activity of the promoter. Mutation in other parts of the promoter regions below may or may not reduce promoter activity.

Genes and operons are often controlled by multiple promoters that may be arranged in tandem or in an overlapping order. The promoters may be convergent or divergent for closely spaced genes and operons. The multiple promoters may be transcribed by the same holoenzyme or more than one type of holoenzyme. In the case of overlapping promoters that control different functions, divergent promoter regions may contain sequences that facilitate the simultaneous regulation of expression of both divergent genes, e.g., an operator site may prevent the expression of both divergent genes. Tandom overlapping promoter regions would allow the expression of two separate genes encoded by the DNA sequence downstream from these promoters depending on which promoter is used.

Overlapping promoters could act as negative or positive regulatory signals. If an RNA polymerase binds to the first of two overlapping promoters and prevents the use of the second promoter, then the first promoter will act as a negative regulating site and the bound RNA polymerase will repress the use of the second promoter. The use of the first promoter could be regulated by the presence or absence of cAMP-CAP and thus the use of the promoters could be involved in a complex regulatory cascade of physiological events. In another mode the first of two overlapping promoters could act in a positive regulatory fashion. In this case the first promoter could act as a "loading or collecting zone" for RNA polymerase molecules that subsequently were shifted to the second promoter where transcription was initiated (Doi, 1991).

**Principle of gene expression**

The yield of the gene product can often be greatly increased. Several factors affect the efficiency of gene expression, some of which are summarized as followed.

1. Number of gene copy of plasmid vector per unit cell (copy number)
2. Efficiency of transcription, the strength of the promoter, segments of DNA which direct RNA polymerase binding.
3. Efficiency of translation, depend upon a strong ribosome binding site (sequence of the RBS) and its distance from the initiation triplet (flaking DNA).
4. Codon choice in the cloned gene.
5. Genetic stability of the polypeptide of recombinant in its novel cellular environment.
6. Proteolysis.

The limiting factor in gene expression is the initiation of protein synthesis. Increasing the number of plasmids per cell increases the number of mRNA molecules transcribed from the cloned gene and this results in increasing protein synthesis. Similarly, the stronger the promoter, the more mRNA molecules are synthesized. The nucleotide sequence of the RBS and the initiating AUG codon are so important that a single base change, addition or deletion, can affect the level of translation up to 1000-fold.

Another important factor is related to the redundancy of the genetic code. There are several trinucleotide codons for most amino acids and different organisms favor different codons in their genes. If gene inserted into cells of another species the utilize codons are rare in the host cell, the host's biosynthetic machinery may be

starved of charged tRNAs. This could result in premature protein chain termination of high error frequency in the amino acid sequence of the protein.

The enzymatic breakdown of protein or the desired product is influenced by the apparent rate of gene expression. Although proteolysis can be reduced, it is difficult to eliminate completely. One approach which is used widely is to protect the desired protein by fusing it to normal cellular protein from which it must subsequently be released (Primrose, 1991, Harwood, 1992, Haima *et al.*, 1990, Hannig and Makridges, 1998).

## 2.7 Regulation of microbial chitinase genes

Chitinase gene expression in microorganisms has been reported to be controlled by a repressor/inducer system in which chitin or other products of degradation act as inducers. High levels of chitinase were found in cultures of *Metarhizium anisopilae* supplied with chitin but not with pectin, xylan or cellulose. Studies with restricted cultures (a carbon-deficient medium with slow feeding of sugars) to prevent catabolic repression revealed that the most efficient inducers of chitinase and chitobiase were *N*-acetylglucosamine and glucosamine respectively. In batch cultures it was found that inducing sugars accumulated to repressive levels even when the extracellular chitinase activity was very low. This leads to the conclusion that the accessibility and amount of chitin relative to other available nutrients (including lipids and proteins) may control the level of chitinase through the repressor/inducer system. Chitobiase was always produced along with chitinase in all media and was not affected by catabolite repression. Chitobiase was very affective in degrading the dimers and trimers of *N*-acetylglucosamine thus contributing to the

release of chitinase inducers. This explains the large constitutive levels of chitinase present and the accumulation of reducing sugars even at low chitinase activity. This is an interesting contrast to the usual mode of regulation, where the expression of one enzyme affects the regulation of another gene (St. Leger *et al.* 1986). Monreal and Reese (1969) found that, in batch cultures of *S. marcescens*, a larger amount of chitinase was produced in the presence of chitin than in the presence of *N*-acetylglucosamine. At low concentrations of *N*-acetylglucosamine (0.2%), a reasonably large amount of chitinase was produced, indicating induction and repression of the chitinase gene. Similarly Young and Carroad (1981), working with the same strain of *S. marcescens* in restricted cultures (carbon concentration controlled by dillution rate), found that chitinase was produced at low concentrations of *N*-acetylglucosamine. The observation of Monreal and Reese (1969) along with that of Young and Carroad (1981) suggests that chitinase of *S. marcescens* is regulated by *N*-acetylglucosamine induction and catabolic repression in much the same way as the chitinase gene is regulated in *M. anisopilae*. Vasseur et al. (1990) found that chitinase gene expression in a chitinase-overproducing mutant *A. album* E3 was induced by *N*-acetylglucosamine while it was repressed by glucose. Chitin or other oligomers did not enhance chitinase produced in the absence of inducers or repressors increased up to 20 h and then decreased to about 50% of the maximum activity. Proteolytic destruction was suggested as a possible reason for this decrease in activity. No definite experimental data were reported to substantiate proteolytic degradation. A similar proteolytic inactivation of *T. harzianum* chitinase during synthesis was observed by Srividya and Panda (Felse and Panda, 1999). On the other hand, in *T. harzianum*, high chitinase activity was found only in cultures supplied

with chitin but not with cellulose, chitosan or chitobiose. Unlike the above cases, in *T. harzianum*, *N*-acetylglucosamine did not enhance the enzyme production but instead repressed its synthesis. Repression of the chitinase gene was also observed in the presence of glucose. Ulhoa and Peberdy (1991) suggested that the signal for the induction of chitinase may result from physical contact between the cell surface and the insoluble substrate. This suggestion implies that the organisms producing chitinase should have a receptor on their surface specific to chitin. However, no experimental verification has proved the involvement of cell signalling in the expression of this enzyme. Similar mechanisms have been proposed for cellulase synthesis (Binder and Ghose 1978). Though the idea that cell signalling is involved in chitinase synthesis is promising, it still remains speculative. Ulhoa and Peberdy (1991) also observed that induction of chitinase was inhibited by 8-hydroxyquinoline (a RNA inhibitor) and cyclohexamide (a protein synthesis inhibitor), thus establishing that the induction of chitinase was dependent on *de novo* transcription and translation of proteins. Basal and constitutive levels of chitinase were reported in cultures of *A. album* E3 and *T. harzianum* even under starvation conditions. These low levels of chitinase may be sufficient to initiate chitin degradation and to release soluble oligomers which, in turn, induce chitinase synthesis (Vasseur *et al.* 1990, Ulhoa and Peberdy 1991). Repression of chitinase synthesis by glucose in almost all organisms indicates that catabolite repression may be involved in the regulation of microbial chitinase genes. The gene coding for a 33-kDa chitinase (*Chi33*) in *T. harzianum* CECT 2413 was strongly repressed by glucose and de-repressed under starvation conditions or in the presence of fungal cell wall containing chitin. The *Chi33* gene was not induced by chitin or its oligomers. Interestingly, as shown by Western blot

analysis, the *Chi33* mRNA was strongly expressed under starvation conditions, but very little chitinase activity was observed, suggesting post-transcriptional regulation of the gene. However, high proteolytic activity reported under similar conditions may also be the reason for low levels of chitinase (Limon *et al.* 1995). Here again, there was no experimental data to explain conclusively either post-transcriptional regulation or proteolytic degradation of the enzyme and this point needs to be explored in detail. Limon *et al.* (1995) also observed that the intracellular level of chitinase was always at a constant basal level irrespective of the substrate. This might indicate that the chitinase required for cellular functions is localised in the cytoplasm. Irene *et al.* (1994) reported that another gene (*Chi42*), coding for a different chitinase of 42 KDa from the same organism, was found to be weakly derepressed under starvation conditions, but the same gene was repressed in the presence of glucose and was induced by chitin and chitin-containing cell wall. Addition of pyruvate induced the chitinase mRNA to a lesser extent. Unlike the *Chi33* gene, the protein and mRNA levels of *Chi42* were similar, implying that no post-transcriptional regulation is involved in the expression of the *Chi42* gene. This difference in expression of the two chitinase genes, *Chi42* and *Chi33* of *T. harzianum* CECT2413, indicates that these genes are independently regulated by induction with chitin and de-repression under starvation conditions (Limon *et al.*, 1995). Expression of the *ech42* chitinase gene of *T. harzianum* IMI206040 was induced by growing the organism in a minimal medium containing chitin as the sole carbon source. The level of the *ech42* mRNA was high after 24 h of incubation, while it was not detectable in cultures grown on glucose even after 24 h of incubation; lower levels of the *ech42* mRNA were detected after 48 h. In cultures grown on chitin, the chitinase activity was 100 times higher than in the

culture grown on glucose. Thus, it has been suggested that the expression of the *ech42* gene can be explained by the presence of an inducer or by the absence of a repressor. Analysis of chitinase profiles in cultures containing chitin revealed that maximum chitinase activity appeared 24 h after the appearance of the maximum *ech42* mRNA. As in the case of *Chi33*, reported by Limon *et al.* (1995), there may be post-transcriptional regulation or proteolytic processing involved in the expression of the *ech42* gene (Carsolio *et al.* 1994).

## 2.8 Cloned chitinase gene expression

Currently, bacterial genes from terrestrial and marine bacteria such as *Serratia marcescens* (Fuchs *et al.*, 1986, Vorgias *et al.*, 1992), *Bacillus circulans* (Wiwat *et al.*, 1999), *Aeromonas hydrophila* (Roffey *et al.*, 1990). *Streptomyces plicatus* (Robbins *et al.*, 1992), *Vibro vulnificus* (Wortman *et al.*, 1986), *V. haryeyi*, and *Altermonas* sp. (Tsujibo *et al.*, 1993) have been cloned and sequenced. However, the mechanism of hydrolysis, the relationship between structure and function and the regulatory system involved in enzyme induction are still unclear (Tsujibo *et al.*, 1993). In most cases, when genes encoding foreign extracellular proteins are cloned in *E. coli*, the precursor is synthesized, processed, and exported across the inner membrane but not the outer membrane. The cloned chitinase enzyme has not secreted into the growth medium, but accumulated in the periplasmic space (Tsujibo *et al.*, 1993, Wortman *et al.*, 1986, Robbins *et al.*, 1988). In contrast both the *S. marcescens* chitinase and *A. hydrophila* chitinase are mostly detected in the culture medium (Fuchs *et al.*, 1986, Vorgias *et al.*, 1992) when they were cloned in *E. coli* (Roffey *et al.*, 1990). These experiments indicated that the signal peptide could be functional in

*E. coli*, but the reason(s) for the occurrence of chitinase activity in the supernatant was unclear (Vorgias *et al.*, 1992, Amann *et al.*, 1983). Presumably a leader sequence at the amino terminal end of the protein directs the synthesized protein to the periplasm. Preliminary observations suggest that during the thermal induction, the outer membrane of bacteria undergoes certain modifications that lead to secretion of the accumulated chitinase into the growth medium (Vorgias *et al.*, 1992).

However, knowledge concerning the origin and processing of the chitinase is still poor. Although some reports indicated that chitinases from *B. circulans*, *Serratia*, and *Streptomyces* are generated by multiple genes, it has also been suggested that certain chitinases from strains of *Altermonas*, *B. circulans*, and *Streptomyces* are encoded by a single gene whose products are processed post-translationally. Post-translational modification of chitinase precursor(s) may occur at either the N-terminus or C-terminus. Young cultures (6-7 h) of *B. licheniformis* TP-1 gave 3 enzyme activity bands in renatured SDS-PAGE. By contrast, old cultures produced only a single band of enzyme activity. Transformed *E. coli* harboring a single cloned TP-1 chitinase gene also produced multiple chitinases of different sizes as determined by SDS-PAGE. Analysis of these chitinases by immunoassay, N-terminal amino acid determination, and amino acid sequence comparison, indicated that they were derived from a single gene by deletion at the C-terminus to yield enzymes which hydrolyzed chitin differently (Tantivanich *et al.*, 1998).

A range of chitinase genes from microorganisms have been cloned and the potential uses of these genetically manipulated organisms are being investigated by various researchers.

**Cloning of fungal and yeast chitinase genes**

A brief summary of the research on cloning of microbial chitinase genes is given in Table 2.9.

**Cloning of chitinase gense of *Saccharomyces* species**

A gene for *Saccharomyces cerevisiae* endochitinase (*CtsI*) was isolated and cloned into a *Sachizosaccharomyces pombe/S. cerevisiae* shuttle vector. Restriction maps of plasmids from multiple isolates were used to localize a glycosidase – overproducing gene and this was subcloned into a *S. pombe/S. cereside* shuttle vector (YEAp24). Transformation of *S. pombe* with a *CtsI* clone resulted in the appearance of about a 5-to 13-fold increase in chitinase activity. High levels of chitinase were observed in the logarithmic phase, the time during which endochitinase is important in the formation of septa, marking the point of separation during budding.

Table 2.9 A brief summary of research done on the cloning of microbial chitinase genes

| Source Organism | Cloning Host | Vector System | Reported information | Reference |
|---|---|---|---|---|
| Actinomycetes | | | | |
| *Streptomyces plicatus* | *Escherichia coli* | Shuttle vector containing *c*DNA library | The signal peptide sequence of the wild-type, containing 30 amino acids, was removed in the recombinant enzyme. The cloned enzyme was secreted into the periplasmic space, but the wild-type enzyme was secreted into the medium | Robbins *et al.*, 1988 |
| | *E. coli* | Plasmid pUC vector | Removal of 150-200 bp from the *Streptomyces* insert resulted in greater expression of the enzyme. Removal of more than 200 bp resulted in incomplete inactivation of the enzyme. | Robbins *et al.*, 1992 |
| *Streptomyces lividans* | *S. lividans* | Multi-copy-number plasmid pIJ702 | The 2-Kb chitinase gene consisted of an open reading frame of 1713 bp coding for a 571-amino acid protein. The region upstream of nucleotide 133 was essential for the expression of chitinase in *S. lividans* | Miyashita *et al.*, 1991 |
| Bacteria | | | | |
| *Serratia marcescens* | *E. coli* and then into *P. fluorescens* and *P. putida* | Cosmid vectors | Resulted in four strains of *Pseudomonas* with enhanced chitinase activity | Gooday, 1990 |
| | *E. coli* and then into *S. marcescens* | High-copy number plasmid vector | *E. coli* containing *S. marcescens* chitinase gene had high chitinase activity and was used as an effective biocontrol agent against fungal infection in plants | Gooday, 1990 |

Table 2.9 A brief summary of research done on the cloning of microbial chitinase genes (continued)

| Source Organism | Cloning Host | Vector System | Reported information | Reference |
|---|---|---|---|---|
| *S. marcescens* | *E. coli* | Broad-host-range cosmid pLAFR1 containing a genomic library | Four independent clones were obtained and all were found to have a common *EcoR*I fragment containing a 57-KDa chitinase gene | Fuchs *et al.*, 1986 |
| | *E. coli* | Broad-host-range cosmid pLAFR3 containing a genomic library of chromosomal DNA | Positive clones were identified on chitin medium and high levels of chitinase were observed following chitinase induction | Sundheim, 1987 |
| | *E. coli* | Plasmid pBR322 containing a chiA DNA fragment. This was further modified by including an operatory promoter of bacteriophage λ, oLpL and the resulting phagemid pLCHIA was introduced into *E. coli* | High levels of chitinase were observed following chitin induction | Shapira *et al.*, 1989 |
| | *Lactobacillus lactis* Subsp. *Lactis* MG1363 and *L. plantarum* E196 | - | The chitinase gene was expressed in *L. lactis* and *L. plantarum* with 9-27-fold increase in chitinase activity | Burberg *et al.*, 1994 |
| *Serrtia liquefaciens* | *E. coli* | Plasmid pBR329 with a chitinase gene insert | The transformed cells were capable of expressing the cloned chitinase gene | Joshi and Kozlowski 1986 |
| | *E. coli* | - | Increased chitinase activity was observed in the transformants | Joshi *et al.*, 1987 |
| | *E. coli* | - | Among the three genes cloned *chiA* and *chiB* coded for chitinase while *chiC* code for chitobiase. Removal of the repressor gene *chiD* led to increased production of chitinase | Joshi *et al.*, 1988 |

Table 2.9 A brief summary of research done on the cloning of microbial chitinase genes (continued)

| Source Organism | Cloning Host | Vector System | Reported information | Reference |
|---|---|---|---|---|
| *Serrtia liquefaciens* | Transposon-Tn5-mediated mutagenesis was used to obtain high-yielding mutants | - | A chitinase-overproducing mutant, which lacked a negative regulatory element; showed about 80% increase in chitinase activity over the wild type | Joshi *et al.*, 1989 |
| *Bacillus circulans* | *E. coli* | Plasmid vector pKK233-3 with chromosomal DNA fragments | The gene coding for chitinase activity contained an ORF of 2097 bp that codes for a precursor protein with signal sequence of 15 amino acids. The recombinant chitinase showed 33% homology with chitinase A of *S. liquefaciens* | Watanabe *et al.*, 1990 |
| | *E. coli* | Site-directed mutagenesis | Glu-204→Gln and Glu-204→Asp mutations decreased chitinase activity. Asp-200→Glu mutation did not affect chitinase activity | Watanabe *et al.*, 1990 |
| *Aeromonas hydrophila* | *E. coli* | Plasmid pJP2512 | The chitinase gene from *Aeromonas hydrophila* consisted of an open reading frame of 2.6 kb and was translated on its own promoter to give a 96-Kda protein. | Roffey and Pomberton 1990 |
| | *E. coli* | | Chitinase in *E. coli* accumulated in the periplasmic space The recombinant chitinase was similar to the wild-type chitinase with a molecular mass of 85 kDa and was subjected to catabolite repression | Chen *et al.*, 1991 |

Table 2.9 A brief summary of research done on the cloning of microbial chitinase genes (continued)

| Source Organism | Cloning Host | Vector System | Reported information | Reference |
|---|---|---|---|---|
| Fungi | | | | |
| *Trichoderma Harzianum* | *T. harzianum* | pGEM-T vector containing a *c*DNA library | The cloned chitinase gene codes for an endochitinase of 42 Kda and was present as a single-copy gene in *T. harzianum*. A putative signal peptide of 22 amino acids and a second peptide of 12 amino acids were cleaved post-translationally to give the mature protein | Irene *et al.*, 1994 |
| | *E. coli* | *c*DNA library | The transcription of the chitinase gene began preferenntially 109 bp upstream of the translation initiation codon. The preproenzyme was subjected to two processing steps between amino acids 23 and 24 and aminoacids 33 and 34 to give mature protein of molecular mass 42 Kda. | Carsolio *et al.*, 1994 |
| *Vibro vulnificus* | *E. coli* | - | *V. vulnificus* chitinase was expressed in *E. coli*, but the enzyme accumulated in the periplasmic space, while in wild-type cells the enzyme was secreted into the medium. | Gooday, 1990 |
| Yeast | | | | |
| *Saccharomyces cerevisiae* | *Schizosaccharomyces Pombe* | *S. pombe*/*S. cerevisiae* shuttle | Introduction of the chitinase gene of *S. cerevisiae* into *S. pombe* resulted in high chitinase activity in host | Kuranda and Robbins, 1987 |

Table 2.9 A brief summary of research done on the cloning of microbial chitinase genes (continued)

| Source Organism | Cloning Host | Vector System | Reported information | Reference |
|---|---|---|---|---|
| *Aphanocladium album* | *S. cerevisiae* | Plasmid containing genomic library | Four distinct domains were identified in chitinase. The signal sequence was cleaved and the serine/threonine-rich region was glycosylated post-translationally to yield the mature protein | Kuranda and Robbins, 1987 |
| | *Fusarium oxysporum* | Plasmid containing *c*DNA library | Introduction of the *A. album* chitinase gene fragment into *F. oxysporum* resulted in high chitinase levels in the host without any effect on growth | Blaiseau *et al.*, 1992 |

Kuranda and Robbins (1991) sequenced the *S. cerevisiae* endochitinase gene following initial cloning by a plasmid-based over-expression. The derived amino acid sequence showed four distinct domains: a signal sequence consisting of 20 amino acids, a catalytic domain made up of 306 amino acids, a serine/theronine-rich region containing 52 amino acids and a chitin-binding domain having 81 amino acids at the carboxy terminus. The signal sequence is cleaved and the serine/theronine-rich region is glycosylated with sugar chains containing mannose residues post-translationally to yield the mature protein. The catalytic domain was found to be homologous to the cucumber chitinase that is active during pathogenesis. The indicates that *S cerevisiae* chitinase may be useful for biocontrol of phytopathogens.

**Cloning of *Trichoderma* chitinase genes**

A cDNA of *T. harzianum (Chi42)* , coding for an endochitinase of 42 KDa, was cloned by lrene *et al.* (1994) using synthetic oligonucleotides corresponding to amino acid sequences of the purified chitinase. Analysis of the N-terminal amino acid sequence of the chitinase and the N-terminal amino acid sequence of the chitinase and comparison with that deduced from the chitinase and comparison with that deduced from the nucleotide sequences revealed post-translational processing of the putative signal peptide of 2 amino acids and a second hydrophillic peptide of 12 amino acids and a second sequence of the 42-KDa chitinase showed high homology with the 40-KDa chitinase of *A. album* and to a lesser extent, with chitinase A1 of *B. circulans,* chitinase A and B of *S. marcescens* and the α-subunit of *Kluveromyces lactis* killer toxin. The fact that chitinase genes of organisms isolated from different environments exhibit a reasonable homology suggests that these organisms may have a close evolutionary relationship. Southern analysis showed that the *Chi42* gene was present

as a single copy gene in *T. harzinum.* This observation reveals that three chitinase genes of *T. harzianum.* (*Chi33*, *Chi37* and *Chi42*) are not a result of proteolytic breakdown of a single common chitinase, but are transformed from three different genes of little or of no homology.

A gene *(ech42)* encoding endochitinase, produced by a biocontrol agent, *T. harzianum* IMI206040, was cloned and characterized by Carsolio *et al.* (1994). Expression of the cDNA clone in *Escherichia coli* resulted in high chitinase activity. The *ech4* gene was assigned to a double chromosomal band (chromosome V or VI) upon electrophoretic separation of *T. harizanum* genomic DNA and Southern analysis of the chromosomes. This is similar to the *pBRI* gene of *T. harizanum* (also involved in mycoparasitism), which was also assigned to the same chromosomal band. It is possible that these two or other mycoparasitic enzymes may be arranged as clusters, but this idea is just a conjecture and needs further investigation. Primer extension analysis indicated that the transcription of the gene begins preferentially 109 bp upstream of the translation initiation codon. Similarly, light-induced sporulation resulted in high levels of the transcript, suggesting developmental regulation of the gene. The *ech42* protein is synthesized as a pre-proenzyme, which undergoes the initial processing of a signal peptide sequence between amino acids 23 and 24. The second putative processing is by an endoproteinase activity between amino acids 33 and 34. The mature protein had a molecular mass of 42 KDa. Limon *et al.*(1995) isolated and sequenced a 33 KDa gene *(chi33)* coding for chitinase of *T. harzianum* to determine the primary structure and expression of the gene. An oligonucletide probe was designed, using the amino-terminal peptide sequence, and this probe was used as a primer to isolate a 1.2-Kb cDNA fragment. The cDNA codes for a protein of 321

amino acids with a poly (A) tail, which includes a putative signal peptide of 19 amino acids. However, the lack of three distinct domains of chitinase, namely, a Ser/Thr-rich region, a chitin-binding region and the C-terminal region (as in *Cts1* of *S. cerevisiae*) indicated that the *Chi33* protein is not involved in cell separation or cell-wall morphogenesis. Instead it may be involved in a mycoparasitic role, as suggested by the extracellular localization of the enzyme and the conservation of a six-cysteine sequence that is usually found in plant chitinases. No significant homology was detected with bacterial or other *Trichoderma* chitinases.

Csaba *et al.* (1996) used the polymerase chain reaction (PCR) to identify a 1424-bp DNA sequence responsible for chitinase synthesis in *Trichoderma hamatum.* High homology was observed between this gene sequence of *T. harzianum.* Chromosomal DNA of five potential biocontrol species of *Trichoderma* was isolated by pulsed-field gel electrophoresis. There was a total of six chromosomes in all cases with sizes ranging between 3.7 Mb and 7.7 Mb, while the genomic size varied between 30.5 Mb and 35.8 Mb. The polymorphic chromosomal location of the highly conserved 42-KDa chitinase gene of *Trichoderma* sp. was indicated by strong but variously located, Southern hybridization with the radiolabelled *Th-ch* gene. Southern hybridization and sequence comparison revealed that the *Th-ch* gene is present as a single-copy fragment, as observed with other chitinase genes of *T. harzianum* (Irene *et al.* 1994; Limon *et al.* 1995).

**Cloning of chitinase genes from other fungal species**

The genes coding for chitinase I and chitinase II of *Rhizopus oligosporus* were isolated and sequenced by PCR amplification to study the various domains present. The *chiI* and *chiII* genes had five domains, with an additional C-terminal domain

present. Two small regions were also found to be conserved with other glycosidases. Comparison of the derived amino acid sequences with the C-terminal amino acid sequences of the purified chitinase indicated posttranslational processing of the precursors at the C-termini. This additional sequence was found to contain two regions. The first was a variable region where the C-terminal processing occurred and its sequence was found to be different for the two chitinases. The second was a prosequence region that is removed during the maturation of the enzyme (Sahai and Manocha 1993). Blaiseau *et al.* (1992) cloned a chitinase gene of *A. album* from a cDNA library. An antiserum raised against chitinase 1 of a chitinase-overproducing mutant of *A. album* E3 was used to select a suitable cDNA clone. Transformation of the putative chitinase genomic fragment into two strains of *F. oxysporum* resulted in high chitinase activity in the culture filtrate over the control and did not inhibit growth. Only a partial amino acid sequence (of 23 amino acids starting from the amino terminal) was obtained by direct sequencing of the chitinase 1 gene. Hence it was not possible to compare this protein sequence with the complete amino acid sequence of other chitinases.

A genomic DNA fragment of *Metarhizium anisopilae* strain CG32, containing 375 bp, was amplified by PCR using chitinase-specific consensus primers. High similarity was observed between the *M. anisopilae* sequence and the catalytic domains of the chitinase genes of *A. album* and *T. harzianum* except for the absence of introns present in both *A. album* and *T. harzianum* (Valadaris-Inglis *et al.* 1997).

**Cloning of bacterial chitinase genes**

**Cloning of chitinase from *Serratia* sp.**

Two chitinase genes *chiA* and *chiB* from random cosmid clones of *S. marcescens* were inserted into *E. coli* and then transformed into *Pseudomonas fluorescens* and *Pseudomonas putida* resulting in four strains of manipulated *Pseudomonas* with enhanced chitinase activity. Attempts have been made to clone the same *S. marcescens* chitinase genes into *E. coli* and then back to *S. mercescens* on a high-chitinase-yielding strain for enhanced bioconversion of shellfish waste. *E. coli* containing the *S. marcescens* chitinase gene was also used as an effective biocontrol agent for various fungal diseases in plants (Gooday 1990).

Wan *et al.* (1997) isolated a DNA fragment (pCHI5422) containing two genes encoding a 54-KDa and a 22-KDa chitinase from a cosmid library of *S. marcescens* KCTC 2172. The 22-KDa chitinase had 227 amino acids coded by a 681-bp nucleotide sequence in an open reading frame, while the 54-KDa chitinase had 499 amino acids coded by a 1497-bp nucleotide sequence in an open reading frame. The genes coding for the 54-KDa and the 22-KDa chitinases were isolated and subcloned in *E. coli.* The individual chitinases were expressed in the host and the specific activity of the 54-KDa chitinase was 300 $\mu$M $min^{-1}$ $mg^{-1}$ while that of 22-KDa chitinase was 17 $\mu$M $min^{-1}$ $mg^{-1}$. Takeshi *et al.* (1997) isolated several Tn5 mutations of *S. marcescens* 2170 with defects in chitinase production and characterized them, to study the genetics of chitin degradation and utilization. Prior to isolation, the mutants secreted four chitinases, A, B, C1, C2 and a 21-KDa protein (CBP2), lacking chitinase activity but having chitin-binding activity. The gene for chitinases A and B showed high similarity to the chitinases of other strains of *S.*

*marcescens.* Characterisation of the mutants revealed that some of them had defects in chitinase excretion, a negative regulatory mechanism for chitinase gene expression, and essential factor for such expression, and a structural gene for the chitinase.

**Cloning of chitinase genes from *Aeromonas* sp.**

The gene encoding extracelluar chitinase from *Aeromonas hydrophila* has been cloned and expressed in *Escherichia coli* using plasmid *pJP2512* with a 3.9-kb chtinase-producing insert (Chen *et al.* 1991). The gene was transcribed from its own promoter, producing a protein of molecular mass 96 KDa. The chitinase open reading frame with an estimated length of 2.6-Kb was subcloned into a 3.0-Kb fragment. This fragment did not carry the functional chitinase promoter. In *E. coli,* the chitinase could not traverse to the outer membrane but accumulated in the periplasmic space and cytoplasm, as revealed by cellular fractionation studies. The chitinase gene in plasmid *pJP2512* is present as single fragment encoding a protein of 96 KDa, the highest molecular mass ever reported for a chitinase. Investigations with plasmid *pJP2530* showed that the region between *ClaI* and the internal *Bam*HI site was responsible for the secretion of the protein across the cytoplasmic membrane. However, the dependence of protein translocation on other factors was not investigated and hence the role of the above region in protein translocation could not be properly established (Roffey and Pemberton 1990). PCR techniques were used to clone the gene encoding chitinase from *A. caviae* into *E. coli* BL21 (DE3). The transformation was carried out using the plasmid *pET20b(t).* Protein sequencing showed that the recombinant chitinase, as compared to the wild-type chitinase, contained an additional 33 amino acids at its N-terminus and 13 amino acids at its C-terminus. The recombinant enzyme was secreted into the cytoplasm as well as into

the culture medium. Activity and protein staining confirmed a single band on a native polyacrylamide gel (Lin *et al.* 1997).

The toxicity of *Bacilius thuringiensis* sub sp. *Kurstalis* (Bt-k) towards diamond black moth larvae was increased by the addition of a crude preparation of chitinase from *B. circulans* 41. This implied that the toxicity of *B. thuringiensis* towards target insects may be enhanced by introducing a chitinase gene from other bacteria. Such genes from *A. hydrophila* (pHYA1) and *Pseudomonas maltophilia* (pHYB1, pHYB2 and pHYB3) were cloned into *E. coli* DH5$\alpha$ from the shuttle vector pHY300PLK. 4-Methylumbellifery $\beta$-*D-N-N'*-diacetyl chitobioside was used as the detecting substrate. The four plasmids were then introduced into *B. thuringiensis* subsp. *israelensis* strain C4Q272 by electroporation. The transformants harbouring chitinase genes were found to be stable for long periods of time, implying that these strains could be used as effective biocontrol agents (Wiwat *et al.* 1997).

**Cloning of chitinase genes from *Bacillus* sp.**

Watanabe *et al.* (1990) cloned a chitinase gene of *B. circulans* WL-12 into *E. coli* by transforming HB101 cells with a recombinant plasmid composed of a chromosomal DNA fragment prepared from *B. circulans* WL-12 and a plasmid vector pKK233-3. DNA sequencing analysis revealed that the region necessary for the normal chitinase activity contained an open reading frame of 2097 bp, which codes for the precursor of chitinase Al. The precursor of chitinase Al contained a long signal sequence of 41 amino acids with an extremely long hydrophilic segment of 15 amino acids. The cloned chitinase produced in *E. coli* had, at its N terminus, an 8-amino-acid sequence that was not found in the mature chitinase A1. This chitinase A1 showed a 33% amino acid match with chitinase A of *S. marcescens.* This region

of chitinase A1 is immediately followed by tandemly repeating 95-amino-acid segments that are homologous to type III fibronectin. The signal peptide of chitinase A1 in *E. coli* was cleaved between Ala-32 and Leu-33, whereas the signal peptide in *B. circulans* WL-12 was cleaved between Ala-40 and Ala-41. This difference in cleavage site probably reflects the different protein export mechanisms in these two bacteria. It is interesting to note that a prokaryotic chitinase shares a homology with a mammalian cell adhesion molecule (fibronectin). This is the first report of its kind and might indicate that type-III or type-III-like sequences are also widespread in microorganisms. A lot of research is yet to be done to substantiate this argument. At present the function of the 95-residue tandem repeats is not very clear. Authors speculate that the nature of the binding of the type III units may indicate possible involvement of these repeats in substrate binding. To elucidate the region of importance for catalytic activity, Watanabe *et al.* (1993) replaced three amino acid residues (Ser-160, Asp-200 and Glu-204) in chitinase Al of *B. circulans* WL-12 by site-directed mutagenesis. It was concluded that Asp-200 and Glu-204 were directly involved in the catalytic activity of chitinase Al. Chitinases with Glu-204→ Gln mutation and Glu-204→Asp mutaion were inactive with $k_{cat}$ values approximately 1/5000 and 1/17000 those of the wild-type chitinase respectively. The Asp-200→Asn mutation decreased the $k_{cat}$ values to 1/350 that of the wild-type enzyme. On the other hand $k_{cat}$ values were not affected by Asp-200→Glu mutation. Thus the authors concluded that the relative disposition of the carboxyl group of Glu-204 is critical for catalytic activity. Similarly, the carboxyl group of Asp-200 is essential but its position is not very critical for chitinase activity. The hydroxyl group of Ser-160 is not directly required for catalytic activity, but it may be involved in structural

modulation of the catalytic site of chitinase A1. Two other amino acid residues, Asp-197 and Asp-202, were highly conserved among chitinases and may be involved in catalytic activity along with Asp-200. The last two conclusions are yet to be confirmed and efforts to do this are underway.

**Cloning of chitinase genes from *Enterobacter* sp.**

The complete nucleotide sequence of the gene *chiA* in *Enterobacter agglomerans,* which codes for an endochitinase was deduced to study the structural construction and homology of the gene. The *chiA* gene consists of an open reading frame, which coded for a 562-amino-acid sequence that formed a 61-kDa precursor protein with a putative leader peptide at its N terminus. The nucleotide sequence of *chiA* showed 86.8% homology with a *chiA* gene of *S. marcescens* chitinase. Homology modelling of the *E. agglomerans* endochitinase three-dimensional structure showed that most amino acid substitutions were at the solvent-accessible sites. The sequenced gene was isolated and transformed into *E. coli* JM109. The transformed host carrying *E. agglomerans chiA* was able to secrete the *E. agglomerans* enzyme. Growth of *R. solani* on plates was inhibited by the transformed strain, indicating that this strain could be used as a potential biocontrol agent against phytopathogenic fungi (Leonid *et al.* 1997). Similarly Kweon *et al.* (1997) amplified and cloned the gene coding for a 60-KDa chitinase from *Enterobacter* sp. Gl into *E. coli* by PCR using a synthetic oligonucleotide sequence corresponding to the derived amino acid sequence of the purified enzyme. Positive clones were indentified by their ability to degrade water-soluble chitin. Nucleotide sequencing showed that *chiA* gene consisted of 1686 bp encoding 562 amino acids. The amino acid sequence of the recombinant chitinase enzyme had 95.7% homology with the amino acid sequence of

chitinase A from *S. marcescens* QMB 1466. Transformation of the *chiA* gene into *E. coli* and subsequent deletion and sequence analysis indicated that its expression in *Enterobacter* sp. Gl was controlled by an inverted repeat sequence located upstream from the promoter region.

Molly and Burke (1997) attempted to link the chitinase gene *(chi69)* from *Janthinobacterium lividans* to the secretion signal of the *K. lactis* killer toxin and then ligate it to the galactose-inducible *CYC-GAL* hybrid promoter of the plasmid *pEMBL Yex4.* The chimera was transformed into *S. cerevisiae* DY-150. The chitinase enzyme was secreted by the transformed organism into the medium and a maximum chitinase activity of 0.7 U $ml^{-1}$ was observed after 24 h of galactose induction. Insertion of the *chi69* gene did not have any deleterious effect on the host. The secretion signal of the killer toxin $\alpha$ subunit contains the sequence Lys-Arg, which is cleaved by the *Saccharomyces* protease Kex2 during secretion. It was also found that the enzyme was glycosylated, but glycosylation is unlikely to affect chitinase activiy because only one of the three glycosylation sites (Asp-37, Phe-374, Ser-375) lies within the catalytically active region.

**Cloning of chitinase genes of *Streptomyces* sp.**

In order to study the genetic control of chitinases in *Streptomyces,* Miyashita *et al.* (1991) cloned a chitinase gene from *Steptomyces lividans* TK64 into a plasmid plJ702 and monitored its expression in a natural host by measuring the increase in chitinase activity. Four chitinases (A, B, C and D), of molecular mass 36, 46, 65 and 41 KDa respectively, were identified and significant differences were observed among chitinases A, B and C in terms of their gene structure and activity and in their mode of action towards the substrate. Chitinases C and D were found to be very

similar in this respect. Four distinct clones with plasmids pEMJ1 (coding for chitinase A), pEMJ5 (coding for chitinase B), pEMJ7 (coding for chitinase C and D) and pEMJ8 were obtained. From orientation studies, the authors concluded that pEMJ5 and pEMJ7 were expressed from their own promoters, while the expression of the pEMJ1 occurred from the *lacZ* promoter found in pUC vectors. The authors further suggested that the difference in expression of the cloned genes may be due to difference in their promoter sequences. Again no experimental verification was given for this suggestion (it was just a conjecture) and hence the difference in promoter sequence, if any, requires detailed investigation. Robbins *et al.* (1988) used the bacteriophage vector $\lambda$ EMBL4 to transform *E. coli* cells with a chitinase gene of *Streptomyces plicatus.* The *Streptomyces* chitinase was secreted into the periplasmic space of *E. coli* and its signal sequence was removed post-translationally to give the mature protein. Sequence analysis of the cloned gene indicated that the nucleotides 7-9 code for the ATG start codon of the chitinase gene. The first 30 amino acids of the chitinase were similar to the signal sequences of secreted proteins of other gram-positive bacteria. The protein purified from the periplasmic space of *E. coli* had a sequence of 15 amino acids that was exactly similar to the already established sequence that started at amino acid 31. Therefore, it was concluded that the *E. coli* signal peptidase cleaves the protein between amino acids 30 and 31 to give the mature protein. A chitinase gene fragment from *S. plicatus* DNA was subcloned into *E. coli* and detectable amount of chitinase was expressed. High-level expression was achieved by restriction of the cloned DNA with *Bal 31,* which was followed by inframe fusion to the N-terminal sequence of β-galactosidase found in the pUC vector. Robbins *et al.* (1992) stated that incomplete removal of λDNA and the

removal of 150-200 bp from the *Streptomyces* insert are the reasons for the high expression of the enzyme. However, the removal of more than 200 bp from the insert led to the incomplete inactivation of the enzyme. The high-level expression plasmid pCT-F1 was chosen for this purpose. *Streptomyces* chitinase was secreted into the periplasmic space of *E. coli* unlike what occurred in the wild type (*Streptomyces* sp.), where the enzyme was secreted into the medium. Miyashita and Fuji (1993) characterized a chitinase gene *(ChiA)* of *S. lividans* 66 and the nucleotide sequence for the 2-Kb *ChiA* fragment showed an open reading frame of 1713 bp to code for a putative protein of 571 amino acids. However, the open reading frame starts with a GTG at nucleotide 77 and ends with TAA at nucleotide 1894. A perfect inverted-repeat sequence of 11 nucleotides situated 17 bp downstream from the TAA termination codon was believed to act as terminator of transcription, although this was not completely established in this study. The 5' end of the insert pEMJ112 did not generate chitinase in *S. lividans,* but did do so in *E. coli* from the *lac* promoter (nucleotide 133). Apparently the region upstream of nucleotide 133 is essential for the expression of chitinase in *S. lividans.* The promoter region of the *chiA* gene was subcloned on a 178-bp fragment into the promoter probe vector pIJ486. One of the subclones detected, which contained a 114-bp sequence upstream from the translation start codon, retained both chitin-stimulated production and glucose repression. Chitin-stimulated production was lost in another mutant detected containing a 104-bp sequence upstream from the translation start codon. This truncation of the promoter sequence revealed that the transcription of the *chiA* gene is directed from its own promoter sequence.

Kiyotaku *et al.* (1997) reported that the structural gene (*chiB*) for one of the chitinases of *S. lividans* 66 consisted of 1830 bp encoding a 610-amino-acid sequence. Comparison of the amino acid sequence, derived from its nucleotide sequence, with those of other bacteria revealed a domain structure of the following order (N termini to C termini): signal peptide, substrate-binding domain, type-III repeating units and catalytic domain. Of the three chitinase genes (*chiA, chiB* and *chiC*) of S. *lividans* 66, the nucleotide sequence of *chiA* and *chiB* showed 49% similarity, while the amino acid sequence of chitinase A and chitinase B showed 59% similarity. The gene sequence of chic showed no similarity with that of *chiA* or with *chiB.* This indicates that *chiA* and *chiB* diverged relatively recently while the *chiC* gene must have evolved much earlier. A 90% homology was found between *chiB* of *S. lividans* and *chi01* of *Streptomyces olivaceoviridis* in terms of both amino acid and nucleotide sequence, while *chiC* of *S. lividans* was almost identical to *chiA* of *S. plicatus.* Southern hybridization of genomic DNA from *Streptomyces* species with *chiA, chiB* and *chiC* of *S. lividans* as probes indicated that these S. *lividans* genes are highly distributed and conserved among the genus *Streptomyces.*

# CHAPTER III

# ISOLATION OF ENDOPHYTIC ACTINOMYCETES AND THEIR ANTIFUNGAL ACTIVITY

## Introduction

Actinomycetes are prokaryotes which have a hyphal (hence fungal-like) morphology. Most of the actinomycetes those are known are soil microorganisms and are active in the decomposition of plant tissues, and thereby in the recycling of carbon and nitrogen. Members of the genus *Frankia,* where the bacterium occurs in root nodules have become recognized in the recent past for their wider association with plants which therefore make them free-living actinomycetes (Baker *et al.* 1980; Knowlton *et al.*1980). However, these reports give no indication of prevalence of different species growing as endophytes in the different tissues of the plants. Several reports refer to actinomycete activity in the protection of the plant host against pathogens and the influence of their metabolic products on plant growth and physiology (Katznelson and Cole 1965; Tahvonen 1982; Williams *et al.* 1984; Drautz and Zahner 1986; Schippers *et al.* 1987). Other reports refer to pathological interactions of endophytic actinomycetes with plants (Alwadi and Baka 2000).

The antifungal activity of endophytic actinomycetes has been a focus in the exploitation of these organisms as excellent biocontrol agents against phytopathogenic fungi. To date, however, much less is known about the antifungal antibiotics produced by endophytic actinomycetes (Sardi *et al.* 1992). This study

involved the isolation of actinomycetes from the tissues of healthy plants, an initial identification of them and an evaluation of the antifungal activity of their secondary metabolites.

**Materials and methods**

**3.1 Isolation of endophytic actinomycetes from the selected plants**

**3.1.1 Sample collection**

Leaf, stem and root tissues were recovered from healthy representatives of herbaceous and woody plants from the environs of Chiang Mai, Thailand during the period September, 2001 – February, 2002.

**3.1.2 Isolation of endophytic actinomycetes**

The samples were dissected into leaves, stems and roots, washed in running tap water and cut into small pieces of ca. 4x4 $mm^2$. Tissue pieces were rinsed in 0.1% Tween20 for 30 sec, then in 1% sodium hypochlorite for 5 min, and then washed in sterile distilled water 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of humic acid-vitamin (HV) agar (Otoguro *et al.* 2001) containing 100 μg $ml^{-1}$ nystatin and cycloheximide; the cultures were incubated at 30°C for about 1 month. The organisms growing from the tissue pieces were inoculated onto to an International Streptomyces Project medium 2 (ISP-2) (Shirling and Gottlieb 1966) for a purification. The isolated colonies were subcultured onto slants of Hickey-Tresner (HT) medium (Redshow *et al.* 1976) to establish stock cultures. Isolate prevalence was calculated as follows (Bussaban *et al.* 2001).

$$\text{Isolate prevalence} = \frac{\text{Number of samples yielding 1 isolate X 100}}{\text{Number of samples in that trial}}$$

### 3.2 Morphological observations

The actinomycete isolates were cultured on ISP-2 agar plates at 30°C for 3 days then a cover slides was placed over the actinomycete colonies and incubated at 30°C for further 5 days. They grew on the cover slides and were stained with crystal violet for 1 min. The morphology of actinomycetes was observed under a light microscope.

Leaf, stem and root materials from *Zingiber officinale* L. (Zingiberaceae) and *Alpinia galanga* L.(Zingiberaceae) were selected for microscopic observation by scanning electron microscopy (SEM) (JEOL-JSM840A SEM, Tokyo, Japan). The specimens were washed several times using distilled water and fixed overnight in 2.5% glutaraldehyde at 4°C. They were then dehydrated in a graded alcohol series (30-95%) followed by treatment in acetone and critical-point drying (Petrolini *et al.* 1986). The specimens from each process were mounted on stubs, splutter-coated with gold, and viewed on the SEM at an accelerating voltage of 20 KV. Photomicrographs were recorded on Kodak VP200 film (New York, USA).

### 3.3 Taxonomic properties

Methods and media described by the International Streptomyces Project (Shirling and Gottlieb 1966) were used to determine most of the cultural and physiological characteristics. For morphological characteristics, the presence of aerial mycelium, spore mass color, distinctive reverse colony color, diffusible pigment, and sporophore and spore chain morphology were recorded after 10 days incubation on ISP-2 medium. Diaminopimelic acid isomers and sugars from whole-cell extract were analyzed for chemotaxonomic studies (Becker *et al.* 1964; Boone and Pine 1968).

### 3.4 Paper chromatography of whole-cell hydrolysates

The actinomycetes were grown in shake culture on ISP-2 medium at 30°C, and the cells were collected at maximal growth after incubation varying from 2 days to 2 weeks. After incubation, the cells were collected by filtration with Whatman No.1 filter paper, washed with distilled water and absolute ethanol, and air-dried at room temperature. Dried cells (10 mg) were hydrolysed for 18 h with 1 ml of 6 N HCl in a screw cap tube held at 100°C in an oven. After cooling, the tubes were opened and the contents were filtered through paper. The solid material on the paper was washed with 3 drops of distilled water. The liquid hydrolysate was dried three consecutive times on a steam bath to remove most of the HCl. The residue was taken up in 0.3 ml of distilled water, and 20 ml of the liquid were spotted on Whatman No.1 paper. A spot of 10 ml of 0.01 M *meso*-diaminopimelic acid (Wako pure chemical industries LTD, Japan) also contained some of the LL-isomer of the same compound, thus acting as a standard for both forms of the acid. Descending chromatography was carried out overnight by irrigation with methanol-water-10 N HCl-pyridine (80:17.5:2.5:10, by volume). Amino acids were detected by dipping the papers in a bath of acetonic ninhydrin (0.1% w/v), followed by heating for 2 min at 100°C. Diaminopimelic acids in the hydrolysate gave purple spots with this reagent and travelled faster than the diaminopimelic acid, some migrating off the paper during the overnight irregation. In this system, *meso*-diaminopimelic acid had an $R_{LL}$-diaminopimelic acid of 0.8.

### 3.5 Antifungal activity of actinomycetes isolates against phytopathogenic fungi on solid media

The endophytic isolates were cultured on plates on ISP-2. Two fungal pathogens *Colletotrichum musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively, were used for screening antifungal activity. They were grown on potato dextrose agar (PDA). Mycelial disks of 6 mm diameter were cut from the plates with the two pathogens and transferred to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony. The plates were incubated at 30°C for 5-7 days. The width of inhibition zones between the pathogen and the actinomycete isolates was measured and evaluated as follows: +++, <20 mm; ++, 11-19 mm; +, 2-10 mm; $\pm$, $\leq$1 mm; -, 0 mm.

## Results

### 3.1 Isolation of endophytic actinomycetes from the selected plants

After 3-4 weeks incubation, the surface of some tissue samples showed hyphal growth that had formed a small colony which subsequently grew out onto the surface of the HV agar (Figure 3.1a, b, c). This process of growth of the actinomycetes through the surfaces of the tissues was observed by SEM (Figure 3.2a, b, c, d). Growth of bacteria and fungi from the tissues was almost completely inhibited by the antibiotics included in HV agar, and only a low level of bacterial contamination (*Bacillus* spp) was observed, leaving the actinomycetes clearly visible.

Some 36 plant species (Table 3.1) were examined using a total of 5400 each of root, stem and leaf tissues. *Streptomyces* were the most common isolates recovered, being most prevalent from roots (3.9%), leaves (1.8%) and less from stems (0.3%). With SEM hyphae of these organisms could recognised in the leaf and root tissues of

both *Z. officinale* and *A. galanga*. Observations on 40 samples of each tissue type confirmed the high incidence of actinomycetes in roots (7.5%) and leaves (5%), but hyphae were not seen in stems. It is clear therefore that roots present a good habitat for these endophytic microorganisms.

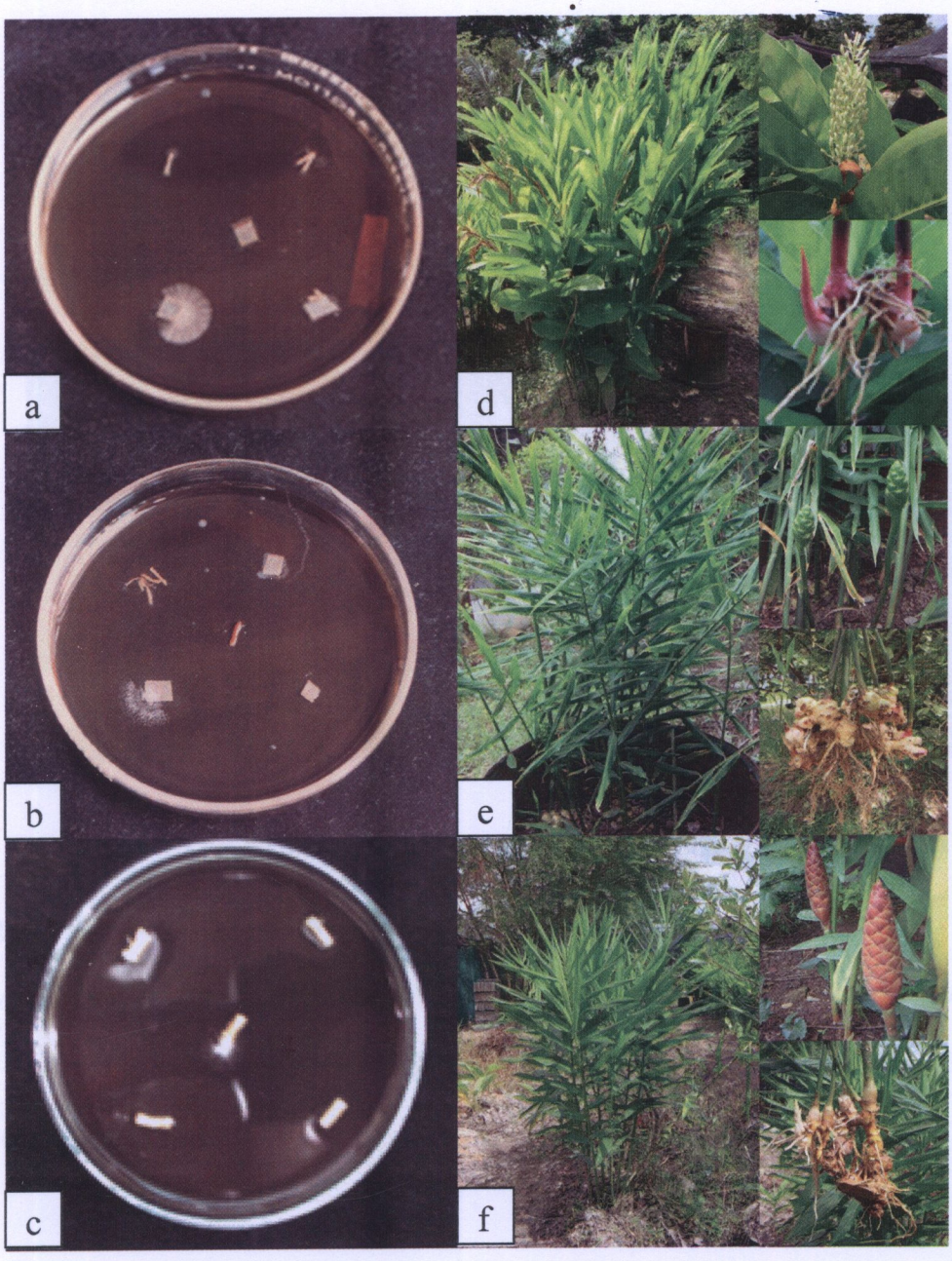


**Figure 3.1** Growth of actinomycete colonies from sterilized blocks of plant tissue; a) leaf of *Alpinia galanga*, b) leaf of *Zingiber officinale*, c) root of *Zingiber cassumunar* on HV agar, these plates were photographed after 3 weeks of incubation. d) *Alpinia galanga*, e) *Zingiber officinale* , f) *Zingiber cassumunar*.

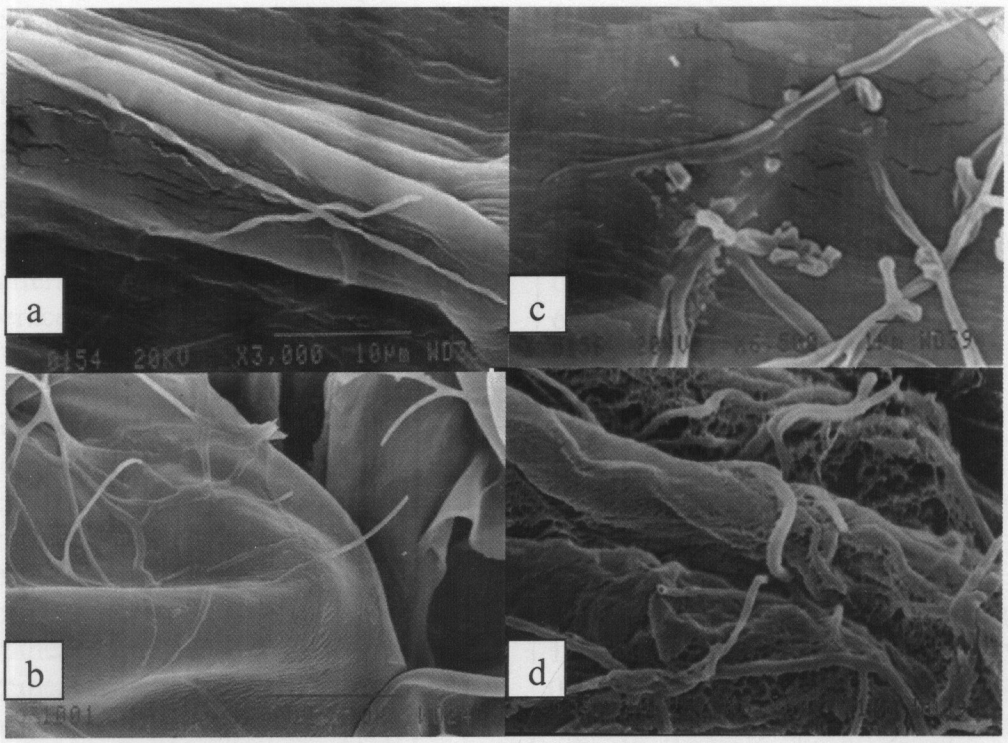


**Figure 3.2** Scanning electron micrographs of aerial hyphae of actinomycetes which have grown through the epidermis of a leaf of *A. galanga*; a), a leaf of *Z. officinale*; b), a root of *A. galanga*; c), a root of *Z. officinale*; d). Magnification: 3,000, 2,500, 6,500, 5,000, respectively.

Table 3.1 Numbers of isolates of endophytic actinomycete per tissue block from leaves, stems and roots or a range of herbaceous and woody plants. Sterilised tissue blocks were placed on HV agar and incubated for up to a month at 30°C.

| Family | Host plant | Leaves (%) | Stems (%) | Roots (%) | Total (%) |
|---|---|---|---|---|---|
| Acanthaceae | *Rhinacanthus communis*[a] | 1 (0.6) | 0 (0.0) | 0 (0.0) | 1 (0.2) |
| Amaranthaceae | *Amaranthus gracilis*[a] | 0 (0.0) | 0 (0.0) | 4 (2.6) | 4 (0.8) |
| Cruciferae | *Brassica juncea*[a] | 0 (0.0) | 0 (0.0) | 1 (0.6) | 1 (0.2) |
| Cruciferae | *Brassica oleracea*[a] | 0 (0.0) | 0 (0.0) | 20 (13.3) | 20 (4.4) |
| Cyperaceae | *Cyperus difformis*[a] | 6 (4.0) | 3 (2.0) | 4 (2.6) | 13 (2.8) |
| Cyperaceae | *Cyperus iria*[a] | 3 (2.0) | 1 (0.6) | 5 (3.3) | 9 (2.0) |
| Cyperaceae | *Cyperus kyllingia*[a] | 5 (3.3) | 3 (2.0) | 5 (3.3) | 13 (2.8) |
| Cyperaceae | *Cyperus malaccensis*[a] | 3 (2.0) | 5 (3.3) | 5 (3.3) | 13 (2.8) |
| Cyperaceae | *Cyperus rotundus*[a] | 5 (3.3) | 1 (0.6) | 4(2.6) | 10 (2.2) |
| Gramineae | *Chloris barbata*[a] | 0 (0.0) | 2 (1.3) | 0 (0.0) | 2 (0.4) |
| Gramineae | *Cymbopogon citratus*[a] | 3 (2.0) | 0 (0.0) | 3 (2.0) | 6 (1.3) |
| Gramineae | *Cymbopogon nardus*[a] | 3 (2.0) | 0 (0.0) | 9 (6.0) | 12 (2.6) |
| Gramineae | *Echinochloa colona*[a] | 3 (2.0) | 0 (0.0) | 1 (0.6) | 4 (0.8) |
| Gramineae | *Echinochloa crusgalli*[a] | 1 (0.6) | 1 (0.6) | 3 (2.0) | 5 (1.1) |
| Gramineae | *Imperata cylindrica*[a] | 0 (0.0) | 0 (0.0) | 8 (5.3) | 8 (1.7) |
| Iridaceae | *Eleutherine palmifolia*[a] | 0 (0.0) | 0 (0.0) | 6 (4.0) | 6 (1.3) |
| Labiatae | *Ocimum tenuiflorum*[a] | 5 (3.3) | 4 (2.6) | 0 (0.0) | 9 (2.0) |
| Rubiaceae | *Coffea arabica*[b] | 2 (1.3) | 0 (0.0) | 2 (1.3) | 4 (0.8) |
| Rutaceae | *Citrus hystrix*[b] | 1 (0.6) | 0 (0.0) | 0 (0.0) | 1 (0.2) |
| Taccaceae | *Tacca chantrieri*[a] | 0 (0.0) | 0 (0.0) | 2 (1.3) | 2 (0.4) |
| Umbelliferae | *Apium graveolens*[a] | 9 (6.0) | 0 (0.0) | 0 (0.0) | 9 (2.0) |
| Umbelliferae | *Coriandrum sativum*[a] | 0 (0.0) | 0 (0.0) | 2 (1.3) | 2 (0.4) |
| Zingiberaceae | *Alpinia blepharocalyx*[a] | 2 (1.3) | 1 (0.6) | 3 (2.0) | 6 (1.3) |
| Zingiberaceae | *Alpinia galanga*[a] | 9 (6.0) | 0 (0.0) | 32 (21.3) | 41 (9.1) |
| Zingiberaceae | *Amomum siamense*[a] | 10 (6.6) | 0 (0.0) | 28 (18.6) | 38 (8.4) |
| Zingiberaceae | *Boesenbergia pandurata*[a] | 4 (2.6) | 0 (0.0) | 19 (12.6) | 23 (5.1) |
| Zingiberaceae | *Curcuma domestica*[a] | 3 (2.0) | 0 (0.0) | 10 (6.6) | 13 (2.8) |
| Zingiberaceae | *Curcuma longa*[a] | 8 (5.3) | 0 (0.0) | 0 (0.0) | 8 (1.7) |
| Zingiberaceae | *Etlingera elatior*[a] | 0 (0.0) | 0 (0.0) | 2 (1.3) | 2 (0.4) |
| Zingiberaceae | *Zingiber cassumunar*[a] | 3 (2.0) | 0 (0.0) | 2 (1.3) | 5 (1.1) |
| Zingiberaceae | *Zingiber officinale*[a] | 8 (5.3) | 0 (0.0) | 33 (22.0) | 40 (8.8) |
| Totals | | 97 (1.7) | 21 (0.3) | 212 (3.9) | 330 (2.0) |

No isolates were recovered from the tree species, *Citrus aurantifolia*[b](Rutaceae), *Streblus asper*[b](Moraceae), *Tamarindus indica*[b](Leguminosae), *Mangifera indica*[b] (Anacardiaceae) and *Dimocarpus longan*[b](Sapindaceae)

[a] : herbaceous plants, [b] : woody plants

In total 330 isolates were recovered, the majority were *Streptomyces* sp. with the remainder identified as *Microbispora* sp., *Nocardia* sp. and *Micromonospora* (Figure 3.3a-h). Four isolates failed to develop sporing structures, although *meso*-diaminopimelic acid was detected in the whole cell extracts confirming an actinomycete status. Twenty three isolates were lost do to drying out during subculture (Figure 3.4). Correspondingly the prevalence of *Streptomyces* sp. was the highest ranging from 6.4% for *Z. officinale* to 0.2% for *Brassica juncea* (Cruciferae) and for *Citrus hystrix* (Rutaceae). Values of *Microbispora* sp., *Nocardia* sp. and *Micromonospora* sp. were much lower (Table 3.2). These results indicate that herbaceous plants are the major host for endophytic actinomycetes with *Streptomyces* sp. being the dominant. In contrast this study has shown that actinomycetes are found only rarely in tree species such as *Citrus aurantifolia* (Rutaceae), *Dimocarpus longana* (Sapindaceae), *Streblus asper* (Moraceae), *Tamarindus indica* (Leguminosae) and *Mangifera indica* (Anacardiaceae).

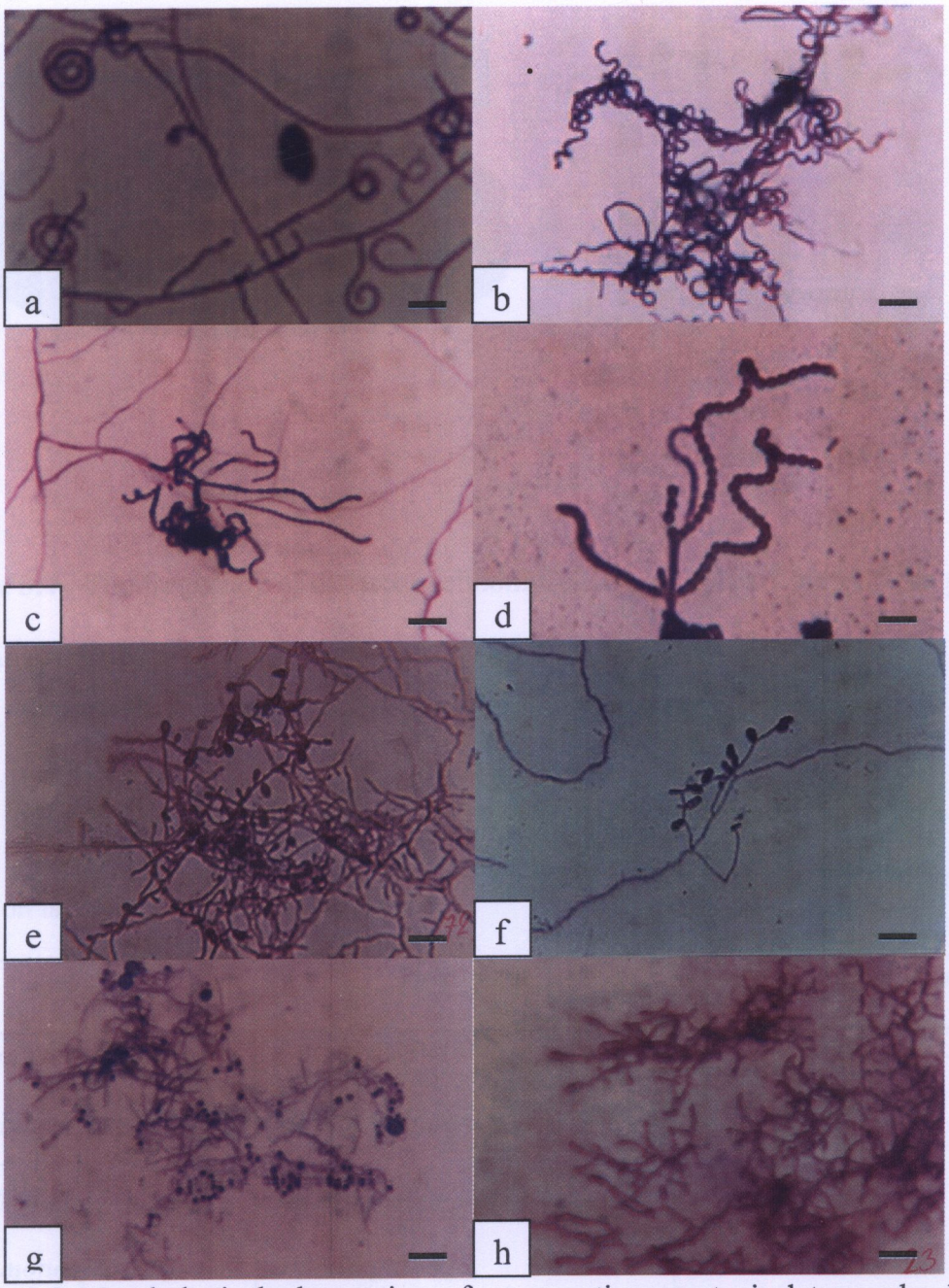


**Figure 3.3** Morphological observation of some actinomycete isolates under light microscopy at 400X magnification. *Streptomyces* sp. morphology; (a: CMUAc53, b: CMUAc77, c: CMUAc160, d: CMUAc271), *Microbispora* sp. morphology; (e: CMUAc72, f: CMUAc42), *Micromonospora* sp. morphology; (g: CMUAc330), *Nocardia* sp. morphology; (h: CMUAc23 ). Bar = 10 μm.

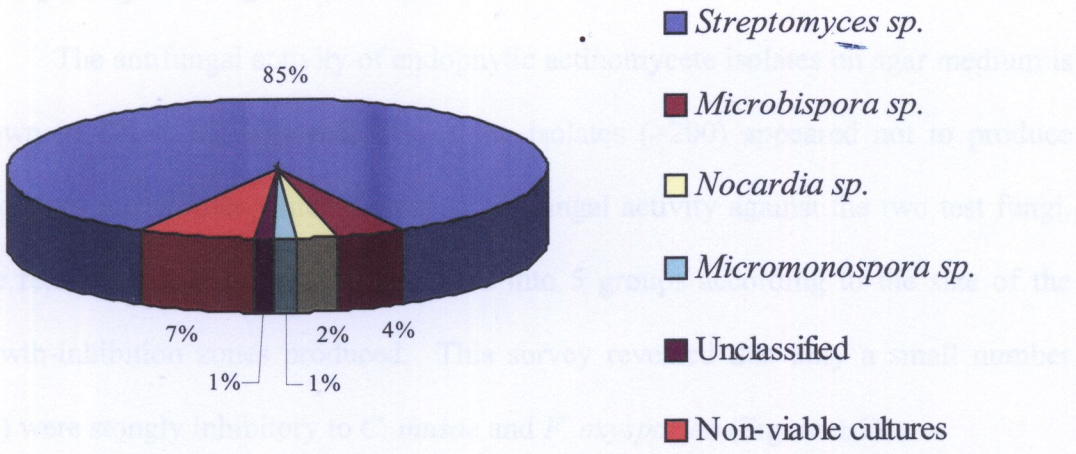


**Figure 3.4** The frequency of different actinomycete types isolated from all the plant types investigated.

**Table 3.2** The highest prevalence of actinomycete isolates and the specific plants, based on isolations made on HV agar

| Isolates[a] | Host plant | Highest prevalence |
|---|---|---|
| *Streptomyces* sp. | *Zingiber officinale* | 6.44% |
| *Microbispora* sp. | *Alpinia galanga* | 0.66% |
| *Nocardia* sp. | *Cyperus malaccensis* | 0.44% |
| *Micromonospora* sp. | *Alpinia galanga*, *Boesenbergia pandurata*, *Curcuma domestica* and *Echinochloa colona* | 0.22% |

[a] The most frequently isolated actinomycetes from the specific plants

### 3.2 Antagonistic action of isolated endophytic actinomycetes to phytopathogenic fungi on solid media

The antifungal activity of endophytic actinomycete isolates on agar medium is shown in Table 3.3. The majority of the isolates (>200) appeared not to produce secondary metabolites which displayed antifungal activity against the two test fungi. The remaining isolates could be divided into 5 groups according to the size of the growth-inhibition zones produced. This survey revealed that only a small number (<5) were stongly inhibitory to *C. musae* and *F. oxysporum* (Figure 3.5).

**Table 3.3** Antifungal activity of endophytic actinomycetes isolates against *C. musae* and *F. oxysporum*. The potential of antifungal activity was evaluated by the zone of fungal growth inhibition on ISP-2 medium after incubation at 30°C for 7 days.

| Potential antifungal activity | Number of endophytic actinomycetes isolates (%) against tested fungi | | |
|---|---|---|---|
| | *Colletotrichum musae* | *Fusarium oxysporum* | *Colletotrichum musae* and *Fusarium oxysporum* |
| +++[a] | 3 (0.9%)[c] | 5 (1.5%)[c] | 2 (0.6%)[c] |
| ++ | 10 (3.0%) | 18 (5.4%) | 8 (2.4%) |
| + | 44 (13.3%) | 53 (16.0%) | 36 (10.9%) |
| ± | 10 (3.0%) | 16 (4.8%) | 8 (2.4%) |
| - | 240 (72.7%) | 215 (65.1%) | 253 (76.6%) |
| ND[b] | 23 (6.9%) | 23 (6.9%) | 23 (6.9%) |

[a] +++ : Width of growth inhibition zone (X) > 20 mm.
++ : 20 mm ≥ X > 10 mm.
+ : 10 mm ≥ X > 1 mm.
± : 1 mm ≥ X
- : X = 0.

[b] Not done : Dried isolates.

[c] These isolates were identified to be *Streptomyces* sp.

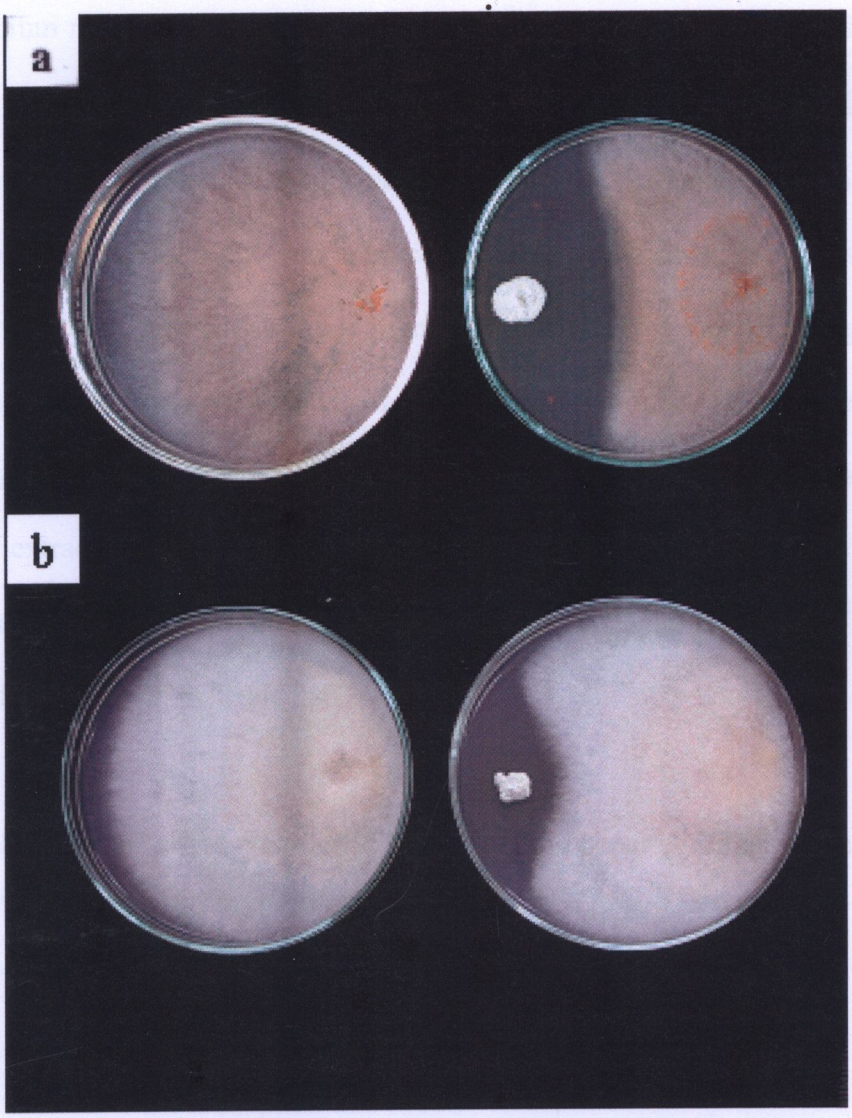


**Figure 3.5** Zones of growth inhibition caused by metabolites from isolate CMUAc130, grown on ISP-2 medium for 7 days, against (a) *C. musae* and (b) *F. oxysporum*.

### 3.3 Identification of the isolate CMUAc130

Morphological observation of 3-day-old cultures of CMUAc130 grown on ISP-2 medium revealed that sporophores to be monopodially branched and flexuous, producing open spirals of spherical to oval-shaped spores (1x1-1.5 μm) (Figure 3.6a, b) with wrinkled surfaces. The substrate mycelium was extensively branched with non-fragmenting hyphae. The aerial mycelium was white changing to ash-gray or dark gray with faint yellowish soluble pigment occasionally discernible (Figure 3.6c). The whole-cell extracts contained LL-type diaminopimelic acid. Based on the morphology of the organism and the presence of LL-type diaminopimelic acid in the whole-cell extracts (Figure 3.7). CMUAc130 was identified as belonging to the genus *Streptomyces*. In this descending chromatography system, the Rf value of all tested amino acids was shown in Table 3.4.

**Table 3.4** Rf value of tested amino acid by descending paper chromatography

| Amino acid | Rf value |
|---|---|
| *meso*-DAP | 0.31 |
| LL-DAP | 0.38 |
| Glutamic acid | 0.60 |
| Lysine | 0.57 |
| Aspartic acid | 0.48 |
| Alanine | 0.67 |
| Glycine | 0.50 |

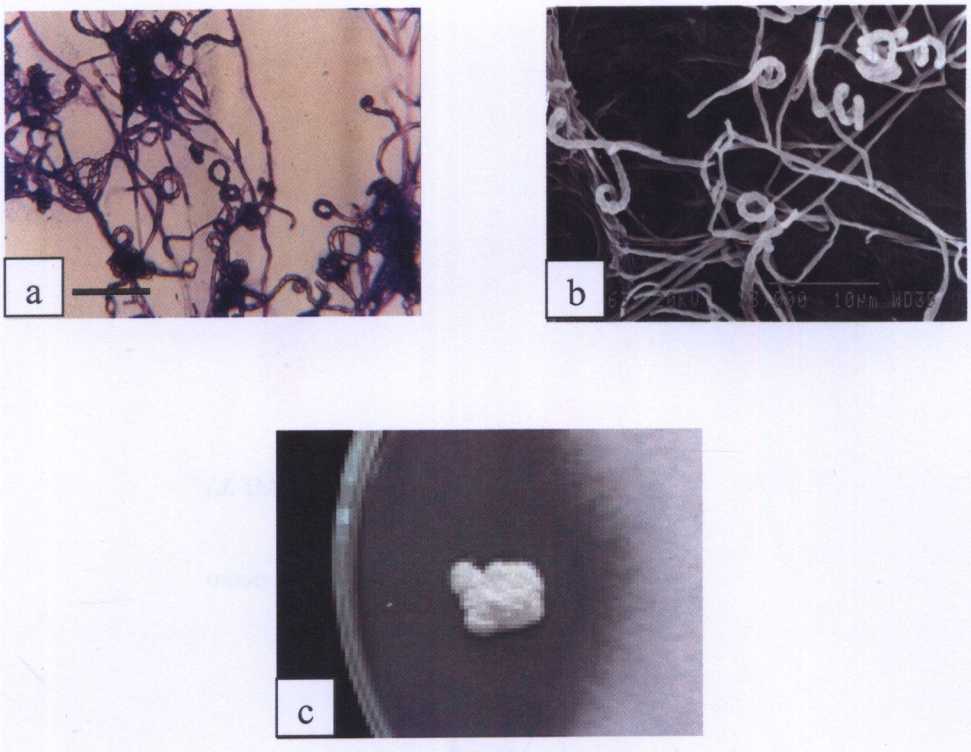


**Figure 3.6** Morphology and colony of the *Streptomyces* sp. CMUAc130. The sporophores have an open spiral form and its spores are spherical to-oval-shaped under light microscopy observation, bar = 10 μm; a) and SEM observation; b). A white colony, changing to ash-gray or dark gray with faint yellowish soluble pigment; c).

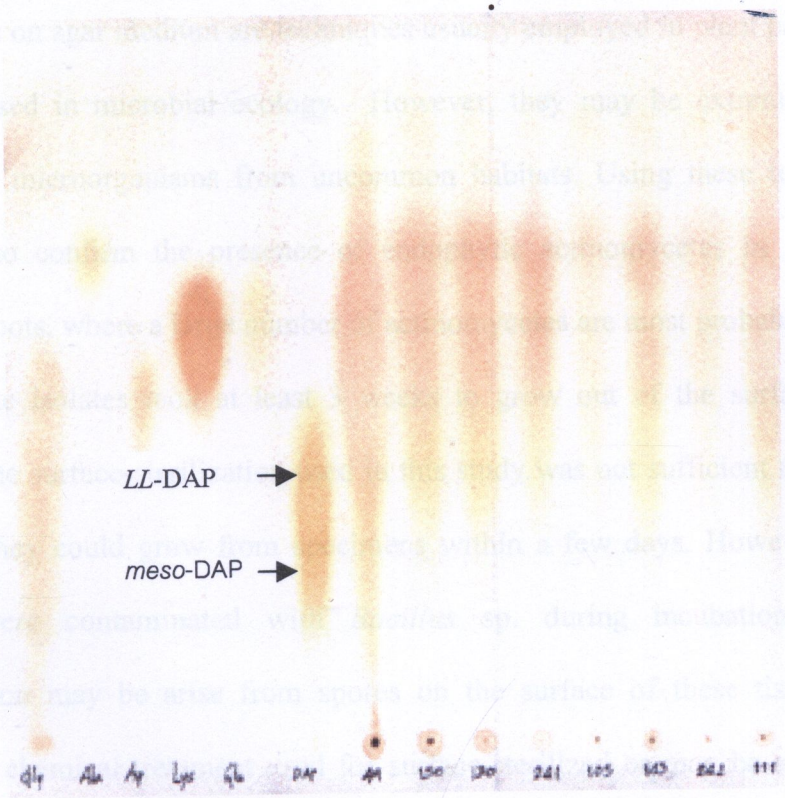


**Figure 3.7** Paper chromatography of whole-cell hydrolysate of *Streptomyces* sp. CMUAc130 showing the amino acid composition of the whole-cell hydrolysate.

**Discussion**

Incubation of surface-sterilized plant parts in a moist chamber and plating of plant tissues on agar medium are techniques usually employed in plant pathology, and not often used in microbial ecology. However, they may be extremely useful in isolation of microorganisms from uncommon habitats. Using these techniques we were able to confirm the presence of endophytic actinomycetes in plant tissues, especially roots, where a large number of actinomycetes are most probably found. The actinomycete isolates took at least 3 weeks to grow out of the surfaces of these tissues. If the surface-sterilization used in this study was not sufficient to kill surface microbes, they could grow from specimens within a few days. However, a few of samples were contaminated with *Bacillus* sp. during incubation time. This contamination may be arise from spores on the surface of these tissues that are resistant to chemical treatment used for surface-sterilized or may be an endophytic *Bacillus* sp. (Garbera *et al.* 2001; Bai *et al.* 2002).

The mycelia of the actinomycete isolates grew out of the tissue blocks onto the surfaces. Thus, these isolates are considered endophytic rather than ectophytic microbes, as discussed by Okazaki *et al* (1995). In the present study, we demonstrated endophytic actinomycetes in *Z. officinale* and *A. galanga* examined by SEM and by isolation on agar plates. Both approaches demonstrated the importance of roots as the habitat for these endophytes. However, the frequency reports from the SEM observations were higher suggesting that the visual observation revealed non-viable hyphae or the consequence of using HV agar which undoubtedly is selective in

its nutrient availability. Future studies should involve the use of several media for isolation.

The isolates were obtained most frequently from roots and less so from other parts. This may relate to the presence of actinomycetes as a large part of the rhizophere microbial flora (Sardi *et al.* 1992) thus enabling easier infection of a host. However, the presence of endophytic actinomycetes in leaves and stems support previous reports (Okazaki *et al.* 1995; Shimizu *et al.* 2000). The presence of endophytic actinomycetes, as shown by their isolation from healthy plants and the SEM investigations on internal tissues, leads to the conclusion that there is a close relationship between these microorganisms and plant tissues in which growth of the former could have a favorable effect on plant growth and development. Their biological activities can affect plant growth either through nutrient supply (Katznelson and Cole 1965; Tahvonen 1982; William *et al.* 1984; Drautz and Zahner 1986; Schippers *et al.* 1987) or the *in situ* production of secondary metabolites which stimulate or depress vegetative development (Mishra *et al.* 1987) and may also protect against phytopathogenic microorganisms (Aba-Allah 2001; Getha and Vikineswary 2002).

In this study most of actinomycetes were obtained from herbaceous plants and very few from woody plants. Similar observations can be drawn from other workers. Okazaki *et al* (1995) obtained 246 isolates from 172 samples of healthy leaves of monocotyledons such as *Cyperus* sp. and *Carex* sp. and in comparison Shimizu *et al* (2000) obtained 10 isolates from *Rhododendron* sp. In our experiment the use of only

one medium for isolation of actinomycetes may be a factor, however, there are also intrinsic differences between woody and herbaceous species and it maybe to these that we have to look for an explanation. Many tree species have mycorrhizal fungi associated with their roots which may form a barrier to infection of these tissue by other endophytic species.

In a similar study, Sardi *et al* (1992) obtained ca 500 isolates from the roots of 13 plant species and most of these were *Streptomyces* sp. They classified these isolates into 72 groups based on their characteristics. After testing antimicrobial activity of 10 groups against *Micrococcues luteus* and *F. oxysporum*, then found that all groups had antimicrobial activity against one or the other organisms, but not to both. Thus most of their isolates had a narrow antimicrobial spectrum. From the present study results of *in vitro* antifungal activity (Table 3), only two endophytic actinomycetes isolates had a strong potential of antifungal activity to *C. musae* and *F. oxysporum*. These results demonstrated that some of endophytic actinomycetes were potent for inhibiting the growth of tested phytopathogenic fungi. However, more detailed investigation is required to demonstrate the potential of these organisms in the biocontrol of plant diseases. Further investigations are therefore necessary to understand the other forms of relationship between endophytic actinomycetes and plant tissues and the usefulness of this phenomenon in agriculture.

# CHAPTER IV

# ACTIVITY OF ENDOPHYTIC *Streptomyces* sp. CMUAc130 AGAINST PHYTOPATHOGENIC FUNGI AND ITS MOLECULAR TAXONOMY

## Introduction

With the continual increase in the world population, scientists are trying to devise new means to increase food production. Unfortunately, severe cereal loss is still inevitable owing to plant diseases, particularly those caused by phytopathogenic fungi. Application of the synthetic fungicides has been considered to be one of the cheapest and most common approaches for the control. However, these chemicals usually take long timelines to be degraded completely causing heavy toxicity to humans, domestic animals and wild life. Like human pathogenic micro-organisms, phytopathogens are also prone to developing resistance to chemical control agents thereby decreasing substantially the effectiveness of these pesticides. Accordingly, there is an urgent need to work towards the invention of safer antifungal agents which are expected to be renewable, non-petrochemical, naturally eco-friendly and easily obtainable. During long-time evolution, plants, on which insects, micro-organisms and mammals are feeding, usually acquire self-defence capabilities by producing a variety of secondary metabolites such as alkaloids, terpenoids, steroids and aromatic compounds which are presumably unpleasant or even toxic to the enemy.

Inside the tissue of nearly all healthy plants, there are many endophytic microorganisms. Endophytes are synergistic to their host, at least some of them are

thought to be making returns for the nutrition from the plant by producing special substances such as secondary metabolites to prevent the host from successfully attacking fungi and pests. The metabolites of endophytes inhibit a number of microorganisms (Gurney and Mantle 1993). Endophytic actinomycetes from the various plant tissues have antifungal activity (Sardi *et al.* 1992; Shimizu *et al.* 2000). Are the endophytes capable of producing antifungal compounds that could be used for controlling the phytopathogenic fungi? We therefore undertook the present study to provide answers to these questions.

**Materials and methods**

**4.1 Antifungal activity of endophytic *Streptomyces* CMUAc130 ferment broths**

Among the 307 isolates of endophytic actinomycetes, the strain designated CMUAc130 was found to be the best producer of antifungal metabolites on ISP-2 agar plate. This isolate was selected for antifungal activity assay by fermentation broth. The inoculum was prepared by introducing the 7-day-old Petri dish culture into 500 ml flasks containing 200 ml of ISP-2 broth, which were shaken (100 rpm) continuously for 3 days at 30°C. The follow-up fermentation was accomplished by adding the inoculum (50 ml) into 1000 ml flasks containing 550 ml of ISP-2 broth, and then shaking for 5 days under the same conditions. The culture broth was filtered, and the filtrate was extracted with ethyl acetate by evaporator (R-124 BUCHI Rotavapor R-124, Switzerland). The extract obtained was tested at a concentration of 0, 5, 10, 30 mg $ml^{-1}$ (dry extract/volume) for the antifungal activity against phytopathogenic fungi and yeast with the whole plate diffusion method (Nishioka *et al.* 1997). The conidia of *C. musae* and *F. oxysporum* were prepared to give a density

of $10^6$ conidia $ml^{-1}$. For *C. albicans*, the cells were inoculated into potato dextrose broth (PDB) and cultured at room temperature with shaking at 100 rev $min^{-1}$ for 48 h. A 1 ml aliquot of conidia suspension or *C. albicans* culture was added to 20 ml of PDA and overlaid onto a PDA plate. Sterile paper disks (diameter, 8 mm; Advantec, Tokyo, Japan) were permeated with 50 ml of serially diluted crude extract samples and placed onto the agar plate immediately after the medium had solidified. The plates were then incubated at 30°C for 5 days for fungi and for 24 h for yeast. Each inhibition experiment was replicated thrice.

### 4.2 Molecular identification of the selected strain

The strain identified as *Streptomyces* sp. CMUAc130 was the most effective in antifungal activity and chitinase production amongst those investigated. So it was selected for species identification based on 16S rDNA sequencing.

#### 4.2.1 16S rDNA gene sequencing

Genomic DNA was isolated from the endophytic actinomycetes CMUAc130 by using a procedure described by Hopwood *et al.* (1985). 16S rDNA was amplified by PCR using *Pfu* DNA polymerase (Promega, USA) and primers A 7-26f (5'-CCGTCGACGAGCTCAGAGTTTGATCCTGGCTCAG-3') and primers B 1523-1504r (5'-CCCGGGTACCAAGCTTAAGGAGGTGATCCAGCCGCA-3'). The conditions used for thermal cycling were as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C 10 min. At the end of the cycles, the reaction mixture was kept at 72°C for 10 min and then cooled to 4°C. The 1.5 Kb amplified 16S rDNA fragment was separated by agarose gel electrophoresis and purified by using a QIAquick gel

extraction kit (QIAGEN, Germany). The purified fragments were directly sequenced using a dRhodamine dye terminator cycle sequencing kit (Applied Biosystems, USA). The sequencing primers were primer A, primer B, primer C 704-685r (5'-TCTGCGCATTTCACCGCTAC-3') and primer D 1115-1100r (5'-AGGGTTGCGCTCGTTG-3'). Sequencing was performed with a model 310 automatic sequencer (Applied Biosystems).

### 3.2.2 Sequencing alignment and phylogenetic analysis

Reference strains were chosen from BLAST (Altschul *et al.* 1997) search results. Multiple alignments of sequence determined in this study together with reference sequences obtained from databases and calculations of levels of sequence similarity were carried out using CLUSTAL W 1.74 (Higgins *et al.* 1992). A phylogenetic tree was reconstructed by using treeing algorithms contained in the PHYLIP package (Felsenstein 1995). The topology of the neighbour-joining phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (Felsenstein 1995) with 1000 replicates.

## Results

### 4.1 Antifungal activity of endophytic *Streptomyces* CMUAc130 in liquid culture

The isolate CMUAc130 showed promising activities against *C. musae* and *F. oxysporum* (Table 3.3). Ethyl acetate extracts of fermentation broths recovered from cultures of CMUAc130 exhibited a broad antifungal spectrum. At a concentration of 10 mg $ml^{-1}$ (dried extract/volume), the extract showed discernible growth inhibition of both *C. musae* and *F. oxysporum* (Figure 4.1). Furthermore, the MICs of the ethyl

acetate extract of the isolate CMUAc130 culture against these fungi was determined to be 5, 10, and 30 mg $ml^{-1}$, respectively (Figure 4.2).

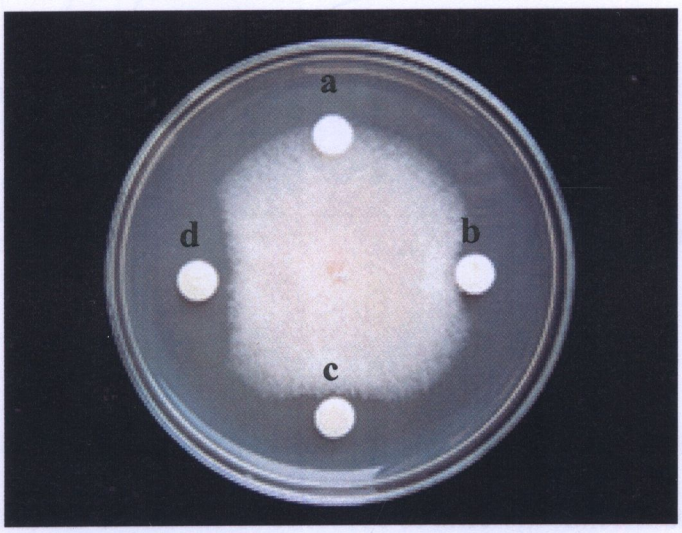


**Figure 4.1** Zone of growth inhibition of *C. musae* by the ethyl acetate extract of the isolate CMUAc130 at different concentration (a: 0, b: 5, c: 10 and d: 30 mg $ml^{-1}$)

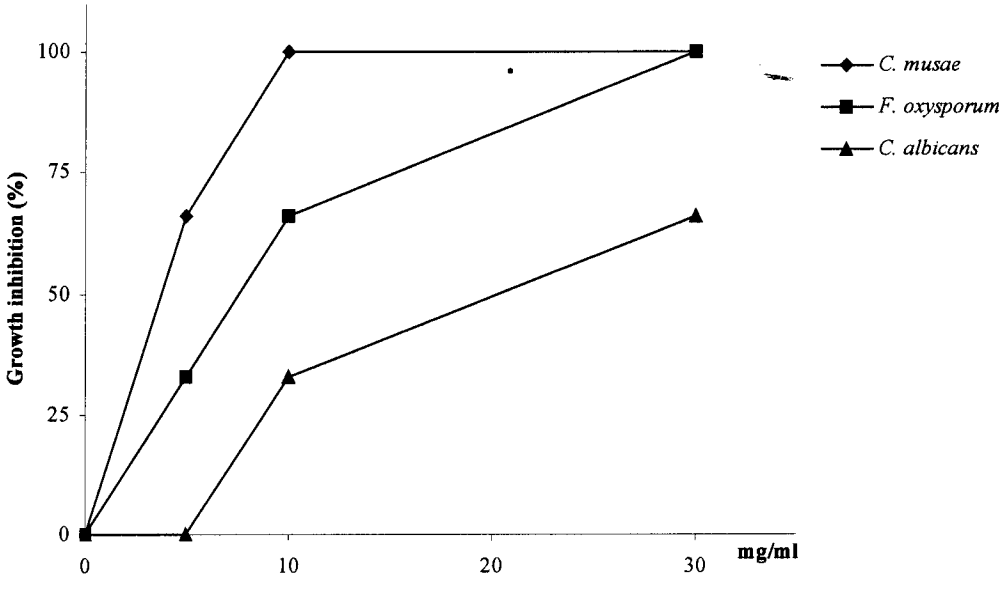


**Figure 4.2** Percentage of growth inhibition of phytopathogenic fungi and yeast by the ethyl acetate extract of the isolate CMUAc130 at different concentrations.

### 4.2 Molecular identification and phylogenetic analysis of 16S rDNA from endophytic *Streptomyces* sp. CMUAc130

The purified 1.5 Kb fragment of 16S rDNA of endophytic *Streptomyces* sp. CMUAc130 was run on agarose gel electrophoresis (Figure 4.3). The DNA sequences of the various primers used are shown in Table 4.1. Almost the complete 16S rDNA sequence was determined for the isolate *Streptomyces* sp. CMUAc130 (>95% of the *Escherichia coli* sequence) from position 8 to position 1523 (*E. coli* numbering system; Brosius *et al.* 1978). BLAST search results for strain CMUAc130 came from non-redundant GenBank + EMBL + DDBJ; when reference sequences were chosen, unidentified and unpublished sequences were excluded. The BLAST search results

and the phylogenetic tree (Figure 4.4) generated from representative strains of the related genera showed that strain CMUAc130 had high levels of sequence similarity to *S. aureofaciens* (accession number: AB045881). 16S rDNA analysis revealed a sequence similarity level of 97% (The strain CMUAc130 is phylogenetically closely related to *S. aureofaciens*). The nucleotide sequence data reported in this thesis has been deposited in the GenBank, EMBL and DDBJ databases with the accession number AB105068.

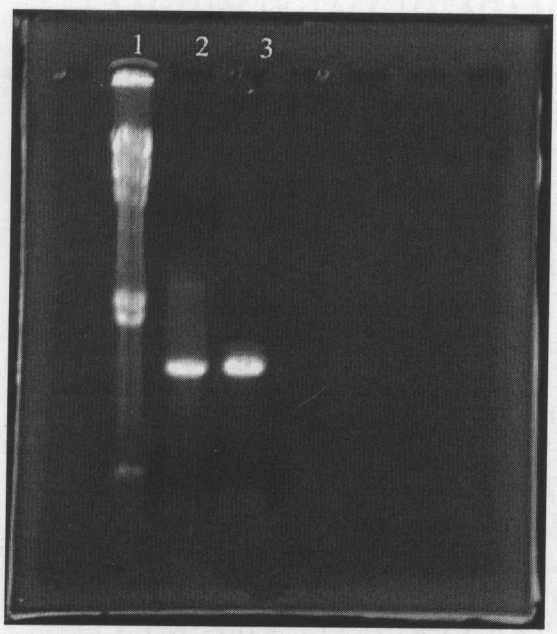


**Figure 4.3** Agarose gel (0.8%) analysis of 1.5Kb PCR product of 16S rDNA from endophytic *Streptomyces* sp.CMUAc130. 1) λDNA/*Hin*dIII marker, 2) 1.5Kb PCR product, 3) Purified PCR product.

**Table 4.1** The DNA sequence of 16S rDNA of endophytic *Streptomyces* sp. CMUAc130 amplified by various primers.

| Used primers | DNA sequence (5'→ 3') |
|---|---|
| Primer A 7-26f | GACGAACGCTGGCGGCGTGCTTAACACATGCAAGT<br>CGAACGGTGAAGCCCTTCGGGGTGGATCAGTGGCG<br>AACGGGTGAGTAACACGTGGGCAATCTGCCCTGCA<br>CTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC<br>GGATATGACCTTCCTCCGCATGGGGGTTGGTGGAAA<br>GCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCA<br>GCTTGTTGGTGGGGTAATAGCCTACCAAGGCGGCG<br>ACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACAC<br>TGCGACTGAGACACGGCCCAGACTCCTACGGGAGG<br>CAGCAGTGGGGAATATTGCACAATGAGCGAAAGCC<br>TGATGCAGCGACGGCCGCGTGAGGGATGACGGCCT<br>TCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGC<br>AAGTGACGGTACCTGTAGAAGAAGCACCGGCNAAN<br>NN |
| Primer B 1523-1504r | ACGGCTACCTCGTTACGACTCCGTCCCAATCGCTGG<br>TCCCACCTCCGACGGCTCCATCCCTTACGGGTTAGG<br>CCACCGGCTTCGGGTGTTACCGACTTTCGTGACGCG<br>ACGAGCGGTGTGTACATGGCCCGGGAACGTATTCA<br>CCGCAGCATGCTGATCTGCGATTACTAGCAACTCCA<br>ACTTCATGGGGTCGAGTTGCAGACCCCAATCCGGAC<br>TGAGGCCGGCCCTTTGGGATTCGCTCCGCCTCACGG<br>CATCGCAGCCCTTTGTACCGACCATTGTAGCACGTG<br>TGCAGCTCAAGACATAAGGGGCATGATGACTTGAC<br>GTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAGT<br>CTCCTGTGAGTCCCCGACATTACTCGTTGGCAACAC<br>AGAACAGGGGTTGCGCTCGTTGCGGGACTTAACCC<br>AACATCTCACGACACGAGCTGACGACANCNNNN |
| Primer C 704-685r | ACTCTAGCCTGCCCGTATCGAATCCAGACCCGAGGT<br>TAAGCCCCGGGCTTTCACATCCGACGCGACAGGCCG<br>CCTACGAGCTTTACGCCCAATAATTCCGGACAACGC<br>TCGCACCCTACGTATTACCGCGGCTGCTGGCACGTA<br>GTTAGCCGGTGCTTCTTCTACAGGTACCGTCACTTG<br>CGCTTCTTCCCTGCTGAAAGAGGTTTACAACCCGAA<br>GGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGC<br>TTTCGCTCATTGTGCAATATTCCCCACTGCTGCCTCC<br>CGTAGGAGTCTGGGCCGTGTCTCAGTCGCAGTGTGG<br>CCGGTCGCCCTCTCAGGCCGGCTACCCGTCGCCGCC<br>TTGGTAGGCTATTACCCCACCAACAAGCTGATAGGC<br>CGCGGGCTCATCCTGCACCGCCGGAGCTTTCCACCA<br>ACCCNCATNNNN |

**Table 4.1** The DNA sequence of 16S rDNA of endophytic *Streptomyces* sp. CMUAc130 amplified by various primers (continued).

| Used primers | DNA sequence (5'→ 3') |
|---|---|
| Primer D 1115-1100r | CGGGACTTAACCCAACATCTCACGACACGAGCTGA<br>CGACAACCATGCACCACCTGTATACCGACCACAAG<br>GAGGCACCCATCTCTGGATGTTTCCGGCATATGTCA<br>AGCCTTGGTAAGGTTCTTCGCGTTGCGTCGAATTAA<br>GCCACATGCTCCGCTGCTTGTGCGGGCCCCCGTCAA<br>TTCCTTTGAGTCTTAGCCTTGCGGCCGTACTCCCCAG<br>GCGGGGAACTCAAGCGAGCTGCGGCACCGACGACG<br>TGGAGTGTCGCCAACACCTAGTTCCCAACGTTTACG<br>GCGTGGACTACCAGGGTATCTAGTCCTGTTCGCTCC<br>CCACGCTTCCGCTCCTCAGCGTCAGTAATGGCCCAG<br>AGATCCGCCTTCGCCGCCGGTGTTCCTCCTGATATC<br>TGGCGCATCTCACCGCTACACCAGGAATTCCGATCT<br>CCCCTACTACACTCTAGCCTGCCCGTATCGAATNCN<br>NNN |

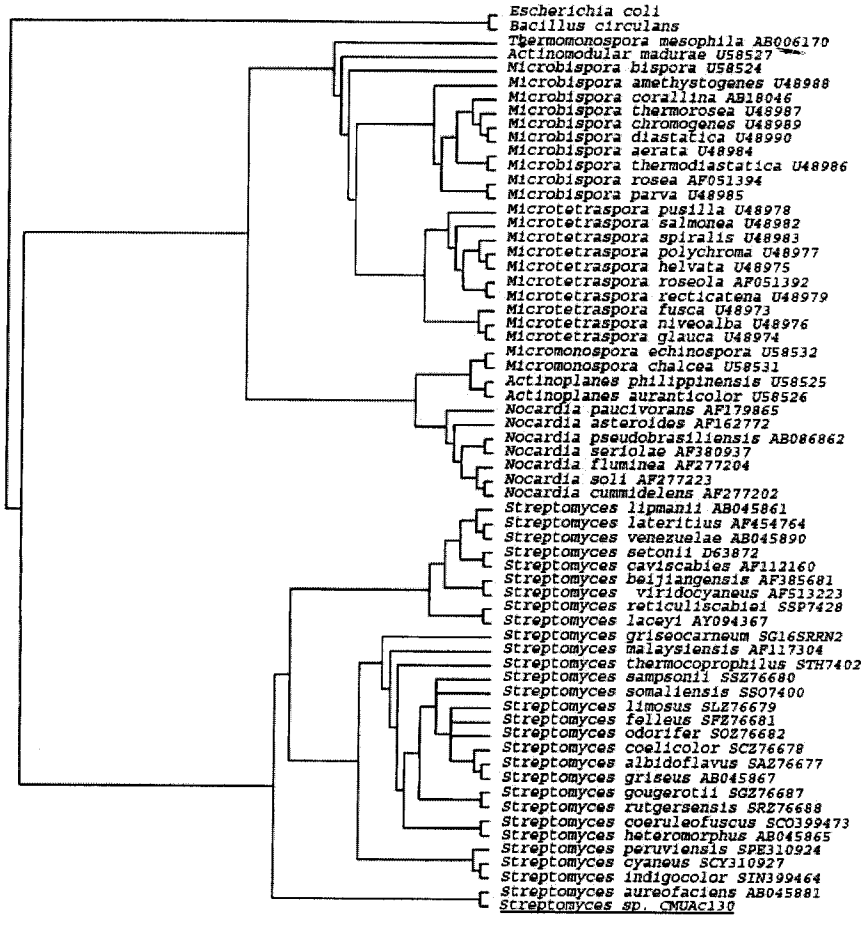


**Figure 4.4** Phylogenetic tree showing the relationships of endophytic *Streptomyces aureofaciens* CMUAc130 (underline), related species of the same genus and other taxa based on 16S rDNA genes sequences.

**Discussion**

Based on results of the morphological observation as well as on the presence of LL-type diaminopimelic acid in the whole-cell extracts, the endophytic actinomycete isolate CMUAc130 was identified as belonging to the genus *Streptomyces*. Almost the complete 16S rDNA sequence was determined (>95% of the *Escherichia coli* sequence) from position 8 to position 1523 (*E. coli* numbering system; Brosius *et al.* 1978). BLAST search results for strain CMUAc130 came from non-redundant GenBank + EMBL + DDBJ; when reference sequences were chosen, unidentified and unpublished sequences were excluded. The BLAST search results and the phylogenetic tree generated from representative strains of the related genera showed that strain CMUAc130 had high levels of sequence similarity (97%) to the species of *S. aureofaciens* (accession number: AB045881). The nucleotide sequence data reported in this thesis appeared in the GenBank, EMBL and DDBJ databases with the accession number AB105068. This isolate produced the most active substance as ethyl acetate extracts of the fermentation broth exhibited a broad antifungal spectrum. Even at a concentration of 10 mg $ml^{-1}$ (dried extract/volume), the extract showed discernible growth inhibition against *C. musae* and *F. oxysporum*. Furthermore, the MICs of ethyl acetate extract of CMUAc130 culture against these fungi was determined to be 5, 10 and 30 mg $ml^{-1}$, respectively.

The assay, therefore, showed promising results for the use of this isolate as an antifungal agent against phytopathogenic fungi. Similar results have been reported previously in actinomycete screening studies (Crawford *et al.* 1993). Strain CMUAc130 was selected for one trait often associated with biocontrol agents, the ability to produce extracellular metabolites active against several phytopathogenic

fungi, including *C. albicans*. In the *in vitro* assay, preinoculation of strain CMUAc130 was done to allow growth and sporulation (see in chapter VI) of the culture prior to inoculation with *C. musae* and *F. oxysporum*. Thus, the antagonism between strain CMUAc130 and the pathogen may have involved production of secondary metabolites in agar. The *in vitro* studies further indicated that culture of CMUAc130 in liquid media produced extracellular metabolites that showed clear zones of inhibition against *C. musae* and *F. oxysporum*. In their crude form, these metabolites inhibited spore germination and hyphal development of *C. musae* and *F. oxysporum* and induced morphological changes such as swollen and distorted germ tubes that branched more freely than normal conidia (see in chapter VI).

In this study, an approach was used to show that strain CMUAc130 produced diffusible metabolites, which have inhibitory effects on the growth of *C. musae* and *F. oxysporum* in solid medium. In the further study, the inhibitory effects of strain CMUAc130 against *C. musae* and *F. oxysporum* should be tested in a soil environment. Rothrock and Gottlieb (1984) had shown that the population of *Streptomyces hygroscopicus* subsp. *geldanus*, a strong antagonist of the root rot-causing *Rhizoctonia solani*, and antibiotic concentration in soil increased with the time of incubation.

In all cases, there was a definite decrease in the development of *C. musae* and *F. oxysporum* in the presence of strain CMUAc130. Some decrease in the degree of inhibition of *C. musae* and *F. oxysporum* was displayed by strain CMUAc130 in solid medium, when compared to the degree of inhibition by the antifungal metabolites of strain CMUAc130 in the paper disc and spore germination assays (see in chapter VI). This was indicated by a slightly more extensive hyphal development of *C. musae* and

*F. oxysporum* in the strain CMUAc130-inoculated solid medium, compared to that observed in the in vitro assays. Variations in the antifungal potential of strain CMUAc130 in agar and in the crude extract might be due to variations in the concentration of the antifungal metabolites produced by strain CMUAc130 under different growth conditions. Medium composition may also cause a difference in the type of bioactive compounds that are produced (Trejo-Estrada *et al.* 1998). For example, different types of carbon sources in the growth medium have been shown to have an important impact on both the production of secondary metabolites by *Streptomyces*, and the interactions of bacterial cells and the fungal hyphal cell surface (Yuan and Crawford 1995).

Evidence for the role of competition and parasitism in the biocontrol of plant diseases has been convincing. However, antibiosis is much less clearly established due to the lack of methods for a meaningful evaluation of the production and function of compounds mediating antibiosis in soil (Rothrock and Gottlieb 1984). Previously, Fravel (1988) considered antibiosis as a type of antagonism mediated by specific or nonspecific metabolites of microbial origin, by lytic agents enzymes, volatile compounds or other toxic substances. Fungal cell wall-degrading enzymes produced by an antagonist were, therefore, thought to be involved simultaneously in parasitism and antibiosis. Antibiosis is particularly considered to provide an advantage in biological disease control because compounds mediating antibiosis can diffuse rapidly in nature, and direct contact between the antagonist and pathogen is not necessary (Hajlaou *et al.* 1994).

Antibiotics have been implicated repeatedly in the antagonism of fungi by actinomyces (Crawford *et al.* 1993, Rothrock and Gottlieb 1984, Yuan and Crawford

1995). There are many reports related to antibiotic substances that induced such malformations such as stunting, distortion, swelling, hyphal protuberances or the highly branched appearance of fungal germ tubes (Gunji *et al.* 1983). Using this criterion, Stevenson (1956) found that antibiotics of some soil actinomycetes caused similar effects on hyphae of *Helminthosporium sativum*, in culture and soil. Swellings and lysis of hyphal tips have also been seen in the presence of antibiotics of other actinomycetes (Richmond 1975). Several species from the *Streptomyces aureofaciens* clade produced antifungal antibiotics such as chlortetracycline (Li *et al.* 2001) and rutamycin (White *et al.* 2001). On the other hand, *Streptomyces* are also known for their ability to cause lysis of fungal hyphae by producing chitinases and glucanases (Mahadevan and Crawford 1997). Chitin in fungal cell walls is normally in a highly rigid, crystalline state. In the hyphal apex, however, the chitin is sensitive to chitinases (Mahadevan and Crawford 1997).

Lockwood and Lingappa (1963) had indicated that the ability of actinomycetes to lyse living mycelium in agar or in soil was apparently related to their ability to inhibit spore germination as determined by production of inhibition zones on agar. According to these authors, the correlation between these two processes suggests that inhibition of spore germination and destruction of mycelium was brought about by a similar mechanism. This could either be a lytic enzyme that destroys germ tubes or otherwise prevents germination, or an antibiotic that induces autolysis of the fungal hyphae. Autolysis results in self-digestion of the protoplasm and cell walls by enzymes of the fungus itself (Lloyd *et al.* 1965). However, autolysis induced by antibiotics and toxins from antagonistic microorganisms may not result in complete lysis of fungal mycelium in soil. Final dissolution of the remains of the empty cell

wall would then depend on hydrolytic enzymes, which in soil would be of microbial origin (Lochwood and Lingappa 1963). The further studies have been done to determine if strain CMUAc130 produced hydrolytic enzymes under the influence of fungal cell walls. The antifungal biocontrol agent, *S. lydicus* WYEC108, produces high levels of chitinase in the presence of chitin from fungal cell walls as carbon source (Mahadevan and Crawford 1997).

This study indirectly demonstrated that antibiosis mediated by diffusible metabolites was likely to be involved in the antagonism of strain CMUAc130 against *C. musae* and *F. oxysporum* in solid medium. Despite all the *in vitro* assays, conclusive evidence as to the exact role of antibiotics and/or hydrolytic enzymes could not be reached in the absence of purified compounds. Secretion of enzymes and antibiotics may interact synergistically in the process (Benyagoub *et al.* 1998). Further studies are needed to characterize the antibiotic substances of strain CMUAc130, study the antagonistic effects of purified compound(s), determine the enzyme production and understand the regulation of their production. These studies will aid in the manipulation and development of strain CMUAc130 as a potential biological control agent for the *Fusarium* wilt pathogen of wheat.

# CHAPTER V

# CHITINASE PRODUCTION BY *Streptomyces aureofaciens* CMUAc130 AND ITS ANTAGONISTIC ACTION AGAINST PHYTOPATHOGENIC FUNGI

## Introduction

Chitin, a 1,4-β-linked polymer of *N*-acetyl-β-D-glucosamine (GlcNAc), is the second most abundant polymer in nature, after cellulose. It is a major structural component of the exoskeleton of insects and crustaceans and it occurs in the cell walls of a variety of fungi. In accordance with the abundance of chitin, chitin-degrading enzymes are found in a variety of organisms, varying from prokaryotes to man. In chitin-containing organisms, chitinases play an important role in normal life cycle functions such as morphogenesis and cell division, whereas plants produce chitinases as part of their defense against fungal pathogens. Many bacteria and fungi contain chitnolytic enzymes to convert chitin into compounds that can serve as energy source.

Chitinases belong to families 18 and 19 of glycosyl hydrolases group of enzymes (Henrissat and Bairoch 1993). Family 18 contains enzymes from a variety of prokaryotic and eukaryotic organisms, whereas family 19 chitinases only have been found in higher plants and in the Gram-positive bacteria belonging to *Streptomyces* sp. (Ohno *et al.* 1996). The two families contain both endochitinases, cleaving randomly in the chitin chain, and exochitinases. The latter cleave chitobiose (GlcNAc)2 ("chitobiosidase"; exo-*N*, *N*'-diacetylchitobiohydrolase) or chitotriose (GlcNAc)3 ("chitotriosidase"; exo-*N*, *N*', *N*''-triacetylchitotriohydrolase) from the

reducing or the nonreducing end of the chitin chain (Jeuniaux 1966). In addition to endo- and exochitinases, chitin-degrading organisms contain chitobiases (*N*-acetyl-β-glucosaminidases), a third class of chitinolytic enzymes that convert GlcNAc dimers into monomers (Chernin *et al.* 1995).

Chitinases are of great biotechnological interest. Firstly, these enzymes may be used to convert chitin-containing biomass into useful (depolymerised) components. Secondly, chitinases may be exploited for the control of fungal and insect pathogens of plants (Shapairo *et al.* 1987). Thirdly, chitinase inhibitors potentially inhibit growth of chitin-containing (plant-) pathogens and plague insects that need chitinases for normal development (Lim *et al.* 1991).

Endophytic actinomycetes have been isolated from various plant tissues and some of them have had a potential as biocontrol agents against phytopathogenic fungi (Sardi *et al.* 1992; Shimizu *et al.* 2000). As to the ability of some strains of endophytic actinomycetes which can produce chitinase, these microorganisms might protect against phytopathogenic fungi in plant tissue by this property. Thus the role of chitinase of endophytic actinomycetes in antifungal activity should be studied.

**Materials and methods**

**5.1 Screening for chitinase producing actinomycetes**

Three hundred and seven endophytic actinomycetes were isolated from leaves, stems and roots tissue from healthy plants (Sardi *et al.*, 1992; Shimizu *et al.*, 2000), and were maintained on International Streptomyces Project medium 2 (ISP-2) agar slants (Shirling and Gottlieb, 1966). Screening for chitinase production of these isolates was by plate agar assay and followed by tests in broth for chitinase producing strains. The colloidal chitin basal medium was required for screening of chitinase

producing actinomycetes. This medium was adjusted to pH 7.0. Practical grade crab shell chitin powder (Sigma) was used to prepare colloidal chitin (Berger and Reynolds, 1958) as a substrate for growth and enzyme assay. Enzyme production was carried out in shake culture (50 ml medium in 250 ml Erlenmeyer flask) incubated at 30°C in the incubator shaker (Sanyo Gallenkamp PLC, England) at 150 rev $min^{-1}$ for 7 d. Spores were inoculated to a concentration of $10^5$ $ml^{-1}$. Triplicate flasks were harvested at day 7. The culture medium was then filtered through Whatman No.1 filter paper and the culture filtrate was centrifuged in a refrigerated centrifuge at 4°C for 20 min at 5000 rev $min^{-1}$ to remove any remaining cell debris. The culture filtrates were harvested at day 7. The culture filtrate thus obtained was a crude enzyme for determining chitinase activity.

**5.2 Enzyme assay**

Colloidal chitin was used as a substrate to assay chitinase activity: 0.5 ml of 1% colloidal chitin in phosphate buffer (pH 7.0) was incubated with 0.5 ml of enzyme at 37°C for 60 min. The reducing sugars in the reaction mixture were measured by Somogyi Nelson's colorimetric method (Wood and Bhat 1988) (see Appendix D). One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of 1 μmol of *N*-acetylglucosamine $ml^{-1}$ in 60 min.

**5.3 Enzyme purification**

The chitinase obtained from *S. aureofaciens* CMUAc130 was found to be a high level chitinase producing strain, so it was studied for enzyme purification and kinetics. Spores ($5x10^7$) of *S. aureofaciens* CMUAc130 were inoculated into 500 ml of colloidal chitin medium and incubated at 30°C in the incubator shaker at 150 rev $min^{-1}$ for 7 days. Triplicate flasks were harvested at daily intervals up to 14 days. The

culture medium was then filtered through Whatman No.1 filter paper and the culture filtrate was centrifuged in a refrigerated centrifuge at 4°C for 20 min at 5000 rev $min^{-1}$ to remove any remaining cell debris. The culture filtrate thus obtained was a crude enzyme for determining chitinase activity.

The crude enzyme sample was precipitated by 80% ammonium sulphate and further purified by Sephadex G-75 column chromatography. The fractions with chitinase activity were pooled, lyophilized by Dura-dry freeze-dryer (FTS systems, USA) and concentrated by ultrafiltration with the Centricon System (Amicon, Japan) at 1200 rev $min^{-1}$, 4°C for 1 h for further biochemical characterization by incubation at different temperatures and pH. The purity of the chitinase protein was determined by SDS-PAGE, using 4% stacking and 12% separating gels, which was performed according to Laemmli (1970). The purified protein (7.5 μg) was loaded per well. The gels were stained with Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (40:10:50, by vol.).

The chitinase obtained from *S. aureofaciens* CMUAc130 was tested for its potential to act as a biocontrol agent against important fungal phytopathogens by virtue of its lytic action on the chitin component of the cell walls.

**5.4 Determination of optimal conditions for chitinase production**

The chitinase production was carried out in colloidal chitin broth for 7 days. Spores were inoculated to a concentration of $10^5$ $ml^{-1}$. A variety of culture conditions were used; temperature (25, 30, 37, 40, 45 and 50°C), pH (4, 5, 5.5, 6, 6.5, 7, 8 and 9), shaking speed (100, 120, 150 and 180 rev $min^{-1}$) and colloidal chitin concentration (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%). After 7 days incubation, the culture filtrates

were harvested. The culture filtrate thus obtained was a crude enzyme for determining chitinase activity.

**5.5 Chitinase production on different substates**

Chitinase production was carried out in different chitin-derived substrate (colloidal chitin (see Appendix B), crude chitin (Sigma), ball milled chitin (see Appendix B) and *Schizosacchromyces* sp. cell wall (a gift from Prof.Toshiki Mogami, Ritsumeikan University, Japan). Spores were inoculated to a concentration of $10^5$ $ml^{-1}$. The shaken cultures (50 ml medium in 250 ml Erlenmeyer flask) were incubated at 30°C in incubator shaker (Sanyo Gallenkamp PLC, England) at 100 rev $min^{-1}$. After 7 days incubation, the culture filtrates were harvested. The culture filtrate thus obtained was a crude enzyme for determining chitinase activity.

**5.6 Effect of sugars and other C-source additive on chitinase production**

Chitinase production was carried out in 1% colloidal chitin broth for 7 days. Spores were inoculated to a concentration of $10^5$ $ml^{-1}$.

Addition of 0.5% mono- and disaccharides including *N*-acetylglucosamine, glucose, fructose, cellobiose, arabinose, raffinose, xylose, sucrose, lactose, mannose and other C-source including 0.5% pectin, starch and carboxymethyl cellulose to 1% colloidal chitin-containing medium was performed. The shaken cultures (50 ml medium in 250 ml Erlenmeyer flask) were incubated at 30°C in incubator shaker (Sanyo Gallenkamp PLC, England) at 100 rev $min^{-1}$. After 7 days incubation, the culture filtrates were harvested. The culture filtrate thus obtained was a crude enzyme for determining chitinase activity.

### 5.7 Determination of optimal temperature for chitinase activity

The optimal temperature for chitinase activity was determined by measuring the release of *N*-acetylglucosamine from colloidal chitin using enzyme assay. A single reaction mix contained 0.5 ml of purified enzyme plus 0.5 ml of 1% colloidal chitin in phosphate buffer (pH 7.0). Chitinase activity at temperatures 25, 30, 37, 40, 45, 50, and 55°C was determined. The reaction tubes were incubated for 1 h at the required temperature and triplicate tubes were used at each temperature.

### 5.8 Determination of optimal pH for chitinase activity

The effect of pH on enzyme activity was determined by varying the pH of the reaction mixture; pH 4, 5 and 5.5 of acetate buffer, pH 5.5, 6, 6.5 and 7 of phosphate buffer and pH 7, 8 and 9 of Tris-base buffer. A single reaction contained 0.5 ml of purified enzyme plus 0.5 ml of 1% colloidal chitin in various pH of the buffer at 37°C. The reaction tubes were incubated for 1 h. Triplicate tests at each pH were performed.

### 5.9 Chitinase activity on different substates and chito-oligosaccharide

Chitinase activity on several chitin-derived substrates and the chito-oligosaccharides was determined. The substrates used for this study were 1% (w/v) of ball milled chitin, *Schizosaccharomyces* sp. cell wall, crude chitin, colloidal chitin and 0.25 mM of chito-oligosaccharide; *N,N'*-diacetylchitobiose, *N,N',N''*-triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose. Each substrate was prepared in 0.05 M sodium phosphate buffer, pH 7.0. A single reaction contained 0.5 ml of purified enzyme plus 0.5 ml of the test substrate and incubated at 37°C for 1 h. The amount of *N*-acetylglucosamine released was determined using the procedure described. Each test was performed in triplicate.

**5.10 Effect of metal ions, EDTA and β-mercaptoethanol on chitinase activity**

The effect of various divalent cations ($Mn^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Mg^{2+}$ and $Hg^{2+}$) and other inhibitors; β-mercaptoethanol and EDTA on chitinase activity was investigated as described by Gupta *et al.* (1995). Purified chitinase was incubated at 37°C with 5 mM of each divalent cations for 10 min and then the chitinase activity was assayed. Triplicate tests for each metal ion and other inhibitors were performed.

**5.11 Preparation of fungal cell wall**

The fungi *Colletotrichum musae*, *Fusarium oxysporum*, *Bipolaris* sp., *Drechslera* sp., *Rhizoctonia* sp., *Sclerotium* sp. and *Candida albicans* ATCC90028 were grown in potato dextrose broth, incubated at 30°C, and then harvested after 72 h growth. Cell walls were obtained from the mycelia of the above species as described by Skujins *et al.* (1965). For *C. albicans*, the culture medium was centrifuged in a refrigerated centrifuge at 5000 rev $min^{-1}$ for 20 min at 4°C. The cells were washed by distilled water for three times. Mycelium of each fungus and the cells of *C. albicans* were autoclaved and subjected to ultrasonic disintegration in ice bath for 6 min in 15 cycles each of 30 sec in ultrasonicator (Karl Kolb, Germany). The pellet obtained by centrifugation at 5000 rev $min^{-1}$ for 30 min at 4°C was washed three times with distilled water and dried.

Colloidal chitin, along with chitin present in the fungal cell wall in a 1:3, 1:0 and 0:3 ratio, was used for enzyme production in the medium. After inoculation with *S. aureofaciens* CMUAc130 and incubation at 30°C in an incubator shaker at 100 rev $min^{-1}$ for 7 days. The culture filtrate thus obtained was tested for enzyme activity as described above.

### 5.12 Dissolution of fungal cell wall

The ability of the culture filtrate to lyse different fungal cell walls and colloidal chitin and thereby release *N*-acetylglucosamine, was tested in a reaction mixture containing 20 mg of fungal cell wall and 50 ml of culture filtrate in a 250-ml flask. The flask containing the reaction mixture was incubated for 24 h at 37°C. *N*-acetylglucosamine was measured as according to the method of Reissig *et al.* (1995) (see Appendix E). Heat-inactivated culture filtrate containing fungal cell walls were used as the control.

### 5.13 Inhibition of fungal growth by crude and purified enzyme

#### 5.13.1 Paper disc diffusion method

*C. musae*, the causative agents of anthracnose of banana was cultured on potato dextrose agar (PDA). Mycelial discs of 6 mm diameter of this fungal pathogen was transferred from PDA onto the ISP-2 medium and colloidal chitin basal medium. Paper discs (Advantec Toyo, Japan) soaked in crude and partially purified enzyme extract were laid on the inoculated plates 3 cm away from mycelium disks; controls were discs soaked in 5 min-boiled enzyme extract. The mycelium extension inhibition of fungal growth were observed over 5 days of incubation at 30°C.

#### 5.13.2 Agar well diffusion method

*F. oxysporum*; the causative agent of wilt of wheat was cultured on potato dextrose agar (PDA). Mycelial discs of 6 mm diameter of this fungal pathogen was transferred from PDA onto the center of PDA plates which was punched of 6 mm diameter of the agar medium 3 cm away from mycelium disks. In the case of colloidal chitin basal medium was punched as the same position of PDA plates but not transfer the mycelium disks onto the center of plates. Fifty microliters of the crude and

partially purified chitinase of *S. aureofaciens* CMUAc130 was placed in the wells at the concentration 5, 2.5 and 1.25 mg $ml^{-1}$. The plates were incubated at 30°C in a moist chamber for 5 days; controls were 5 min-boiled enzyme extract. Inhibition of mycelium growth was observed over 5 days of incubation.

**5.14 Microscope observation of fungal cell wall lysis**

*F. oxysporum* was cultured on potato dextrose agar (PDA). A mycelial disk of 6-mm diameter of *F. oxysporum* was transferred onto a microslide and incubated at 30°C in moist chamber. After 48 h growth, a mycelial disk was removed, a sterilized solution of the crude and purified chitinase of *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40_Sau (5 U $ml^{-1}$) was overlaid and incubated at 37°C for 12 h in moist chamber. Morphological modification of the mycelial structures of the fungus were observed under a light microscope (400X) and compared with a 5 min-boiled chitinase control.

**5.15 Quantitative methods for protein determination**

Protein concentration of crude concentrated culture filtrates and partially purified enzyme of *S. aureofaciens* CMUAc130 and *E. coli* JM109/ pChi40_Sau was determined by a dye-binding method using dye reagent from BIO-RAD, catalog 500-0006 (California, USA). The method was carried out with standard procedure using various concentrations of BSA (0.5, 1, 2, 3, 4, and 5 μg $ml^{-1}$) to set the standard curve.

**5.16 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

Vertical SDS-PAGE was done by using the discontinuous buffer system of Laemmli with 12% polyacrylamide (Laemmli, 1970), which could be able to separate the proteins in the range of 15-200 KDa.

## Results

### 5.1 Screening for chitinase production of endophytic actinomycetes

Three hundred and seven isolates of endophytic actinomycetes were grown on colloidal chitin basal medium. After 7 days chitinolytic zones around the colonies were observed (Figure 5.1) and measured (Table 5.1). Fourteen isolates produced the chitinolytic zone > 5 mm diameter, these isolates were selected for further study of chitinase production in colloidal chitin broth. The cultures were grown at 30°C, with shaking at 150 rev $min^{-1}$. After 7 days the cultures were harvested and chitinase activity present in the culture filtrates was determined. All fourteen actinomycetes isolates tested produced chitinase (Table 5.2). The best strain was *S. aureofaciens* CMUAc130, which produced nearly 2-6 times more enzyme activity than any other isolate. All subsequent experiments were carried out with this strain.

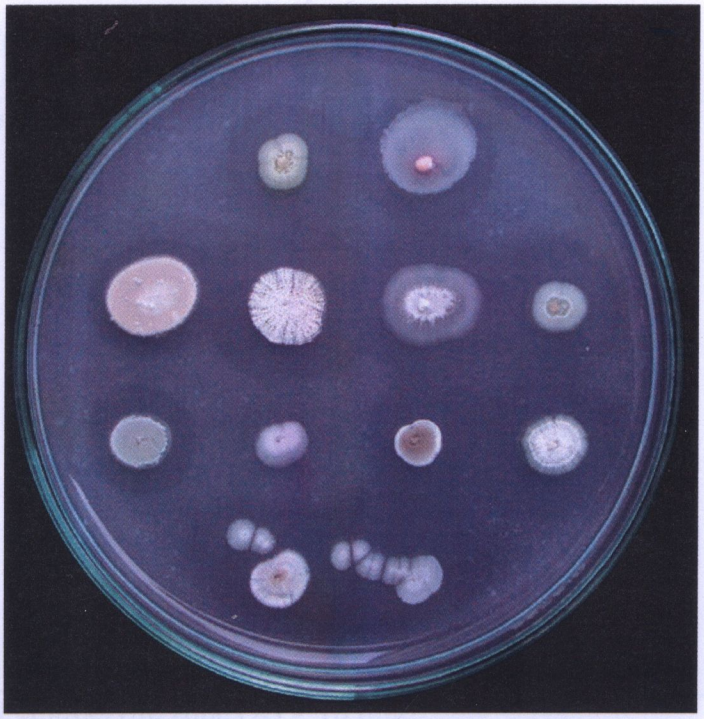

**Figure 5.1** Growth of endophytic actinomycetes on 1% colloidal chitin after incubation at 30°C for 7 days. The clear zone around each colony indicating chitin hydrolysis.

**Table 5.1** Chitinolytic activity of actinomycete isolates. The potential of chitinolytic activity was evaluated by the chitinolytic on colloidal chitin agar plate after incubation at 30°C for 7 days.

| Potential of chitinolytic activity | Number of endophytic actinomycetes isolates (%) |
|---|---|
| ++++[a] | 2 (0.65%)[b] |
| +++ | 12 (3.91%)[b] |
| ++ | 42 (13.68%) |
| + | 72 (23.45%) |
| ± | 45 (14.66%) |
| - | 134 (43.65%) |

[a] ++++ : Width of chitinolytic zone (X) > 10 mm.

+++ : 10 mm ≥ X > 5 mm.

++ : 5 mm ≥ X > 3 mm.

+ : 3 mm ≥ X > 1 mm.

± : 1 mm ≥ X

- : X = 0 mm.

[b] These isolates were identified to be *Streptomyces* sp.

**Table 5.2** Screening of endophytic actinomycetes isolates for production of chitinase in broth containing colloidal chitin. The isolates were assessed for chitinase activity in colloidal chitin broth after incubation at 30°C for 7 days. The results are means of three replicates ± SD.

| Endophytic actinomycete isolates | Enzyme activity (mU/ml) |
|---|---|
| CMUAc036 | 0.0176 ± 0.0035 |
| CMUAc042 | 0.0233 ± 0.0041 |
| CMUAc075 | 0.0320 ± 0.0045 |
| CMUAc130 (*S. aureofaciens*) | 0.0833 ± 0.0045 |
| CMUAc169 | 0.0266 ± 0.0025 |
| CMUAc174 | 0.0416 ± 0.0025 |
| CMUAc196 | 0.0420 ± 0.0020 |
| CMUAc197 | 0.0250 ± 0.0026 |
| CMUAc230 | 0.0386 ± 0.0040 |
| CMUAc237 | 0.0450 ± 0.0040 |
| CMUAc249 | 0.0226 ± 0.0045 |
| CMUAc259 | 0.0420 ± 0.0036 |
| CMUAc292 | 0.0346 ± 0.0032 |
| CMUAc294 | 0.0393 ± .0025 |

## 5.2 Physiological optimization of chitinase production

Maximum levels of ***S. aureofaciens*** CMUAc130 chitinase were achieved by addition of 1% colloidal chitin, at 30-40°C with 100-150 rev $min^{-1}$ shaking in pH 6.5-7.0 culture after 7 days incubation (Figure 5.2a, b, Figure 5.3 a, b). A decrease in enzyme production was observed with increasing concentration of reducing sugars in the culture filtrate after 7 days incubation (Figure 5.4). The effect of various sugar additions along with 1% colloidal chitin on chitinase production was tested. Chitinase production was much lower with all sugars tested, except NAG (Figure 5.5). Addition of 0.1-0.5% NAG plus colloidal chitin increased chitinase production but addition of more than 1% NAG failed to induce enzyme production (Figure 5.6). Several monosaccharides and disaccharides including glucose, mannose, cellobiose, arabinose, raffinose, xylose, lactose, fructose and sucrose showed complete suppression of chitinase production but 0.3% addition of pectin, starch and carboxymethyl cellulose to the colloidal chitin-containing medium, increased chitinase production (Figure 5.7). Among the different chitin substrates, colloidal chitin proved to be the best substrate for chitinase production (Figure 5.8).

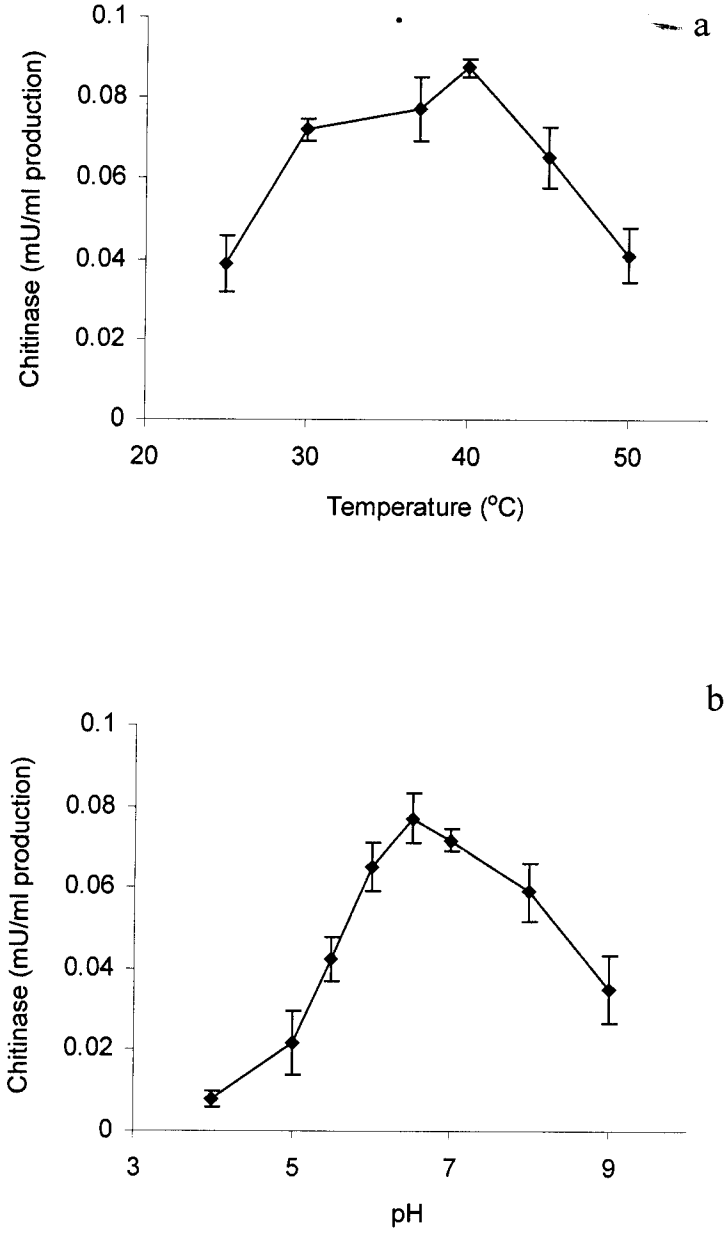


**Figure 5.2** Optimization of chitinase production by endophytic *Streptomyces aureofaciens* CMUAc130. Effect of : (a) temperature (°C); (b) pH. Each value is the mean of triplicate tests with SD error bars indicated.

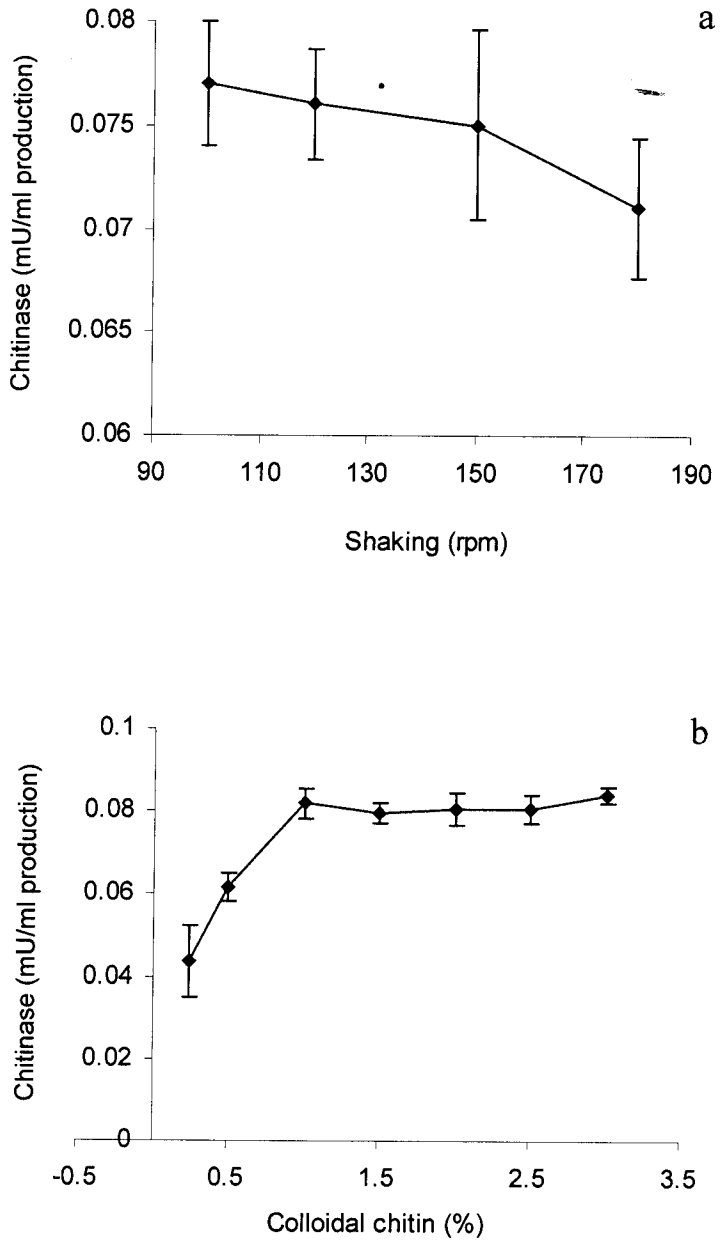


**Figure 5.3** Optimization of chitinase production by endophytic *Streptomyces aureofaciens* CMUAc130. Effect of : (a) shaking; (b) different concentrations of colloidal chitin (%). Each value is the mean of triplicate tests with SD error bars indicated.

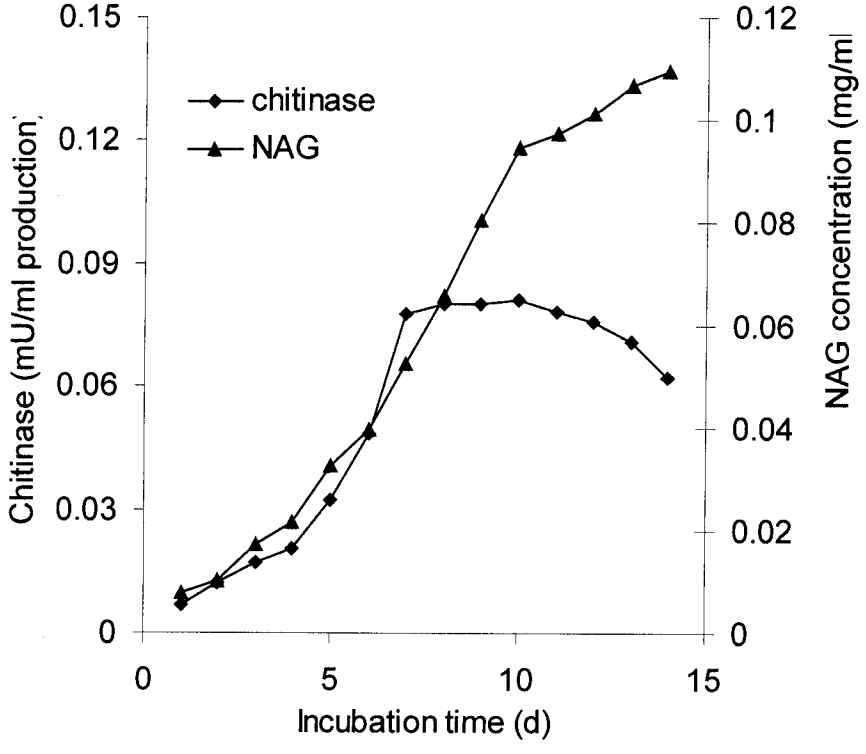


**Figure 5.4** Time course of changes in chitinase activity and the concentration of reducing sugar during the culture of endophytic *Streptomyces aureofaciens* CMUAc130 in colloidal chitin medium at 30°C incubation.

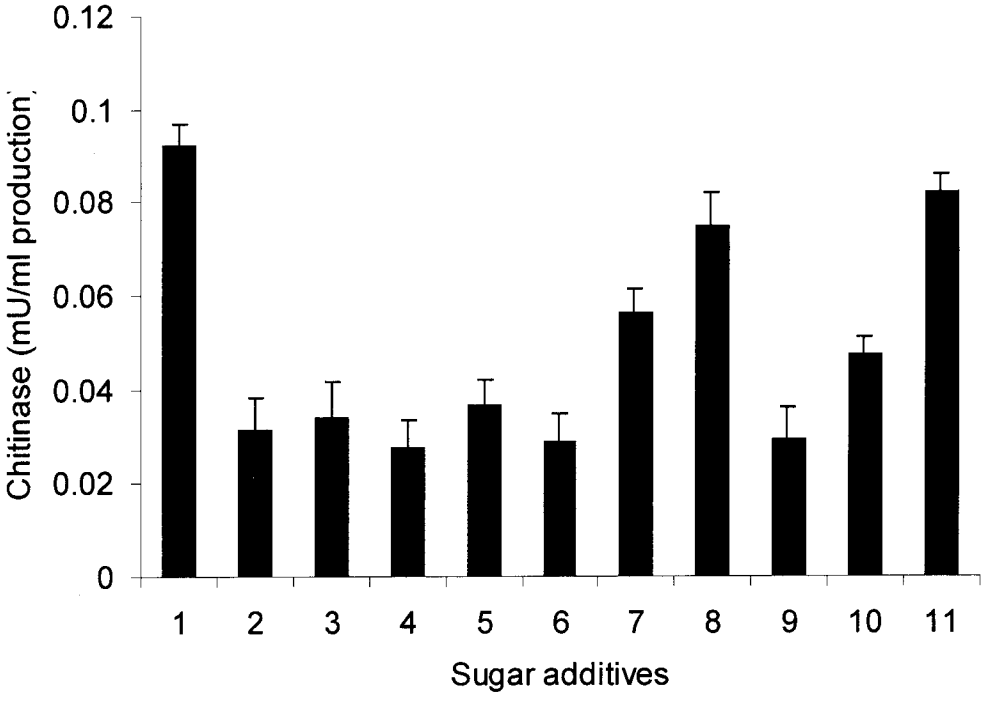


**Figure 5.5** Effect of sugar additives (0.5%) (1; NAG, 2; arabinose, 3; cellobiose, 4; fructose, 5; glucose, 6; lactose, 7; mannose, 8; raffinose, 9; sucrose, 10; xylose, and 11; control) with 1% colloidal chitin on chitinase production. Each value is the mean of triplicate tests with SD error bars indicated.

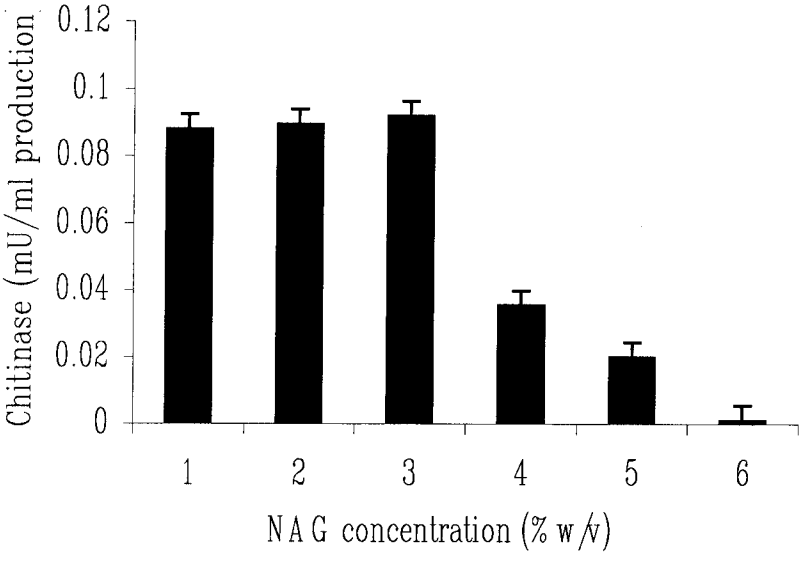


**Figure 5.6** Effect of different concentrations of NAG in 1% colloidal chitin on chitinase production after incubation at 30°C for 7 d. (1); 0%, (2); 0.1%, (3); 0.5%, (4); 1%, (5); 1.5% of NAG, and (6) 0.5% of NAG without colloidal chitin. Each value is the mean of triplicate tests with SD error bars indicated.

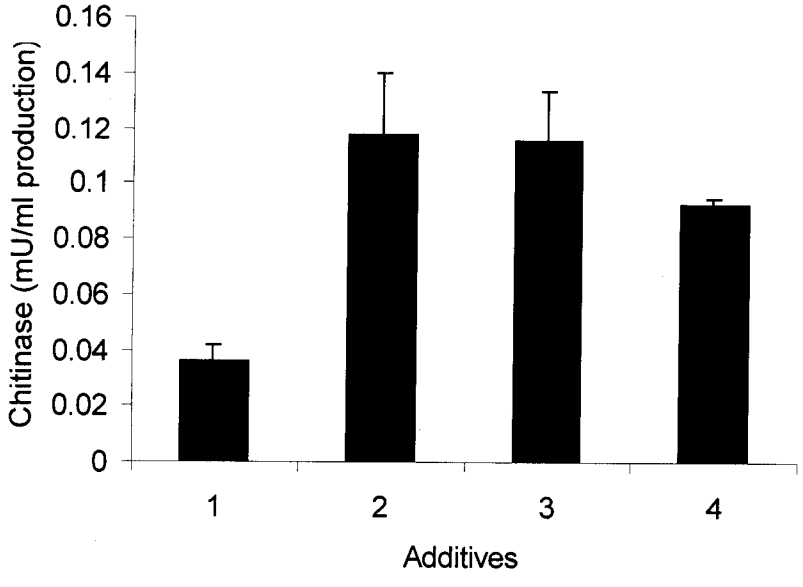


**Figure 5.7** Effect of other C-source additives (0.3%) (1; glucose, 2; CM cellulose, 3; starch, and 4; pectin) with 1% colloidal chitin on chitinase production after incubation at 30°C for 7 days. Each value is the mean of triplicate tests with SD error bars indicated.

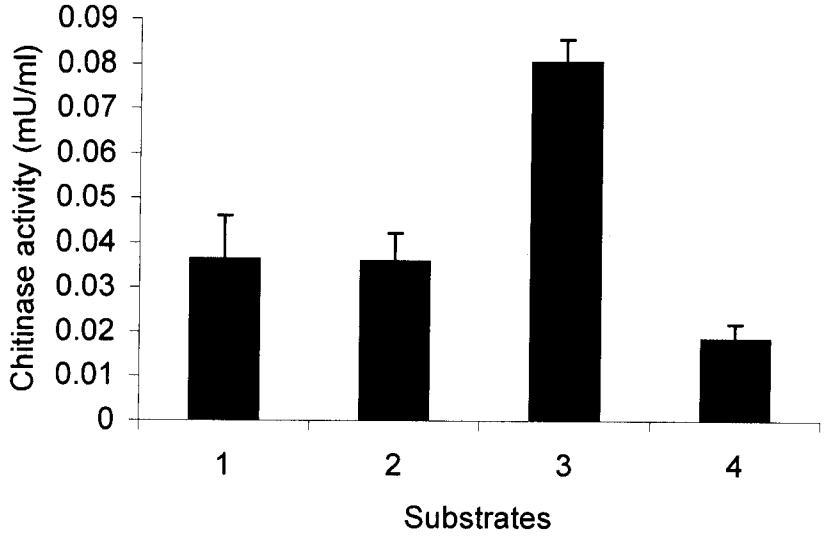


**Figure 5.8** Effect of different chitin substrate (1%) on chitinase production (1; ball milled chitin, 2; crude chitin, 3; colloidal chitin, and 4; *Schizosacchromyces* sp. cell walls). Each value is the mean of triplicate tests with SD error bars indicated.

**5.3 Purification and characterization of chitinase**

The enzyme was concentrated by precipitation with 80% $(NH_4)_2SO_4$ and then further subjected to Sephadex G-75 column purification. The purification faction showed three protein peaks. Of these peaks, a peak showed maximum chitinase activity (Figure 5.9). The other protein peaks were devoid of chitinase activity. Purified enzyme showed a wide range of temperature (30-50°C) (Figure 5.10) and pH activity (5.5-8) (Figure 5.11) with optima at 37°C and pH 7. No significant difference inhibition between the crude and purified enzyme was found among various divalent cations except $Cd^{2+}$, $Ni^{2+}$ and $Hg^{2+}$, while $Mg^{2+}$ enhanced the chitinase activity of both the crude and purified enzyme (Figure 5.12). The purified fraction with chitinase activity exhibited a single band on a 12% SDS-PAGE gel by staining with Coomassie blue (Figure 5.13). One protein band, with an apparent molecular weight of 40 KDa, was found only when chitin was used as carbon source. This protein has the same apparent molecular weight as the purified chitinase from *S. aureofaciens* CMUAc130 recovered by Sephadex G-75 gel filtration. The chitinase specific activity of various treatment steps was shown in Table 5.3. The chitinase activity on several chitin derived substrates and chitooligosaccharides was investigated and presented in Figure 5.14. The substrates most readily hydrolysed were colloidal chitin, ball milled chitin, *N,N',N''*-triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose; methylcellulose and chitobiose were not hydrolysed.

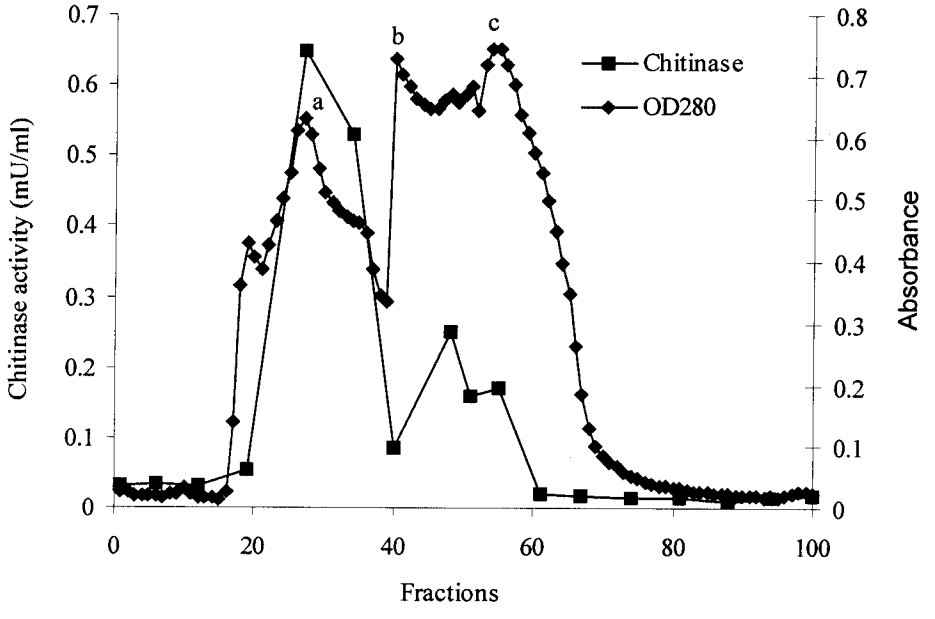


**Figure 5.9** Chitinase activity of different protein fractions obtained on Sephadex G-75 gel chromatography from crude chitinase of *S. aureofaciens* CMUAc130.

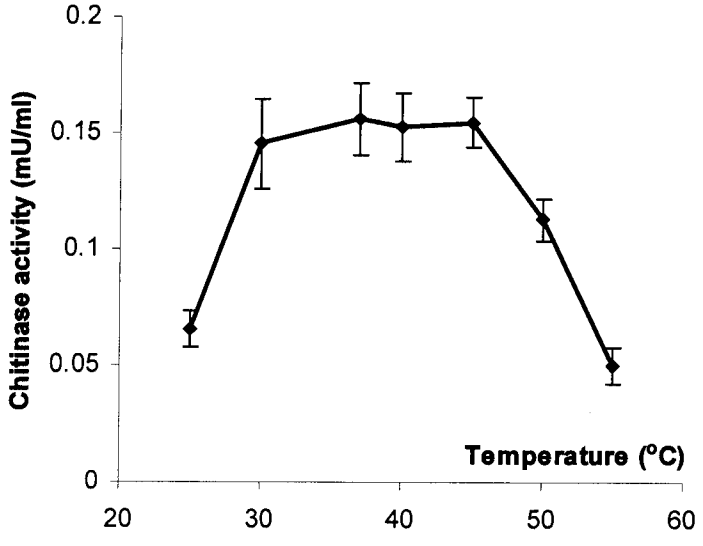


**Figure 5.10** Effect of different temperature on chitinase activity of purified chitinase of *S. aureofaciens* CMUAc130. Each value is the mean of triplicate tests with SD error bars indicated.

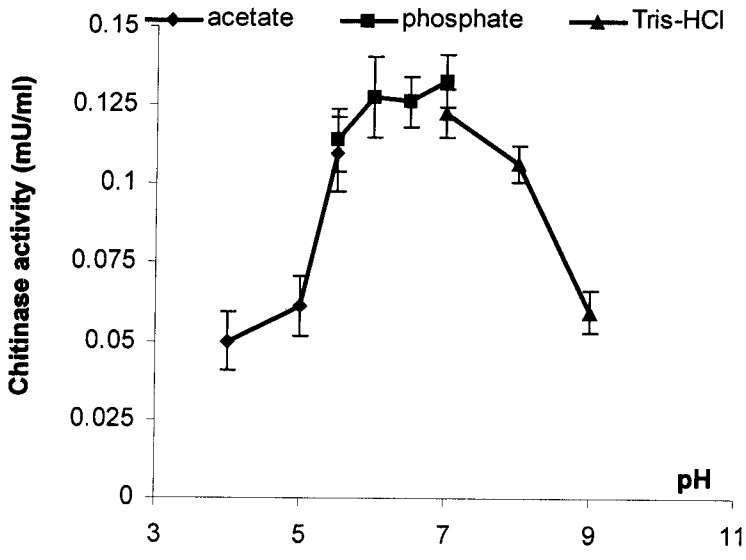


**Figure 5.11** Effect of different pH on chitinase activity of purified chitinase from *S. aureofaciens* CMUAc130. Each value is the mean of triplicate tests with SD error bars indicated.

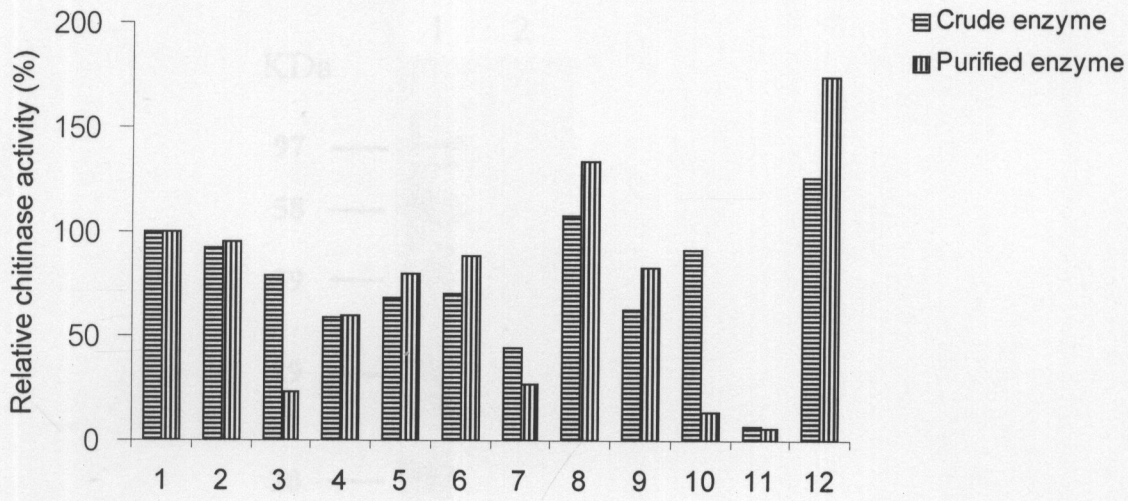


**Figure 5.12** Effect of metal ions, β-mercaptoethanol and EDTA on chitinase activity. Relative values of enzyme activity in the crude and purified enzyme samples in the presence of 5mM different divalent cations (1-10), EDTA (11) and β-mercaptoethanol (12), all at 5 mM. (1) control; (2) $CaCl_2$; (3) $CdCl_2$; (4) $CoCl_2$; (5) $CuSO_4$; (6) $FeCl_2$; (7) $HgCl_2$; (8) $MgCl_2$; (9) $MnCl_2$; (10) $NiCl_2$.

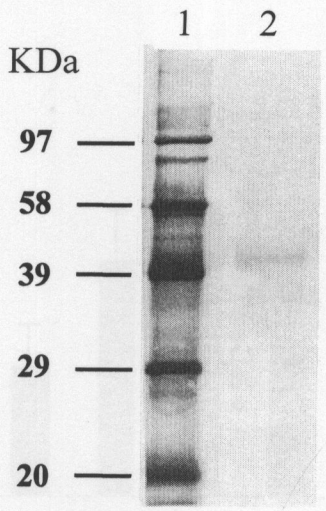


**Figure 5.13** SDS-PAGE of purified chitinase from *S. aureofaciens* CMUAc130. Lane 1: MW marker, lane 2: purified chitinase

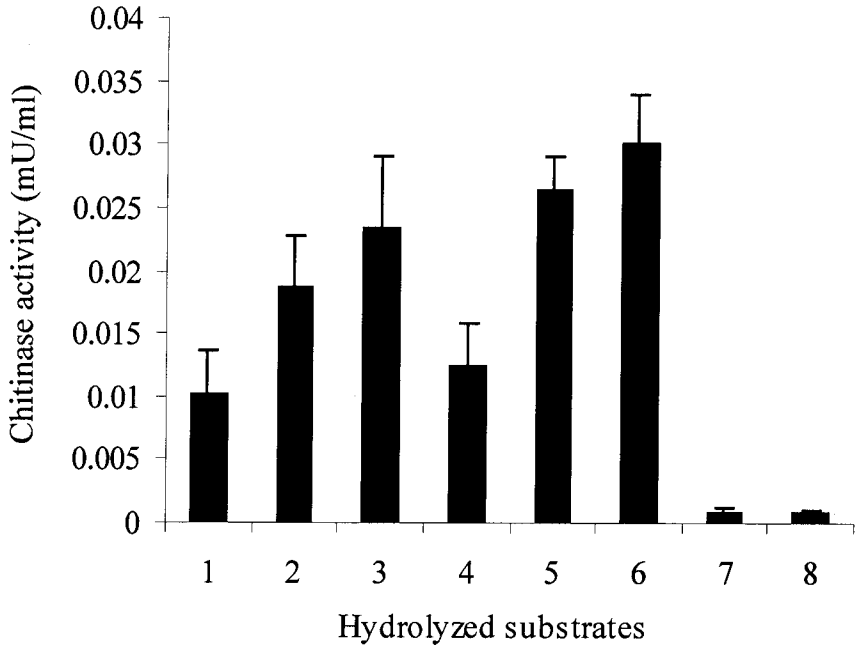


**Figure 5.14** Effect of different substrates on the activity of purified chitinase from *S. aureofaciens* CMUAc130. (1); crude chitin, (2); ball milled chitin, (3); Colloidal chitin, (4); *Schizosaccharomyces* sp. cell walls, (5); *N,N',N''*-triacetylchitotriose , (6); *N,N',N'',N'''*-tetraacetylchitotetraose, (7); *N,N'*-diacetylchitobiose, and (8); CM-cellulose. Each value is the mean of triplicate tests with SD error bars indicated.

**Table 5.3** Specific activity of *S. aureofaciens* CMUAc130 chitinase at various treatment steps.

| Treatment step | Volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (mU/ml) | Total activity (mU) | Specific activity (mU/mg) | Yield (%) |
|---|---|---|---|---|---|---|---|
| Culture filtrate | 1000 | 1.54 | 1540.0 | 0.09 | 89.32 | 0.058 | 100.00 |
| $(NH_4)_2SO_4$ treatment | 15 | 70.62 | 1059.3 | 4.62 | 68.85 | 0.065 | 77.08 |
| Sephadex G-75 | 30 | 1.36 | 40.8 | 1.28 | 38.39 | 0.941 | 47.98 |
| Freez dried | 3 | 11.05 | 33.1 | 11.75 | 35.24 | 1.063 | 39.45 |
| Ultrafiltration | 1 | 30.80 | 30.8 | 33.50 | 33.48 | 1.087 | 37.48 |

## 5.4 Lysis of the fungal cell wall

The crude enzyme extract could digest colloidal chitin and chitin derived from fungal cell walls as shown by Beyer and Diekmann (2), Gupta *et al.* (10), Mahadevan and Crawford (15), and Lima *et al.* (13). Improved levels of chitinase production were observed when fungal cell wall chitin was used along with colloidal chitin in a 1:3 ratio (Figure 5.15). The culture filtrate thus obtained had chitinase activity. Both colloidal chitin and chitin from fungal cell walls were hydrolyzed. The maximum digestion of fungal cell walls by the crude enzyme extract occurred after a 24 h incubation of the reaction mixtures. Crude chitinase preparations from *S. aureofaciens* CMUAc130 cultures grown for 7 days in a medium containing a 1:3 ratio of cell walls to colloidal chitin hydrolyzed chitin prepared from the cell walls of *C. musae* at activity of 0.03 mU $ml^{-1}$ which was the highest chitinase activity among the chitinase produced on fungal cell walls and colloidal chitin (Table 5.4).

Morphological changes were observed in microslide cultures of *F. oxysporum* treated with crude and purified chitinase. Inhibition of spore germination and fungal cell wall lysis was noted (Figure 5.16a) when compared with the control (Figure 5.16b).

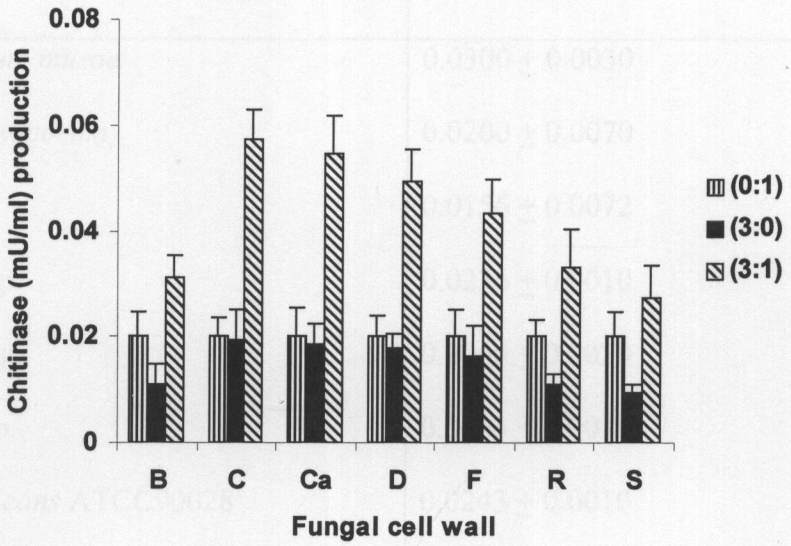


**Figure 5.15** Production of *S. aureofaciens* CMUAc130 chitinase on mixed substrate containing colloidal chitin and chitin derived from different fungal cell walls.(B; *Bipolaris* sp., C; *Colletotrichum musae*, Ca; *Candida albicans*, D; *Drechslera* sp., F; *Fusarium oxysporum*, R; *Rhizoctonia* sp. and S; *Sclerotium* sp.). Each value is the mean of triplicate tests with SD error bars indicated. Ratios shown are expressed as fungal cell walls : colloidal chitin.

**Table 5.4** Activity of *S. aureofaciens* CMUAc130 chitinase produced on *C. musae* cell wall and colloidal chitin (3:1) on dissolution of cell wall material from different fungi. The reactivity was measured after incubation the reaction mixture at 37°C for 24 hours. The results are means of three replicates ± SD.

| Fungal cell wall substrate for dissolution | *N*-aectylglucosamine (μg $ml^{-1}$) |
|---|---|
| *Colletotrichum musae* | 0.0300 ± 0.0030 |
| *Fusarium oxysporum* | 0.0200 ± 0.0070 |
| *Bipolaris* sp. | 0.0156 ± 0.0072 |
| *Drechslera* sp. | 0.0236 ± 0.0010 |
| *Rhizoctonia* sp. | 0.0066 ± 0.0020 |
| *Sclerotium* sp. | 0.0053 ± 0.0025 |
| *Candida albicans* ATCC90028 | 0.0243 ± 0.0010 |

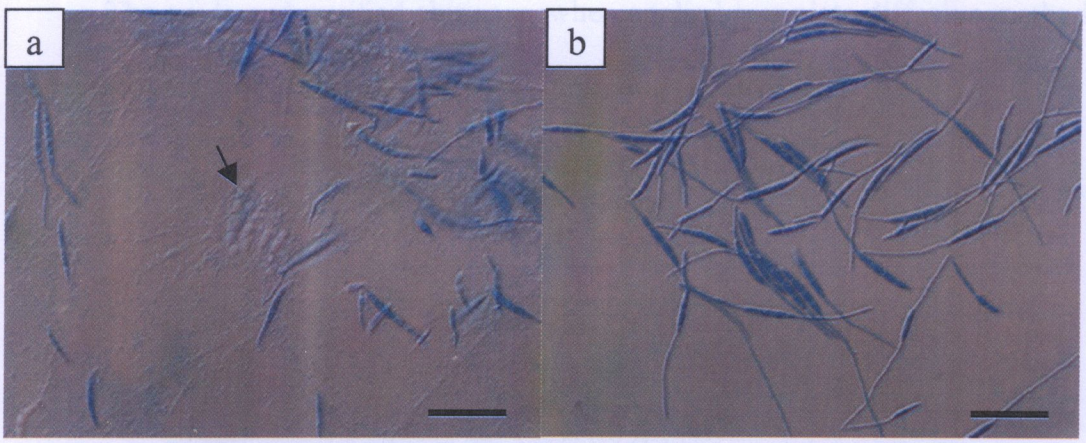


**Figure 5.16** Morphological changes of *F. oxysporum* mycelium after treatment with crude chitinase (a), and heat inactivated crude chitinase (b) of *S. aureofaciens* CMUAc130 for 12 h at 37°C, arrow shown bursting of spore and hyphae.

Bar = 100 μm.

**5.5 Fungal growth inhibition by the crude and purified chitinase**

The crude enzyme extract and purified chitinase from *S. aureofaciens* CMUAc130 were tested for antifungal activity by their ability to inhibit hyphal extention growth of *C. musae*. The inhibition of fungal growth was observed on an agar plate with discs coated with chitinase enzyme (Figure 5.17).

The crude and purified chitinase, when applied to a plate diffusion method of *F. oxysporum* culture, caused the growth inhibition (Figure 5.18).

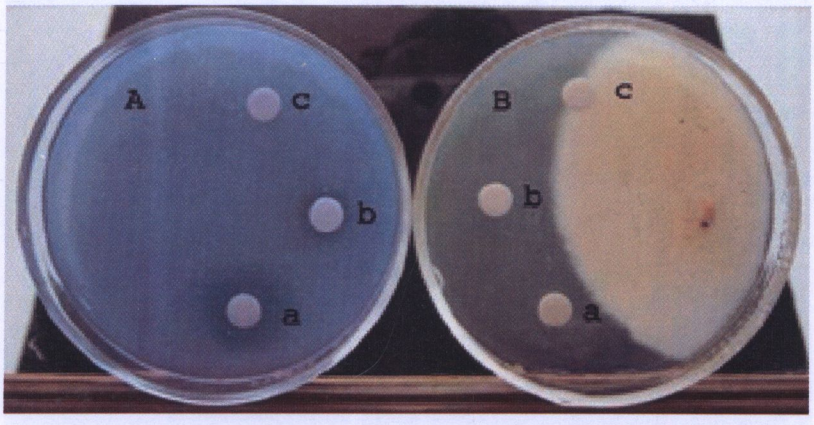


**Figure 5.17** Effects of crude and purified chitinase on inhibition of *C. musae* growth (A) and chitinolytic activity on colloidal chitin agar (B) with disces coated with (a) crude, (b) purified, (c) heat inactivated purified chitinase.

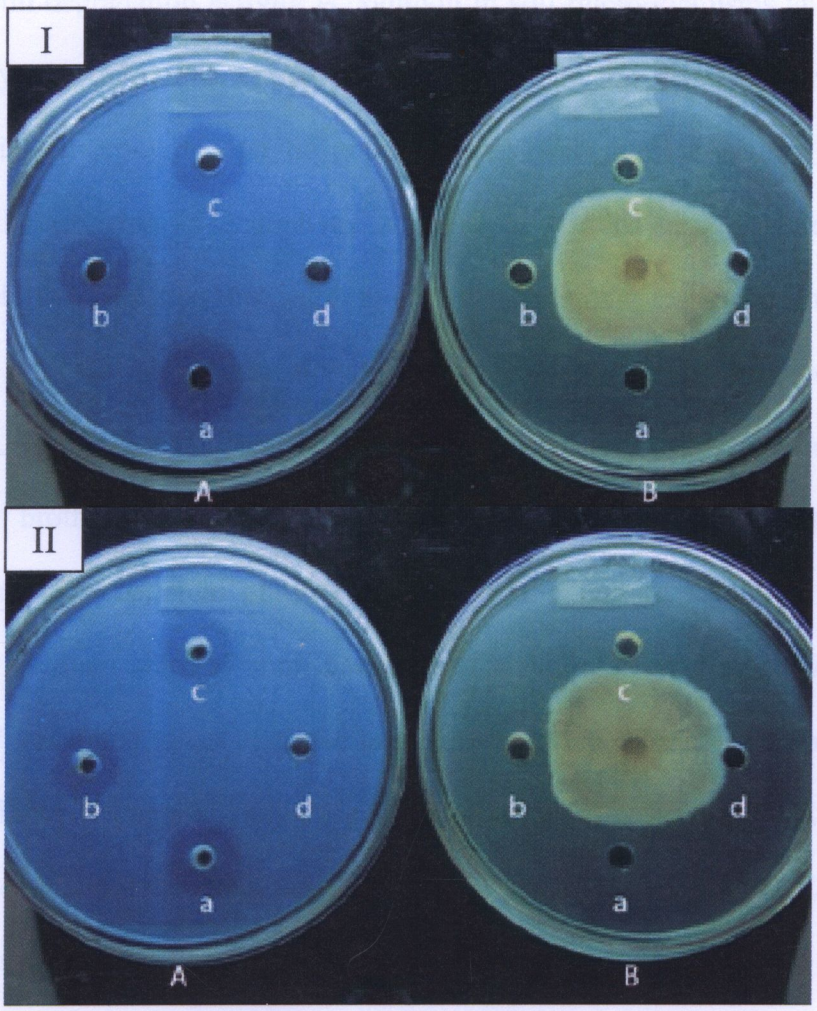


**Figure 5.18** Effects of crude (I) and purified (II) chitinase of *S. aureofaciens* CMUAc130 on chitinolytic activity on colloidal chitin agar (A) and inhibition of *F. oxysporum* growth by punched agar diffusion method. (B) with (a) 5 mg $ml^{-1}$, (b) 2.5 mg $ml^{-1}$, (c) 1.25 mg $ml^{-1}$ of crude chitinase and (d) 5 mg $ml^{-1}$ of 5 min-boiled crude chitinase.

**Discussion**

Chitin degradation activity is common in soil microorganisms and especially in actinomycetes (Williams and Robison 1981). All of the studies described in this thesis originated from endophytic actinomycetes screening procedure which was based on the isolation of endophytic actinomyces strains giving a clear zone of colloidal chitin utilization on solid medium. The strain examined in this work hydrolysed colloidal chitin very slowly, it took at least 1 week to get a clear zone appearance. The size of the clear zone is not according to the chitinase activity from the culture broth. *S. aureofaciens* CMUAc130 exhibited much higher chitinase activity when 1% colloidal chitin was used in the culture broth but this strain exhibited a medium sized (7 mm diameter) of clear zone. While *Streptomyces* sp. CMUAc196 exhibited lower chitinase activity but giving a large clear zone (12 mm diameter). This phenomenon may depend on the different molecule of chitinase from individual strains, because there are many chitinase (between 25 to 92 KDa) among the *Streptomyces* strains (Romaguera *et al.* 1992; Tsujibo *et al.* 2000) or it depends on the chitinase property of the individual strains.

Although many strains of endophytic actinomycetes produce chitinase, their chitinase productivity differs greatly. We selected the strain *S. aureofaciens* CMUAc130 because of its very high chitinase productivity and its potential role in antifungal activity (Taechowisan *et al.* 2003). This study has shown a probable role for chitinase in the antifungal activity of *S. aureofaciens* CMUAc130. However, antifungal antibiotics produced by *S. aureofaciens* CMUAc130 probably also play a role.

Maximum enzyme production at 30-40°C is consistent with results reports by Skujins *et al.* (1965), Gupta *et al.* (1995) Mahadevan and Crawford (1997) and Gomes *et al.* (2001) for chitinases from *Streptomyces* sp. Negligible levels of enzyme were seen upon growth of *S. aureofaciens* CMUAc130 in the absence of chitin. Low constitutive levels probably help induce enzyme production in the presence of chitin (Vasseur *et al.* 1990). Most of the chitinolytic systems reported in the literature are inducible (Gupta *et al.* 1995; Mahadevan and Crawford 1997; Ulhoa and Peberdy 1991). Chitinase production by *S. aureofaciens* CMUAc130 was induced by colloidal chitin as well as by low levels of *N*-acetylglucosamine and pectin, starch and carboxymethyl cellulose, confirming the findings of Mahadevan and Crawford (1997). *S. aureofaciens* CMUAc130 has also produced amylase pectinase and cellulase. Among the monosaccharide and disaccharide as additives to colloidal chitin, almost all of them repressed enzyme production. Overall, these data show that *S. aureofaciens* CMUAc130 chitinase is inducible with low constitutive levels and is subject to catabolite repression by sugar. It has been reported that *Streptomyces* chitinases are induction and repression (Gupta *et al.* 1995; Mahadevan and Crawford 1997).

The preferred substrate for chitinase production was colloidal chitin. With crude chitin from crab shells, ball milled chitin and *Schizosaccharomyces* sp. cell walls, inaccessibility of termini may play a role in the inability of the enzyme to attack whereas there are numerous termini available for the enzyme to hydrolyse in colloidal chitin for utilization. This property confirms that chitinase produced by

*Acremonium obclavatum* could hydrolysed colloidal chitin more rapidly than crude chitin or isolated *A. obclavatum* cell walls (Gunaratna and Balasubramanian 1994).

The substrate specificity of the *S. aureofaciens* CMUAc130 chitinase was investigated. Its action was highest on the colloidal chitin. Based on this data, colloidal chitin may prove to be a useful substrate for determination of chitinase activity. The enzyme was also capable of hydrolysing several insoluble chitin substrate. Its action was higher on ball milled chitin than crude chitin and *Schizosaccharomyces* sp. cell walls. Ball milled chitin was prepared by milling of crude chitin in a Pascall ball mill resulting power. This physical treatment made a smaller chitin molecule than crude chitin and *Schizosaccharomyces* sp. cell walls providing a more accessible substrate for enzyme hydrolysis. Deane *et al.* (1998) demonstrated a low activity of the *Trichoderma harzianum* chitinase on chitosan and glycol chitosan, however, the activity of the enzyme on this substrate was not investigated. The weaker action on these substrates was probably due to the partial deacetylation of chitosan allowing enzyme activity only on the stretches of the polysaccharide chain that had conserved the acetyl group. The enzyme was also active on the chito-oligosaccharides, Chitotriose and chitotetraose, but was inactive towards chitobiose and CM-cellulose. These results indicated that the *S. aureofaciens* CMUAc130 chitinase isolated and purified in this work is specific only for *N*-acetylglucosamine oligomers of more than two units in length. This purified chitinase could be classified into endochitinase (Oppenheim and Chet 1992).

As the molecular weight of the *Streptomyces* chitinases is known to be in the range of 25-92 KDa (Romaguera *et al.* 1992; Tsujibo *et al.* 2000), a G-75 Sephadex column was used. The protein profile of the fraction revealed three peaks, a peak, with good chitinolytic activity, confirming the findings of Gupta *et al.* (1995). An apparent molecular weight of 40 KDa was calculated for the purified chitinase by SDS-PAGE.

The purified enzyme was completely inhibited by 5 mM divalent cations. The increase in activity with mercaptoethanol indicates presence of sulhydryl groups on the active site of the enzyme, confirmed by total inhibition by $Hg^{2+}$. Similar inhibition and mercaptoethanol enhancement has been reported by Pegg (1982), Ueno *et al.* (1990) and Gupta *et al.* (1995). The activity of the enzyme was also inhibited by the metal ion chelating compound EDTA, suggesting that metals may be important in enzyme stability and/or activity which was difference from the report of Gomes *et al.* (2001).

Fungal cell walls often contain chitin as a major component, chitinases are well known to lyse fungal cell walls (Gomes *et al.* 2001; Gupta *et al.* 1995; Mahadevan and Crawford 1997; Ueno *et al.* 1990). Antagonistic activity of several *Streptomyces* spp. against a number of fungal pathogenic species has been known for a long time (Crawford *et al.* 1993). Also, in greenhouse experiments, *Streptomyces* spp., as well as other actinomycete genera, have conferred various degrees of protection on different plant species against soil-borne pathogenic fungi (Yuan and Crawford 1995). However, the exact mechanism of this process has not yet been

completely clarified. Several reports have shown that laminarinases, glucanases, or other hydrolytic enzymes such as proteases, could also be involved in fungal antagonism. Nevertheless, the role of chitinase activity against the fungal cell wall is evident (Shapira *et al.* 1989; Lim *et al.* 1991). In the present study, *S. aureofaciens* CMUAc130 was shown to produce a high level of chitinase when grown in the presence of fungal cell wall chitin, specifically with chitins prepared from the walls of *C. musae*. The chitinase produced using the walls of *C. musae* was active against all other fungi as measured by the release of sugars from their cell walls. Beyer and Diekmann (1985), Ordentlich *et al.* (1988), Gupta *et al.* (1995), Mahadavan and Crawford (1997), and Mansour and Mohamedin (2001) have reported that incubation of *Streptomyces* chitinases with several fungi could release sugar from cell walls. On the other hand, fungal cell wall lysis by α-glucanases of *Streptomyces* strains had been reported (Gacto *et al.* 2000). Fayad *et al.* (1992) pointed out the involvement of β-1,6-glucanases of a *Streptomyces* sp. EF-14 in cell wall lysis of *Candida utilis*, more over Tagawa and Okazaki (1991) reported that chitinase and α-glucanases of *Streptomyces* strains had lytic activity towards *Aspergillus niger* cell wall.

The crude and purified chitinase, when applied to a microslide-culture of *F. oxysporum* and observed under the light microscope, caused fungal cell wall lysis, including inhibition of spore germination and possibly, conidial damage. This may be due to enzymatic digestion of the cell wall region rich in chitin. Hyphal width reduction was also observed. Different morphological effects on the fungal cell wall have been reported in studies using *Streptomyces* sp. or other microbial chitinases. These effects were mainly spore germination inhibition, bursting of spore and hyphal

tips and germ tube elongation (Lorito *et al.* 1993; Gomes *et al.* 2001). The differences in the effects can be attributed to the different experimental conditions of the tests. The results of the present investigation using crude and partial purified enzyme in controlling fungal growth are a promising method of biocontrol of some plant pathogens.

# CHAPTER VI

# MOLECULAR CLONING AND HETEROLOGOUS EXPRESSION IN *Escherichia coli* OF A CHITINASE GENE FROM ENDOPHYTIC *Streptomyces aureofaciens* CMUAc130 FOR ANTIFUNGAL ACTIVITY IMPROVEMENT

## Introduction

Many bacteria, fungi and plants are able to hydrolyze chitin to oligomeric derivatives of *N*-acetyl-β-D-glucosamine (GlcNAc), including disaccharides and monosaccharide of the amino sugar (Gooday *et al.*, 1992). The structural genes encoding chitinases have been cloned from a wide variety of organisms, including bacteria (Felse and Panda 1999). Chitinolytic bacteria from the genus *Serratia marcescens* (Ordentlich *et al.*, 1988) have been shown to be potential agents for the biological control of plant diseases caused by various phytopathogenic fungi whose cell walls contain chitin as a major structural component. Several different chitinase genes have been cloned from *Streptomyces* spp., *chi63* of *Streptomyces plicatus* (Bobbins *et al.*, 1992), *chiC* of *Streptomyces lividans* (Miyashita *et al.*, 1991), *chi40, chi35* and *chi25* of family 19 bacterial chitinases of *Streptomyces thermoviolaceus* (Tsujibo *et al.*, 1993; Tsujibo *et al.*, 2000), *chiA* and *chiB* (Miyashita and Fujii 1993) of *S. lividans* are genes of the group B chitinases of family 18, *chi92* of *Streptomyces olivaceoviridis* (Li *et al.*, 2000) encode the largest chitinase of *Streptomyces* spp., *chiC* of *Streptomyces griseus* is the bacterial chitinase gene the product of which belongs to family 19 chitinases (Ohno *et al.*, 1996).

However, there are no reports of cloning chitinase gene from the endophytic actinomycetes. The chitinolytic enzyme of the endophytic *S. aureofaciens* CMUAc130 has been studied, which can be used for fungal cell wall degradation. The chitinases obtained from *Streptomyces* have been reported for antifungal activity (Tsujibo *et al.*, 2000). The PCR cloning and sequencing of *Chi40* from *S. aureofaciens* CMUAc130 and the characterization of its translated products should be studied. The activity of the expressed *Chi40* in the hydrolysis of insoluble chitin and its antagonism against phytopathogenic fungi should also be studied.

**Materials and methods**

**6.1 pUC18**

The pUC18 plasmid is a 2686 bp *E. coli* cloning vector (Figure 6.1). It includes the *Pvu* II/*Eco*R I fragment of pBR322 that carries the β-lactamase gene (ampicillin resistance, $Ap^r$) and an origin of replication. A *Hae* II fragment (position 239-6684), containing the α-peptide of the *lacZ* (β-galactosidase) gene provide a multiple cloning site of one of the M13mp vectors. Insertion of DNA at the multiple cloning site results in interruption of the *lacZ* α-peptide, producing colorless, rather than blue, colonies on medium containing ampicillin and X-gal or Bluo-gal. Plasmid pUC18 and pUC19 differ only in the orientation of the multiple cloning site: in pUC18 the *Hin*d III site is closest to the M13/pUC Forward 23-Base Sequencing Primer binding site;

in pUC19 the *Eco*RI site is closest. This permits DNA fragments with two different restriction ends to be "forced" into the multiple cloning site with a specified and adjacent to the promoter.

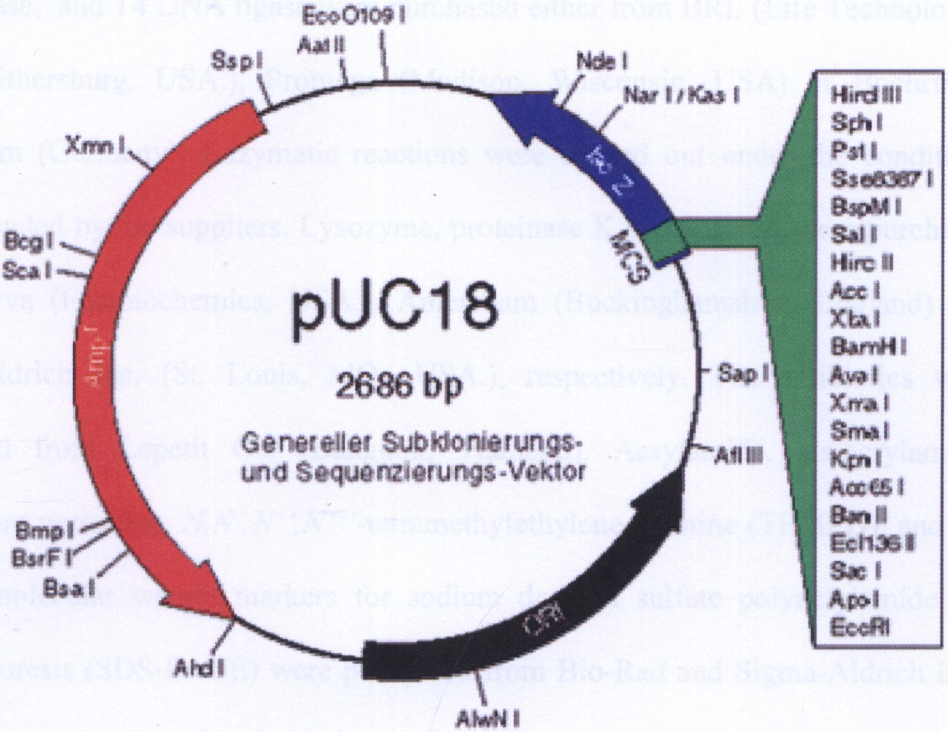


**Figure 6.1** Physical and genetic map of pUC18 (From Gibco BRL Products).

## 6.2 Bacterial host

*E. coli* JM109 was purchased from BRL, USA. It was used as the cloning host for recombinant plasmids. It was grown at 37°C on Luria-Bertani (LB) medium for the selection of transformants. LB medium was supplemented with appropriate antibiotics such as 100 μg $ml^{-1}$ of ampicillin (Ap), for the selection of the transformants harboring pUC18 or their derivative. The bacterial cultures were kept as stocks in 15% glycerol at -70°C.

## 6.3 Chemicals and reagents

All microbiological media used in this study were obtained from Difco, Detroit, USA. Agarose, restriction endonucleases, *Taq* DNA polymerase, *Pfu* DNA polymerase, and T4 DNA ligase were purchased either from BRL (Life Technologies Inc., Gaithersburg, USA.), Promega (Madison, Wisconsin, USA) or Boehringer Mannheim (Germany). Enzymatic reactions were carried out under the conditions recommended by the suppliers. Lysozyme, proteinase K and RnaseA were purchased from Serva (Feinbiochemica, USA.), Amersham (Buckinghamshire, England) and Sigma-Aldrich Inc. (St. Louis, MO., USA.), respectively. The antibiotics were purchased from Lepetit Co. (Bangkok, Thailand). Acrylamide, bis-acrylamide, ammonium persulfate, *N,N',N'',N'''*-tetramethylethylene diamine (TEMED), and the protein molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad and Sigma-Aldrich Inc., USA. 2-mercaptoethanol was purchased from Sigma-Aldrich Inc. Sodium dodecyl sulfate (SDS) and methanol were purchased from Merck, Darmstadt, Germany. Glacial acetic acid, glycine and bromophenol blue were purchased from BDH Chemicals Ltd., Poole, England and Coomassie brilliant blue R-250 was purchased from Fluka Chemie AG, Switzerland.

Other chemicals were obtained from Merck (Darmstadt, Germany), Bio-Rad (California, USA.), Carlo Erba (RPE), J.T. Baker Inc., Phillipsberg, USA., Fluka (Switzerland).

## 6.4 Preparation of plasmid DNA and chromosomal DNA

### 6.4.1 Extraction of plasmid DNA from *E. coli*

#### Small scale extraction of plasmid DNA

Plasmid DNA was extracted from *E. coli* using a modification of the method of Holmes and Quigley (1988). This method can be used with most *E. coli* strains except those express endonuclease A (*endA*+ strain: eg. : HB101). The overnight bacterial cultures were grown in 5 ml LB-broth containing the appropriate antibiotics at 37°C with continuous shaking at 150 rev $min^{-1}$. The cell pellet was collected by centrifugation at 5,000 rev $min^{-1}$ for 3 min. The pellet was suspended in 500 µl STET solution (Sucrose 40 g, Triton X-100 25 ml, 0.5 M EDTA 50 ml and 2 M Tris-HCl, pH 8.0 12.5 ml). One hundred µl of freshly prepared 10 mg $ml^{-1}$ lysozyme was added to the STET solution and mixed by vortexing. The mixture was boiled for 40 sec, chilled on ice for 5 min and centrifuged at 6,000 rev $min^{-1}$ for 30 min. The slimy pellet was removed by sterile toothpick, 0.7 volumes of isopropanol was added. The pellet was collected by centrifugation at 6,000 rev $min^{-1}$ for 30 min and washed twice with 70% ethanol. The pellet was dried and resuspended with 50 µl TE buffer, pH 8.0. RNA was removed by adding 5 µl of Rnase (10 mg $ml^{-1}$) and incubated at 37°C for 30 min.

#### Large scale plasmid extraction of plasmid DNA

Large scale extraction of plasmid DNA from *E. coli* was done using the alkaline lysis method described by Birnboim and Doly ( 1979). The overnight bacterial culture was grown in 5 ml of LB-broth containing the appropriate antibiotics at 37°C in a water bath with continuous shaking at 150 rev $min^{-1}$. The cell pellet was harvested by centrifugation of 1 ml of culture, at 6,000 rev $min^{-1}$ for 1 min

at room temperature. The cells were then resuspended in 100 µl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0 and 10 mg $ml^{-1}$ of lysozyme in 10 mM Tris-HCl, pH 8.0) and incubated at 37°C for 30 min. After incubation, freshly prepared solution II (1% SDS and 0.2 M NaOH) was added and gently mixed. The mixture was placed on ice for 10 min before adding 150 µl of 3 M sodium acetate, pH 5.0, and mixing the solutions. The mixture was placed on ice for 30 min and then the clear supernatant was collected by centrifugation at 6,000 rev $min^{-1}$ for 30 min. DNA was precipitated with 2 vol of absolute ethanol at -20°C for 1 h. The pellet was collected by centrifugation at 6,000 rev $min^{-1}$ for 10 min. The precipitate was dissolved with 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and 50 µl of 7.5 M ammonium acetate was added. Protein was separated from the DNA solution by centrifugation at 6,000 rev $min^{-1}$ for 10 min, the supernatant was transferred to a new Eppendorf tube and precipitated at -20°C for 1 h with 2 volumes of absolute ethanol. The pellet was washed twice with 70% ethanol, dried and dissolved with 10 µl of TE buffer, pH 8.0. RNA was digested by adding 1 µl of RNase (5-10 mg $ml^{-1}$) and incubated at 37°C for 30 min.

### 6.4.2 Purification of plasmid DNA

Two methods of purification were used to prepare DNA for cloning and sequencing.

#### Purification with phenol

For large scale plasmid preparation the DNA was purified by phenol-chloroform extraction. An equal volume of phenol : chloroform was added to the plasmid DNA in a polypropylene tube with a plastic cap and the contents mixed until an emulsion was formed. The solution was centrifuged at 6,000 rev $min^{-1}$ for 20 min

at room temperature. If the organic and aqueous phases were not well-separated, the samples were centrifuged again for a longer time or at a higher speed. Normally, the aqueous phase forms the upper phase. The organic phase is easily identified because of the yellow color. The aqueous phase was transferred to a fresh tube and extraction was repeated until no protein was visible at the interface of the organic and aqueous phases. The aqueous phase was next transferred to a new tube and an equal volume of chloroform was added to remove the residual phenol. The pellet of plasmid DNA was recovered by precipitation with ethanol.

### 6.4.3 Genomic DNA extraction

The salting out procedure (Hopwood *et al.* 1985) was used for genomic DNA extraction from *S. aureofaciens* CMUAc130. The cell pellet was resuspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8.0, 20 mM Tris-HCl pH 7.5), 100 µl of 50 mg $ml^{-1}$ lysozyme solution and then incubated at 37°C for 1 h. One hundred and forty µl of 20 mg $ml^{-1}$ proteinase K solution and 600 µl of 10% SDS were added, mixed by inversion and then incubated at 55°C for 2 h. Two ml of 5 M NaCl was added and mixed thoroughly by inversion. Finally 5 ml chloroform was added and the solution was mixed by inversion for 30 min. The mixture was centrifuged 6000 rev $min^{-1}$ at 4°C for 15 min and transferred the supernatant to a fresh tube. Isopropanal (0.6 vol) was added and mixed by inversion for 3 min. The DNA was spooled onto Eppendorf tube, rinsed in 5 ml of 70% ethanol, air dried, and dissolved in 100 µl TE.

## 6.5 DNA analysis

### Restriction endonuclease digestion

Digestion of plasmid DNA by restriction endonucleases was performed by methods described by manufacturers. Enzyme activity was stopped by heating at 65°C for 15 min.

### Agarose gel electrophoresis

Agarose gels were cast by melting the agarose in the Tris-borate-EDTA buffer (TBE, 89 mM Tris-HCl, 89 mM boric acid and 25 mM EDTA pH 8.0) until the solution was clear. When the agarose gel is hardened, it forms a matrix the density of which varies relative to the concentration of the agarose. When an electric field is applied across the gel, DNA which is negatively charged at neutral pH, migrates toward the anode.

The solution of plasmid DNA (10 μl) was mixed with loading buffer (50% sucrose, 0.05% bromophenol blue and 50 mM EDTA pH 8.0). The mixture was loaded into the slots of horizontal gels (0.7% agarose gel in TBE buffer). The horizontal type minigel electrophoresis set (Mupid, Tokyo Co. Ltd, Japan) was routinely used. Electrophoresis was run at a constant voltage of 50 or 100 V at room temperature until the tracking dye was near the edge of gels (approximately 30 or 60 min). The gels were stained in 1 μg $ml^{-1}$ of ethidium bromide solution for 5-10 min and destained in distilled water for 10-20 min. DNA patterns were visualized by UV light transilluminator (RM-AEC2-2, FOTODYNE, Hartland, USA) and photographed with video graphic printer (UP-890CE, Sony Corporation, Tokyo, Japan).

To estimate the molecular weight of plasmid DNA fragments, λDNA digested with *Hin*dIII was used as standard markers.

### 6.6 PCR cloning of a chitinase gene from *S. aureofaciens* CMUAc 130

Two oligonucleotide primers were used in a PCR with genomic DNA isolated from *S. aureofaciens* CMUAc130. The 5'-TTGACCCAGTGGTCCAGACC-3' (forward primer) and 5'-GTGTGCTGCTCACGCCAG-3' (reverse primer), designed on the basis of published sequence data of the chitinase gene *Chi40* of *S. thermoviolaceus* OPC-520 (GenBank accession no. D14536), were identical to residues 139 to 158 of the sense strand and 1466 to 1483 of the antisense strand of the 2089-bp sequence, respectively. A standard PCR was performed in a total volume of 100 µl, containing 100 mM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 50 pmol of each PCR primer, 1.5 U of *Pfu* DNA polymerase (Promega Co., Madison, Wis.) and 50 ng of genomic DNA from *S. aureofaciens* CMUAc130. The reaction mixture was overlaid with mineral oil, and thermal cycling was achieved in a GeneAmp PCR System 9700 (PE Applied Biosystems Inc., California, USA) according to the following program: 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 10 min and final extension at 72°C for 10 min. PCR products were resolved on a 0.8% agarose gel in TBE buffer. The PCR products were purified with a QIAquick gel extraction kit (Qiagen, Germany).

### 6.7 Restriction endonuclease digestion of plasmid vector

The pUC18 plasmid vector was digested with *Sma*I. The 20 µl reaction mixture contained 10 µg DNA in 1X *Sma*I buffer and *Sma*I (5 U $\mu g^{-1}$ DNA). The reactions were incubated at 37°C overnight and then stopped for electrophoretic analysis.

## 6.8 Ligation of DNA fragments into plasmid vector

The DNA fragments and plasmid vector were mixed together in a ratio of DNA : vector from 2:1. The mixture was incubated at 65°C for 10 min and chilled on ice for 5 min. Then 4 µl of 5X T4 DNA ligase buffer and 1 µl of T4 DNA ligase were added. The ligation mixture was adjusted to 20 µl with deionized distilled water. The ligation mixture was incubated at 16°C overnight.

## 6.9 Introduction of plasmid DNA into host cell

### Preparation of competent *E. coli* cells

An isolated colony of *E. coli* was inoculated into 5 ml of LB broth and shaken at 150 rev $min^{-1}$ at 37°C overnight. 1% of the culture broth was transferred into 50 ml of LB medium and shaken at 150 rev $min^{-1}$ at 37°C for 3 hr. The culture was transferred into the 50 ml centrifuge tube, kept on ice for 15 min and centrifuged at 4,000 rev $min^{-1}$ for 6 min, 4°C (Refrigerated centrifuge Universal RF, Hettich). The pellet was suspended with 10 ml of ice cold FSB buffer (10 mM potassium acetate pH 7.5, 4.5 mM $MnCl_2$, 10 mM $CaCl_2$, 100 mM KCl, 10% glycerol, sterilized by membrane filtration) by gentle vortexing, placed on ice for 10 min and centrifuged 4,000 rev $min^{-1}$ for 6 min at 4°C. The pellet was resuspended with 2 ml of ice cold FSB and placed on ice for 10 min. 70 µl of DMSO was added per 2 ml of resuspended cells and mixed gently by swirling. The suspension was stored on ice for 15 min. The suspensions were dispensed as aliquots into 200 µl portion in Eppendorf tubes and kept at -80°C.

### Transformation by heat shock

The plasmid or ligated product was gently mixed with 100 µl of thawed competent cells and placed on ice for 30 min, then immediately placed in the 42°C

water bath for 90 sec and kept on ice for 5 min. 1 ml of LB broth was added to the suspension, mixed and incubated at 37°C for 50 min. After that the cells were briefly centrifuged at 7,000 rev $min^{-1}$ for 1 min. The supernatant was discarded and the cell suspension was spread onto LB agar containing ampicillin (100 μg $ml^{-1}$). For the transformation of blue-white selection vector, 35 μl of X-gal (20 mg $ml^{-1}$) was spread onto the medium before spreading of cell suspension. The inoculated plate was incubated at 37°C overnight.

**6.10 Selection of the recombinant clone**

The transformants which were grown on LB-agar containing 100 μg $ml^{-1}$ ampicillin and 1% colloidal chitin. Colonies showing clear halo formation were selected for plasmid isolation with a QIAGEN plasmid mini kit (Qiagen, Germany). The purified plasmid was digested with appropriate restriction endonuclease and the digestion products were analyzed by electrophoresis on 1% agarose gels.

**6.11 Restriction endonuclease treatment of *Chi40* gene recombinant plasmid**

Digestion of the plasmid with putative chitinase clone by restriction endonucleases, for example *Bam*HI, *Eco*RI and *Nde*I, was performed by methods described by manufacturers.

**6.12 Subcloning of *Chi40* gene recombinant plasmid**

Clone pUC18 containing the chitinase gene was digested by two restriction endonucleases (*Bam*HI and *Eco*RI) to obtain various sizes of recombinant chitinase gene. The large fragment obtained from *Eco*RI digestion was separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (QIAGEN, Germany). The purified fragment was religated to a circular form and transformed into *E. coli* JM109 by heat shock. The recombinant subclone was confirmed by

digestion with appropriate restriction endonucleases and analysed by electrophoresis on 1% agarose gels.

**6.13 DNA sequencing and sequence analysis**

Plasmid DNA was analyzed by cycle sequencing, using the BigDye terminator cycle sequencing kit on a GeneAmp PCR system 9700. Sequencing reaction products were purified as recommended by the manufacturer and analyzed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems Inc., California, USA) BLAST and CLUSTAL W were used for nucleotide and protein homology search as well as for multiple alignment analysis, respectively. For DNA analysis, the DNASTAR software package (DNASTAR Inc., Madison, WI, USA) was used.

**6.14 Heterologous expression of *E. coli* harbouring *Chi40* gene**

*E. coli* carrying the *Chi40* gene-recombinant plasmid was grown overnight and expression was induced by adding 1 mM IPTG, and incubated at 37°C for 3 h. The culture broth as a crude enzyme, was precipitated with 80% ammonium sulphate and dialysed in distilled overnight against water. The crude enzyme sample was further purified by Sephadex G-75 column chromatography. The fractions with chitinase activity were pooled and lyophilized in a Dura-dry freeze-dryer (FTS systems, USA). The purified chitinase was concentrated by ultrafiltration with a Centricon system (Amicon, Japan) at 1200 rev $min^{-1}$, 4°C for 1 h. The protein concentration and chitinase activity of these fractions were plotted and compared with the results of crude enzyme sample from the original strain (*S. aureofaciens* CMUAc130).

### 6.15 SDS-PAGE and Western-blot analysis

SDS-PAGE was done according to Laemmli (1970). For Western-blot analysis recombinant proteins (crude chitinase) were taken up in SDS-PAGE in 12% (w/v) slabs, and then immunologically detected using a polyclonal antibody raised against *chi40* chitinase according to Towbin *et al.* (1979). The secreted protein containing *chi40* was used as the positive control. Fifty μg of total protein was load per lane. Protein was assayed by the method of Bradford (1976).

### 6.16 Preparation of antibody against chi40

Immunization was done essentially as described by Harlow and Lane (1988). After the secreted protein had been separated by Sephadex G-75, chi40 chitinase was concentrated (1 mg/ml), mixed with synthetic adjuvants (M6661, Sigma) at 1:1 ratio (v/v) and 1 ml was injected intramuscularly into six mice for 1 ml (stain blabC) on day 1, 3, 7 and 14. After day 18, serum was collected and stored at 4°C until used.

### 6.17 Transfer of proteins from gels to nitrocellulose membrane

After electrophoresis, the proteins in gels were electrophoretically transferred to nitrocellulose membrane by the method of Towbin (Towbin *et al.*, 1979). Nitrocellulose membrane (0.45 μm of pore size Hybond-C Amersham, Buckinghamshire, UK) and filter papers (Whatman No.1) was cut into pieces the same size as the gel. One piece of nitrocellulose membrane, 8 pieces of filter papers and 2 pieces of support pad were needed for each gel. The membrane, filter papers and support pads were soaked with cold transfer buffer for 15-20 min. The gels removed from the glass plates were also immersed in cold transfer buffer around 10 min, for desalting. All materials prepared above were tightly assembled. All air

bubbles were carefully rolled out in each step. The assembled sandwiches and ice until were installed in transfer apparatus (Mini Trans-blot Electrophoretic Transfer cells, BIO-RAD) and chilled transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine containing 20% methanol) was added to the chamber. Transfer was done under a constant voltage of 110 V for 45-50 min at 4°C (initial) to 25°C (ending). After transfer, the transblotted nitrocellulose membranes were washed with PBS for 30 min.

**6.18 Immunogenic protein detection**

Before the blots could be processed for protein detection, they had to be blocked by soaking with blocking buffer (5% skimmed milk in PBS pH 7.2) overnight at 4°C for saturation of free binding sites. After the blots were washed 2 times for 10 min with PBS-T (PBS pH 7.2 containing 0.05% tween-20) by slow agitation at room temperature, they were incubated with diluted mice anti-Chi40 antiserum (dilution 1:500 in PBS-T) and pre-immune mice serum (dilution 1:100 in PBS-T) for 1 h at room temperature with slow agitation. The blots were washed in PBS-T 4 times for 20 min. Subsequently, the blots were exposed to diluted horseradish peroxidase conjugated rabbit anti-mouse IgG (dilution 1: 1000 in blocking buffer) for 1 h at room temperature with slow agitation and washed in PBS-T 4 times for 20 min. The antigen-antibody complexes were visualized by staining for peroxidase activity with 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Sigma) as substrate. This substrate solution (60 µl of stock solution of 40 mg $ml^{-1}$ DAB in 3 ml of PBS and 15 µl of $CoCl_2$ and 9 µl of 30% $H_2O_2$) was freshly prepared. The color reaction was stopped by washing with PBS after maximum incubation time of 10 min and allowed to air dry.

## 6.19 Antifungal activity of recombinant chitinase

### 6.19.1 Hyphal extension-inhibition by *E. coli* JM109/pChi40_Sau

Antifungal activity was estimated using the hyphal extension-inhibition assay of Roberts and Selitrennikoff (1986). Two fungal pathogens *Colletotrichum musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively, were used as the test fungi. They were grown on potato dextrose agar (PDA). Mycelial disks of 6 mm diameter were cut from the plate cultures of the two with the two pathogens and transferred to the ISP-2 plates and positioned 3 cm away from the inoculum of *E. coli* JM109/pChi40_Sau. The plates were incubated at 30°C for 3 days and inhibition of hyphal extension was observed by the naked eye.

### 6.19.2 Fungal spore germination-inhibition by recombinant chitinase

Fungal spore germination assays were performed according to the procedure described by Lorito *et al.* (1993) with minor variations, using *F. oxysporum* as the test fungus and the crude secreted enzyme preparation from *S. aureofaciens* CMUAc130, *E. coli* JM109/pChi40_Sau, or *E. coli* JM109 as the test solutions. A stock suspension of ca. $10^6$ conidia of the fungus $ml^{-1}$ in 30% glycerol was prepared and kept at –20°C. Equal volumes (20 µl) of spore suspension, 3X potato dextrose broth, and the test solution (about 5 U of chitinase activity) were mixed in sterile Eppendorf tubes. The test solutions were replaced with sterile water in control samples. Tubes were incubated at 30°C for 24 h. A drop of the mixture from each tube was placed on a microscope slide, and the percentage of conidial germination was determined from the first 100 spores chosen at random. Data were transformed into values representing the treatments as a percentage of the control (in which % inhibition of control = 0) by the following equation: $\%I = (1 - \%St/\%Sc) \times 100$, in which $\%I$ represented the percent

inhibition, *%St* represented the percentage of spores germinating in the treatment of interest, and *%Sc* represented the percentage of spores germinating in the control. The results of each experiment are reported as the average of three replications.

### 6.19.3 Fungal cell wall lysis by recombinant chitinase

For fungal cell wall lysis assays, *Fusarium oxysporum* was cultured on potato dextrose agar (PDA). Mycelial disks of 6-mm diameter of *F. oxysporum* were transferred onto microslides and incubated at 30°C in moist chamber. After 48 h growth, the disks were removed, a sterilized solution (50 µl) of the crude and purified chitinase of *E. coli* JM109/pChi40_Sau (5 U $ml^{-1}$) was overlaid and incubation at 37°C for 12 h in moist chamber was contained. Morphological modification of the mycelial structures of the fungus were observed under a light microscope (400X) and compared with a 5 min-boiled chitinase control.

### 6.19.4 Plate assay of chitinase activity and fungal growth-inhibition

For inhibition of fungal growth by crude and purified recombinant chitinase, *Fusarium oxysporum* was cultured on potato dextrose agar (PDA). The level of chitinase produced by recombinant *E. coli* was assayed using agar plates as described on page 214. To determine the extent of the antifungal activity of the enzyme, plate of PDA were inoculated at the center with a 6 mm disk taken from a plate culture of *F. oxysporum*. Wells were cut into the agar at 3 cm radii from the inoculum. Fifty µl of the crude and partially purified recombinant chitinase from *E. coli* JM109/pChi40_Sau (5 U $ml^{-1}$) was poured in the wells at the concentration 5, 2.5 and 1.25 mg $ml^{-1}$ and incubated at 30°C in moist chamber for 5 days; controls were 5 min-boiled enzyme extract. The inhibition of hyphal extension and fungal growth was observed over 5 days of incubation.

## Results

### 6.1 Construction of recombinant plasmid containing chitinase gene

#### 6.1.1 Expression vector pChi40-Sau

The expression vector pChi40_Sau (4.0kb) (Figure 6.2) was constructed from pUC18 (2.69 Kb). Plasmid pUC18 was digested with *Sma*I to generate the linear blunt end plasmid (Figure 6.3) which was dephosphorylated at the 5' end and ligated to the PCR purified product of the *chi40* gene (1.3 Kb) (Figure 6.4). The completed ligation was assumed by using ligated λDNA/*Hind*III as a control (Figure 6.5). After ligation, the recombinant plasmid was transformed into *E. coli* JM109 by the heat shock method.

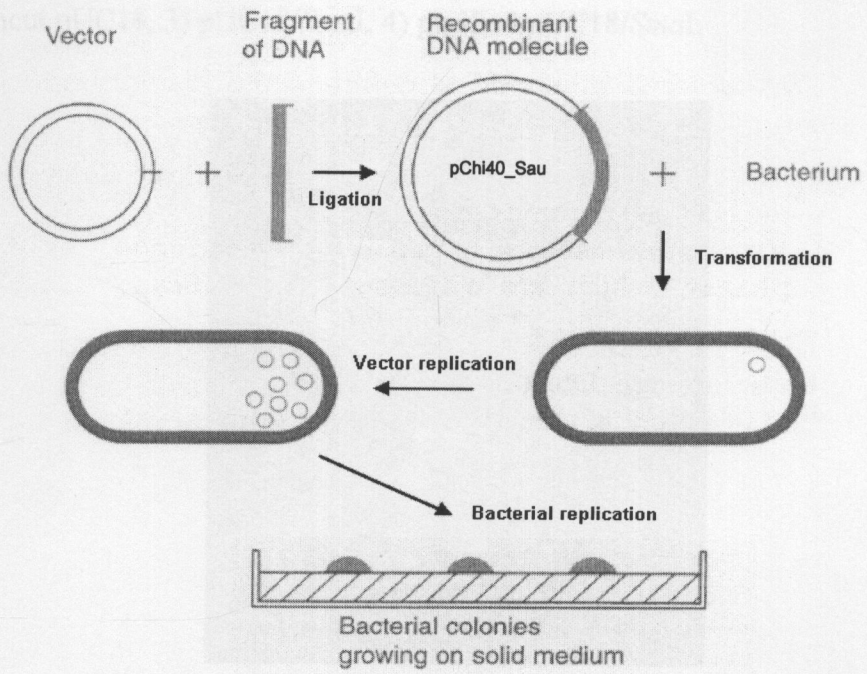


**Figure 6.2** Construction of pChi40_Sau by ligating the purified PCR product into *Sma*I site of pUC18. The transformants which were grown on LB-agar containing 100 μg $ml^{-1}$ amplicillin and 1% colloidal chitin (From Gibco BRL products).

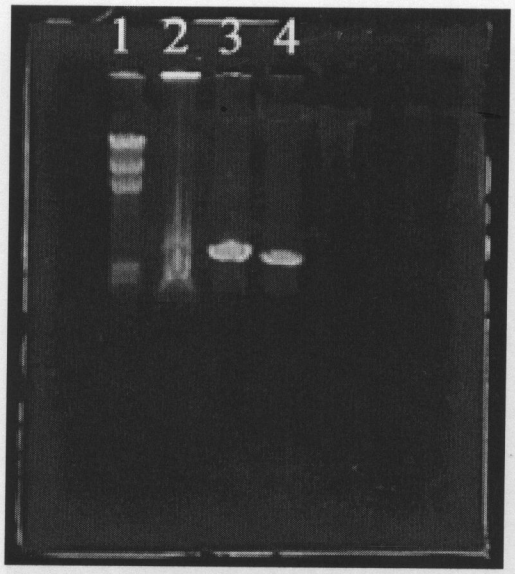


**Figure 6.3** Electrophoretic (agarose gel; 0.8%) analysis of DNA, 1) λDNA/*Hin*dIII marker, 2) Uncut pUC18, 3) pUC18/*Sma*I, 4) purified pUC18/*Sma*I.

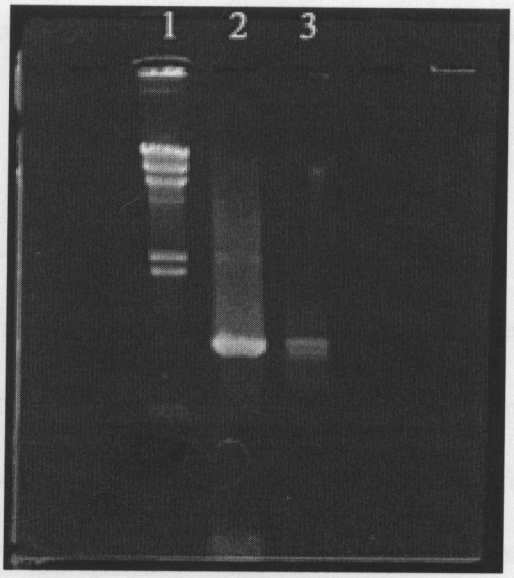


**Figure 6.4** Electrophoretic (agarose gel; 0.8%) analysis of 1.3Kb PCR product of Chi40 gene from *Streptomyces aureofaciens* CMUAc130. 1) λDNA/*Hin*dIII marker, 2) 1.3 Kb PCR product, 3) Purified PCR product.

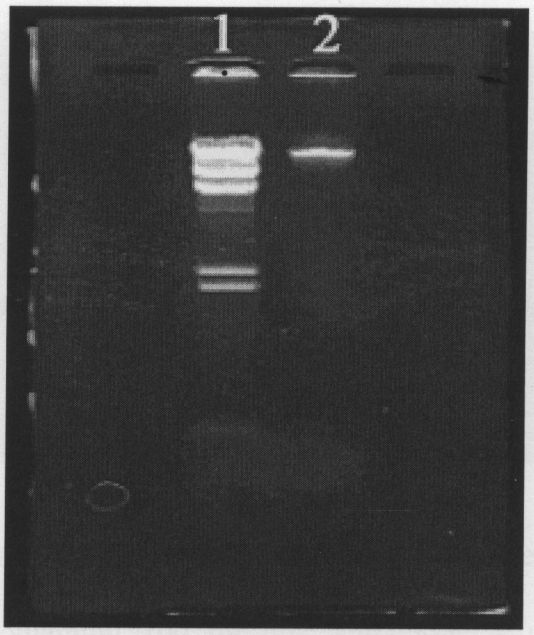


**Figure 6.5** Electrophoretic (agarose gel; 0.8%) analysis of control ligation of λ DNA/*Hin*dIII. 1) λDNA/*Hin*dIII marker, 2) ligation of λDNA/*Hin*dIII by T4 DNA ligase at 14°C for over night.

### 6.1.2 Screening for pChi40_Sau

The transformants which were grown on LB-agar plates containing 100 μg $ml^{-1}$ ampicillin and 1% colloidal chitin and showed clear halo formation around the colony (Figure 6.6) were selected for plasmid isolation. The transformants hosted a 4.0 Kb plasmid (Figure 6.7) which was selected for sequencing and expression.

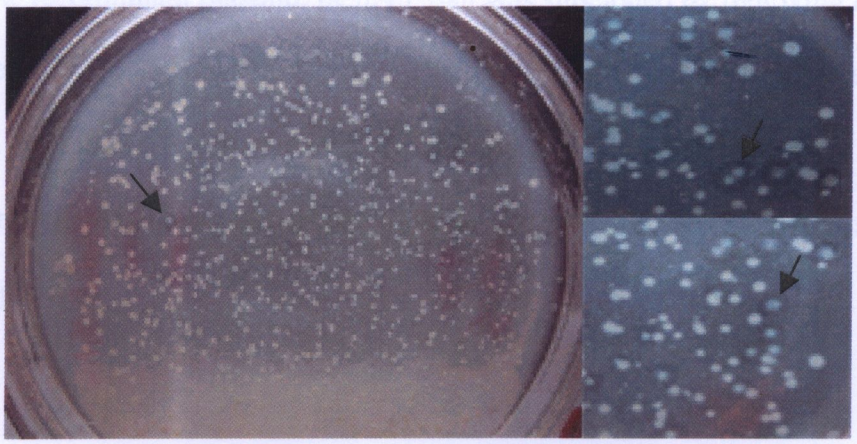

**Figure 6.6** Transformant cells showed clear halo formation around the colony (arrow) after growth on LB-agar containing 100 μg $ml^{-1}$ ampicillin, 1 mM IPTG and 1%colloidal chitin at 37°C for 48 h.

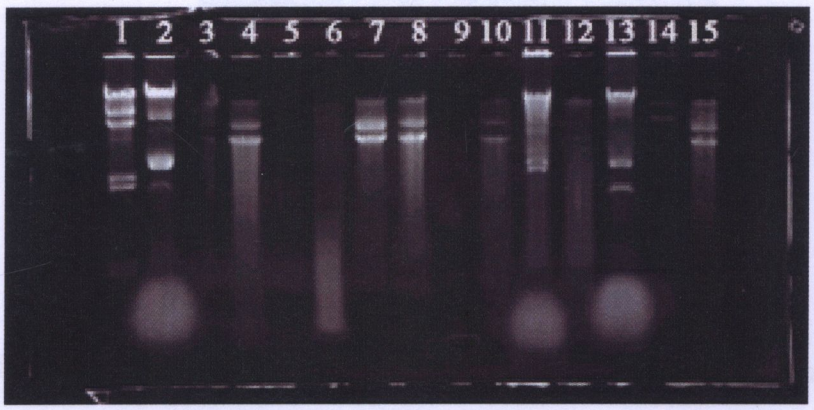


**Figure 6.7** Electrophoretic (agarose gel; 0.8%) analysis of plasmid DNA from selected clone. 1) λDNA/*Hin*dIII, 2) negative control pUC18, 3)-15) clone 1-13, respectively.

### 6.1.3 Subcloning of pChi40_Sau.sub1

The recombinant plasmid, pChi40_Sau was subcloned by removing the small *Eco*RI fragment (0.85 Kb) and was designated as pChi40_Sau.sub1. A large fragment of *Eco*RI digestion (Figure 6.8) of the pChi40_sau was cut, recovered from the agarose and ligated. The transformant was selected based on the 3.2 Kb in size of the extracted plasmid.

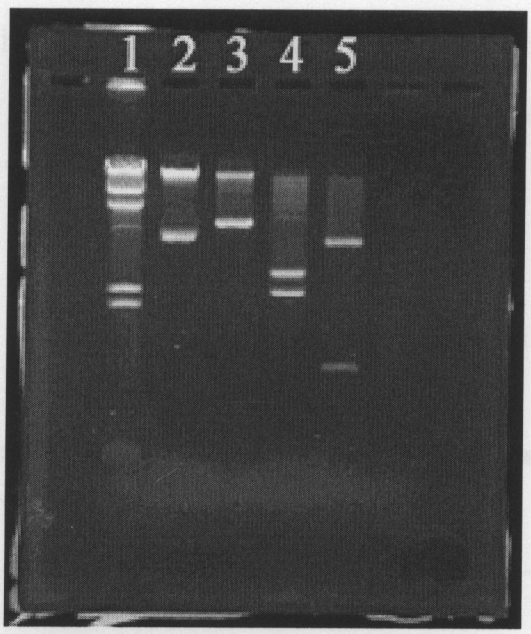


**Figure 6.8** Fragment analysis of digested clone 1 plasmid with various enzymes. 1) λ DNA/*Hin*dIII, 2) uncut pChi40_Sau, 3) partial digestion of pChi40_Sau with *Nde*I, 4) digested pChi40_Sau with *Bam*HI, 5) digested pChi40_Sau with *Eco*RI. (0.8% agarose gel)

## 6.2 Nucleotide sequencing and sequence assembly of Chi40_Sau gene

The pChi40_Sau and pChi40_Sau.sub1 were subjected for sequencing by dye terminator cycle sequencing method. Nucleotide sequences were joined by DNASIS program.

## 6.3 Restriction mapping of Chi40_Sau gene

The restriction mapping of Chi40_Sau gene of *S. aureofaciens* CMUAc130 was done to obtain more information of its restriction sites. The restriction map, of chitinase gene from pChi40_Sau, was analysed by Webcutter 2.0 program from the completed sequence of chitinase gene, and shown in Figure 6.9. In addition, the restriction sites for *Eco*RI, *Bam*HI and *Nde*I were confirmed as shown in Figure 6.8.

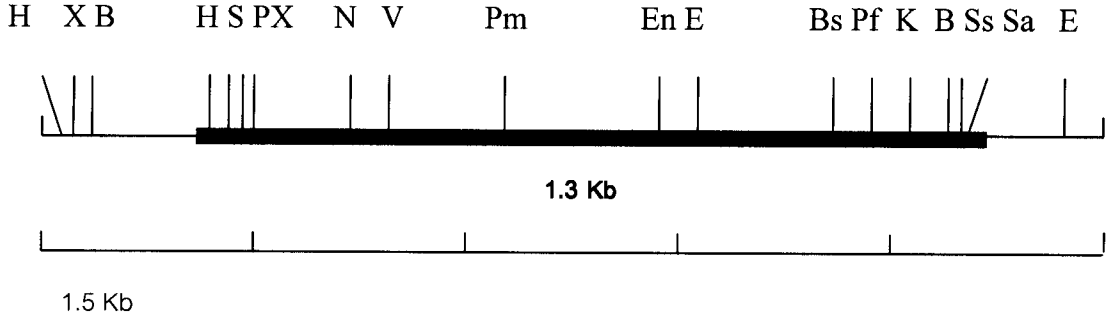


**Figure 6.9** Restriction map of the pChi40_Sau. The thick line indicates the chitinase gene of *Streptomyces aureofaciens* CMUAc130, and the thin line indicates a part of the plasmid vector pUC18. Abbreviation; B, *Bam*HI; Bs, *Bsa*BI; E, *Eco*RI; En, *Eco*NI; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; P, *Pst*I; Pf, *Pfl*MI; Pm, *Pml*I; S, *Sph*I; Sa, *Sac*I; Ss, *Sst*I; V, *Vne*I; X, *Xba*I.

## 6.4 Nucleotide and amino acid analyses of the cloned Chi40_Sau

The chitinase gene of *S. aureofaciens* CMUAc130 was cloned using two primers whose design was based on the Family 19 Chitinase gene of *Streptomyces* sp. The PCR product obtained was ligated into the *Sma*I restriction site of pUC18, and recombinant clones were isolated in *E. coli* JM109. A clone showing clear halo formation was selected for further studies.

The complete 1.4-kb sequence of the insert was determined (Table 6.1), and the deduced amino acid sequence of Chi40_Sau yielded an open reading frame of 1242 nucleotides, coding for a protein of 413 amino acids with an estimated molecular mass of 40 KDa (Figure 6.10). The putative –10 and –35 regions, which showed relatively weak homology with the consensus sequence in *E. coli*, were detected upstream from the putative ribosome-binding site sequence.

A database search revealed that the deduced Chi40_Sau protein amino acids sequence was 87, 87, 31, 29 and 28% identical to those of Chit_Strpl from *S. plicatus* ATCC27800 (Robbins *et al.* 1992), Chia_Strth from *S. thermoviolaceus* OPC-520 (Tsujibo *et al.* 1993a), Chi1_Bacci from *Bacillus circulans* WL-12 (Watanabe *et al.* 1993), Chia_Altso from *Alteromonas* sp. strain O-7 (Tsujibo *et al.* 1993b) and Chia_Serma from *Serratia marcescens* (Jones *et al.* 1986) (Figure 6.11). Chi40_Sau protein also showed high homology with *S. lividans* Chit_Strli (Miyashita and Fujii 1993) and *S. thermoviolaceus* Chi40_Strth (Tsujibo *et al.* 1993a). The precursor protein of Chi40_Sau had a leader peptide identical to that of Chit_Strli (Miyashita and Fujii 1993) and Chit_Strpl (Robbins *et al.* 1992), suggesting that it is also cleaved at the same site. Thus, the mature protein probably starts at Ala31 (Figure 6.11).

**Table 6.1** The DNA sequence of *Chi40_Sau* gene of endophytic *S. aureofaciens* CMUAc130 amplified by various primers and templates.

| Primers | Templates | DNA sequence (5'→ 3') |
|---|---|---|
| M13 Forward 23-base sequencing primer | pChi40_Sau | CGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAG<br>GATCCCCGTGTGCTGCTCACGCCAGGCCGGTGTTGATGGCGC<br>TCACCAGTTCGCCGTTCGCGGTGTCGCCGCTGAAGTCCCAGA<br>CGAAGGCGCCGCCGAGACCCTGCTCGTTCGCCCAGTCCATCT<br>TCGACTTGATGGTGTCCGGGGTGTCGTAGATCCACCAGTTGG<br>AGCCGCAGTGGGCGTACGCGGTGCCGGCGACGGTGCCGGTG<br>GCCGGGCAGCTGTTCTTGAGGACCTTGTAGTCCTCGATGCCG<br>GCCTCGTAGGTGCCGGCCGCCGGGCCGGTGGCGGTGCCGCCG<br>GGCGCGGACTGCGTGCAGCCGGTCCAGCCGCGGCCGTAGAA<br>GCCGATGCCGATCAGGAGCTTGTAGGCCGGGACGCCCTTGGC<br>CTTGAACTTCGCTATCGCGTCGGCGGAGGTGAAGCCCTGCTG<br>CGGGATGCCGTCGTACGAGGTGAGCGGCGAGTGCCCCGCGG<br>TCGGGCCGTTCTTCGCCCAGGCGCCGAAGAAGTCGTACGTCA<br>TCACGTTGTACCAGTCGATGTACTTCGAGGCCTCGCCGTAGT<br>CGGTGGCGTCGATCTTGCCGCCGTCCGAGCCGTCGGCGGTGA<br>CGGCCGCGGTGACCAGGTTGTCGCCGAACTCGGCGCGCACG<br>GCCTTCATCNNGNNNN |
| M13 Forward 23-base sequencing primer | pChi40_Sau. sub1 | CGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAG<br>GATCCCCGTGTGCTGCTCACGCCAGGCCGGTGTTGATGGCGC<br>TCACCAGTTCGCCGTTCGCGGTGTCGCCGCTGAAGTCCCAGA<br>CGAAGGCGCCGCCGAGACCCTGCTCGTTCGCCCAGTCCATCT<br>TCGACTTGATGGTGTCCGGGGTGTCGTAGATCCACCAGTTGG<br>AGCCGCAGTGGGCGTACGCGGTGCCGGCGACGGTGCCGGTG<br>GCCGGGCAGCTGTTCTTGAGGACCTTGTAGTCCTCGATGCCG<br>GCCTCGTAGGTGCCGGCCGCCGGGCCGGTGGCGGTGCCGCCG<br>GGCGCGGACTGCGTGCAGCCGGTCCAGCCGCGGCCGTAGAA<br>GCCGATGCCGATCAGGAGCTTGTAGGCCGGGACGCCCTTGGC<br>CTTGAACTTCGCTATCGCGTCGGCGGAGGTGAAGCCCTGCTG<br>CGGGATGCCGTCGTACGAGGTGAGCGGCGAGTGCCCCGCGG<br>TCGGGCCGTTCTTCGCCCAGGCGCCGAAGAAGTCGTACGTCA<br>TCACGTTGTACCAGTCGATGTACTTCGAGGCCTCGCCGTAGT<br>CGGTGGCGTCGATCTTGCCGCCGTCCGAGCCGTCGGCGGTGA<br>CGNCCNNN |
| M13 Reverse 23-base sequencing primer | pChi40_Sau | AACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCTT<br>GACCCAGTGGTCCAGACCTTTCTATATTCGCGCCACGGGCGT<br>GCTGACCGTCATGCCCCTGACATCCCCCCCGCACACAGAGGA<br>GGCGCTTCATGCGCTTCAGACACAAAGCCGCGGCACTCGCAG<br>CGACCCTGGCGCTTCCCCTCGCCGGCCTGGTCGGCCTCGCGA<br>GCCCGGCCCAGGCGGCCACCAGCGCGACGGCCACCTTCCAG<br>AAGACCTCGGACTGGGGCACCGGCACGGTCAAGCTGGGCTA<br>CTTCACCAACTGGGGCGTCTACGGGCGCAACTACCACGTGAA<br>GAACCTGGTCACCTCCGGCTCCGCCGACAAGATCACGCACAT<br>CAACTATGCCTTCGGCAACGTCCAGGGCGGGAATTCCACCAT<br>CGGCGACTCCTACGCCGACTACGACAAGGCGTACACCGCCG<br>ACCAGTCCGTCGACGGCGTCGCGGACACCTGGGACCAGCCG<br>CTGCGCGCAAACTTCAACCAGCTGCGCAAGTTGAAGGCCAA<br>GTACCCGCACATCAAGGTCCTCTACTCCTTCGGCGGCTGGAC<br>CTGGTCCGGCGGCTTCCCCGACGCCGTGAAGAACCCGGCCGC<br>GTTCGCGCAGTCCTGCTACGACCTGGTCGAGGACCCGCGCTG<br>GGCCGACGTCTTCGACGGCATCGACTGGGAGTACCCCAACGC<br>CTGCGGTCTCAGCTGTGACACCAGCGGCCCCAACGCCTTCAG<br>CAACATGATGAAGGCCGTGCGCGCCGAGTTCGGCGACAACC<br>TGGTCACCGCGGCCGTCACCGCCGACGGCTCGGANGNCNNN |

**Table 6.1** The DNA sequence of *Chi40_Sau* gene of endophytic *S. aureofaciens* CMUAc130 amplified by various primers and templates (continued).

| Primers | Templates | DNA sequence (5'→ 3') |
|---|---|---|
| M13 Reverse 23-base sequencing primer | pChi40_Sau. sub1 | AACAGCTATGACCATGATTACGAATTCAATTCCACCATCGGC<br>GACTCCTACGCCGACTACGACAAGGCGTACACCGCCGACCA<br>GTCCGTCGACGGCGTCGCGGACACCTGGGACCAGCCGCTGCG<br>CGCAAACTTCAACCAGCTGCGCAAGTTGAAGGCCAAGTACCC<br>GCACATCAAGGTCCTCTACTCCTTCGGCGGCTGGACCTGGTC<br>CGGCGGCTTCCCCGACGCCGTGAAGAACCCGGCCGCGTTCGC<br>GCAGTCCTGCTACGACCTGGTCGAGGACCCGCGCTGGGCCGA<br>CGTCTTCGACGGCATCGACTGGGAGTACCCCAACGCCTGCGG<br>TCTCAGCTGTGACACCAGCGGCCCCAACGCCTTCAGCAACAT<br>GATGAAGGCCGTGCGCGCCGAGTTCGGCGACAACCTGGTCA<br>CCGCGGCCGTCACCGCCGACGGCTCGGACGGCGGCAAGATC<br>GACGCCACCGACTACGGCGAGGCCTCGAAGTACATCGACTG<br>GTACAACGTGATGACGTACGACTTCTTCGGCGCCTGGGCGAA<br>GAACGGCCCGACCGCCCCGCACTCGCCGCTCACCTCGTACGA<br>CGGCATCCCGCAGCAGGGCTTCACCTCCGCCGACGCGATAGC<br>GAAGTTCAAGGCCAAGGGCGTCCCGGCCGACAAGCTCCTGA<br>TCGGCATCGGCTTCTACGGCCGCGGCTGGACCGGCGTCACGC<br>AGTCCGCGNNCGNNNN |

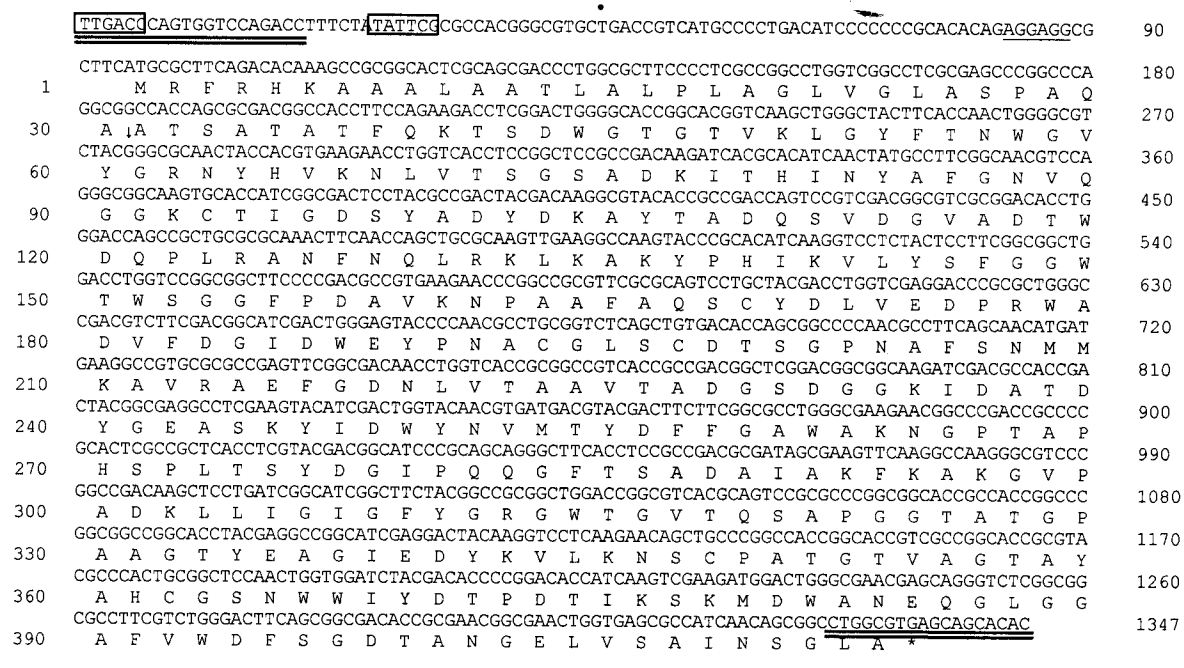


**Figure 6.10** Nucleotide sequence of the 1.35-kb DNA fragment and the deduced amino acid sequence of Chi40_Sau. The putative ribosome-binding site (AGGAGG) is underlined and the -10 and -35 regions of a possible promoter sequence is boxed. The signal peptide cleavage site is shown with an arrow (↓) and the stop codon is indicated by an asterisk. The doubly underlined nucleotide sequences denote the conserved regions of *Streptomyces* sp. family 19 chitinase used to design the PCR primers.

```
Chia_Strau      ATSATATFQKTSDWGTGT------------------------------------------ 18
Chit_Strpl      ATSATATFQKTSDWGTGFGGKWTVKNTGTTSLSSWTVEWDFPSGTKVTSAWDATVTNSAD 60
Chia_Strth      ---------AAADNGT------------------------------------------- 7
Chi1_Bacci      ------------------------------------------------------------
Chia_Altso      ----AAPSTPTLDWQPQQYSFVEVNVDGLGSYKQLVKAKDVVDISIKWNAWSGSGGDNYK 56
Chia_Serma      ----AAPGKPTIAWGNTKFAIVEVDQAAT-AYNNLVKVKNAADVSVSWNLWNGDAGTTAK 55

Chia_Strau      ------------------------------------------------------------
Chit_Strpl      HWTAKNVGWNGTLAPGASVSFGFNGSGPGSPSGCKINGGSCDGSSVPGDEAPSAPGTPTA 120
Chia_Strth      ------------------------------------------------------------
Chi1_Bacci      ------------------------------------------------------------
Chia_Altso      VYFDDLLVNQGSLPAGTKSGVVQFPYTKSGRHQLYLELCEGTVCARSAGKEIVIADTDG- 115
Chia_Serma      ILLNGKEAWSG--PSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIVVADTDG- 112

Chia_Strau      ------------------------------------------------------------
Chit_Strpl      SNITDTSVKLSWSAATDDKGVKNYDVLRDGATVATVTGTTYTDNGLTKGTDYSYSVKARD 180
Chia_Strth      ------------------------------------------------------------
Chi1_Bacci      ------------------------------------------------------------
Chia_Altso      ------------------------------------------------------------
Chia_Serma      ------------------------------------------------------------

Chia_Strau      ------------------------------VKLGYFTNWGVYGRNYHVKNLVTSGSADK 47
Chit_Strpl      TGDQTGPASGSVKVTTTGGDGGEPNPNPGAEVKMGYFTNWGVYGRNYHVKNLVTSGSAEK 240
Chia_Strth      ------------------------------VKLGYFTEWGTYDRNFNVKNLDTSGTAAK 36
Chi1_Bacci      ----------------------------DSYKIVGYYPSWAAYGRNYNVADIDPT----K 28
Chia_Altso      --------AHLAPLPMNVDPNNRNNGTIPGRVTGAYFVEWGIYGRNYDVTKIPAHN---- 163
Chia_Serma      --------SHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDKIPAQN---- 160
                                                *   *  * **  *

Chia_Strau      ITHINYAFG-----NVQGGKCTIGDSYADYDKAYTADQSVDG------------------ 84
Chit_Strpl      ITHINLRFG-----NVQGGKCTIGDAYADYDKAYTADQSVDG------------------ 277
Chia_Strth      ITHINYAFG-----NVTGGKCAIGDSYADYDKAFTADQSVSG------------------ 73
Chi1_Bacci      VTHINYAFADICWNGIHGNPDPSGPNPVTWTCQNEKSQTINVPNGTIVLGDPWIDTGKTF 88
Chia_Altso      LSHILYGFIPICGPN---ESLKSIEIGNSWRALQTACADSQD-YEVVIHDPWAAVQKSMP 219
Chia_Serma      LTHLLYGFIPICGGNGINDSLKEIEG--SFQALQRSCQGRED-FKVSIHDPFAALQKAQK 217
                  *    *

Chia_Strau      VADTWDQPLRANFNQLRKLKAKYPHIKVLYSFGGWTWSGGFPDAVKNPAAFAQSCYDLVE 144
Chit_Strpl      VADTWDQPLRANFNQLRNLKAEYPHIKILYSFGGWTWSGGFPDAVKNPAAFAKSCHDLVE 337
Chia_Strth      QADTWDQPLRANFNQLRQLKAKYPHIKVLWSFGGWTWSGGFADAAKRPAAFAQSCYNLVH 133
Chi1_Bacci      AGDTWDQPIAGNINQLNKLKQTNPNLKTIISVGGWTWSNRFSDVAAT-AATREVFANSAV 147
Chia_Altso      GVDAKD-PIRGVYSQLMALKQRYPDLKILPSVGGWTLSDPFHGFTNK-ANRDTFVASVKQ 277
Chia_Serma      GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDK-VKRDRFVGSVKE 276
                    * *      **  **   *  *   * **** *  *

Chia_Strau      DPRWADVFDGID--WEYPNACGLSCD--TSGPN------AFSNMMKAVRAEFG-DNLVTA 193
Chit_Strpl      DPRWADVFDGIDLDWEYPNACGLSCDE-TSAPN------AFSSMMKAMRAEFGQDYLITA 390
Chia_Strth      DPRWDGVFDGID--WEYPNACGLTCD--SSGPD------AFRNLMAAVRSTFG-DELVTA 182
Chi1_Bacci      DFLRKYNFDGVDLDWEYPVSGGLDGNS-KRPEDKQNYTLLLSKIREKLDAAGAVDGKKYL 206
Chia_Altso      FLKTWKFYDGVDIDWEFPGGDGPNPDLGDPINDGPAYVALMQELRAMLDELEAETGRQYE 337
Chia_Serma      FLQTWKFFDGVDIDWEFPGGKGANPNLGSPQ-DGETYVLLMKELRAMLDQLSAETGRKYE 335
                      ** *  ** *   *

Chia_Strau      AVTADGSDGGKIDATDYGEASKYIDWYNVMTYDFFGAWAKNGPTAP---HSPLTSYDG-- 248
Chit_Strpl      AVTADGSDGGKIDAADYGEASKYIDWYNVMTYDFFGAWAKNGPTAP---HSPLNAYDG-- 445
Chia_Strth      AVTADGTPGGKIEATDYAGAAQYVDWYNVMTYDFFGAWDAQGPTAP---HSPLTSYDG-- 237
Chi1_Bacci      LTIASGASATYAANTELAKIAAIVDWININTYDFNGAWQKISAHNAPLNYDPAASAAG-- 264
Chia_Altso      LTSAIGAGYDKIEDVDYQAAQQYMDYIFAMTYDFYGAWN-NETGHQTGIYCGSHLSTDEC 396
Chia_Serma      LTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGPFDLKNLGHQTALNAP-------- 387
                  *                   *    * *** *

Chia_Strau      ----------IPQQ-GFTSADAIAKFKAKGVPADKLLIGIGFYGRGWTGVTQSAPG---- 293
Chit_Strpl      ----------IPQQ-GFTTADAMAKFKSKGVPADKLLIGIGFYGRGWTGVTQSAPG---- 490
Chia_Strth      ----------IPKQ-GFTSADAIAAFKAQGVPADKLLLGIGFYGRGWTGVTQDAPG---- 282
Chi1_Bacci      ----------VPDANTFNVAAGAQGHLDAGVPAAKLVLGVPFYGRGWDGCAQAGNGQY-- 312
Chia_Altso      NGTGVDDNGVPRKGPAYTGDHAIQLLLQQGVQPSKLVMGVAMYGRGWEGVLDANAAIPGN 456
Chia_Serma      ---------AWKPDTAYTTVNGVNALLAQGVKPGKVVVGTAMYGRGWTGVNGYQNNIP-- 436
                                        **   *   *   ***** *

Chia_Strau      ---GTATGPAAG-----TYEAGIEDYKVLKNSCPATG------------TVAGTAYAHCG 333
Chit_Strpl      ---GTATGPAAG-----TYEAGIEDYKVLKNSCPATG------------TVAGTAYAHCG 530
Chia_Strth      ---GTATGPAAG-----TWEQGIEDYKILKNTCPVTG------------TVAGTAYAHCG 322
Chi1_Bacci      --QTCTGGSSVG-----TWEAGSFDFYDLEANYINKNG-----YTRYWNDTAKVPYLYNA 360
Chia_Altso      PMTAPGNGPLTGSTSEGVWEPGIMDYKAIAANAVGQGGSGVNGYEVGYDEQAQAAYVWNR 516
Chia_Serma      -FTGTATGPVKG-----TWKNGIVDYRQIAGQFMSG------EWQYTYDATAEAPYVFKP 484
                      *   *         *  *                          *   *
```

```
Chia_Strau      SN--WWIYDTPDTIKSKMDWANEQGLGGAFVWDFSG-----------DTANGELVSAIN 379
Chit_Strpl      TN--WWSYDTPATIKSKMDWAEQQGLGGAFFWEFSG-----------DTTNGELVSAID 576
Chia_Strth      SN--WWIYDTPDTIASKMAWANDQGLRGAFVWDFSG-----------DTATGELIAALS 368
Chi1_Bacci      SNKRFISYDDAESVGYKTAYIKSKGLGGAMFWELSGDRNKTLQNKLKADLPTGGTVPPVD 420
Chia_Altso      SNGKLITYDSPRSVIAKGQYANTHQLAGLFGWEIDAD-------------NGDILNAMY 562
Chia_Serma      STGDLITFDDARSVQAKGKYVLDKQLGGLFSWEIDAD-------------NGDILNSMN 530
                        *       *       * *   *                     *
Chia_Strau      SGLA------------------------------------------------------- 383
Chit_Strpl      SGLK------------------------------------------------------- 580
Chia_Strth      NGLA------------------------------------------------------- 372
Chi1_Bacci      TTAPSVPGNARSTGVTANSVTLAWNASTDNVGVTGYNVYNGANLATSVTGTTATISG-LT 479
Chia_Altso      DGLTAGEIPNRAPTIGVSGPINVTSGQVVNVDAQASDLDNDPLTYSWVAAPGLALSANNT 622
Chia_Serma      ASLGN--------SAGVQ----------------------------------------- 540
```

**Figure 6.11** Alignment of deduced amino acid sequences of some bacterial chitinases. The amino acid sequences of the chitinases from *S. aureofaciens* (Chia_Strau, deduced from the translation of the DNA sequence obtained in this study), *S. plicatus* (Chit_Strpl, from the Swiss-Prot database, accession no. P11220), *S. thermoviolaceus* (Chia_Strth, from the GenBank DNA sequence, accession no. JC2135) *B. circulans* (Chi1_Bacci, from the Swiss-Prot database, accession no. P20533), an *Alteromonas* sp. (Chia_Altso, from the Swiss-Prot database, accession no. P32823) and *S. marcescens* (Chia_Serma, from the Swiss-Prot database, accession no. P07254) were aligned using the Clustal W 1.82 program (European Bioinformatics Institute, Cambridge, UK). Numbering starts from the first residue of the mature protein. Asterisks indicate identical residues in all proteins shown. The type-III homology unit of fibronectin is underlined. The amino acid Asp156 and Glu158 that are identical in the active site of lysozyme are in boldface.

Computer analysis revealed 87% identity between the *Chi40_Sau* gene of *S. aureofaciens* CMUAc130 and the *Chit_Strpl* gene of *S. plicatus* ATCC27800. In both genes, a Shine-Dalgarno sequence, AGGAGG, preceded the start codon by six bases (underlined in Figure 6.9). However, the stop codons differed (UGA in the Chi40_Sau gene vs. UAG in the *Chit_Strpl* gene).

**6.5 Chitinolytic activity of *E. coli* carrying the Chi40_Sau gene**

The chitinolytic activity found in the culture medium of the transformant JM109/pChi40_Sau was active in the hydrolysis of the colloidal chitin with 1 mM IPTG but very low in the absent of IPTG (Table 6.2). These data indicate that the chitinase was regulated by the *lacZ* promoter. A significantly decreased level of chitinolytic activity was found in the intracellular fraction of the transformant. No activity was detected in the secreted proteins or extracts of strain JM109 or its transformant carrying the pUC18 vector.

To demonstrate directly that the transformed *E. coli* strain produced and secreted the Chi40_Sau chitinase, the immunological detection of secreted proteins of JM109/pChi40_Sau, renaturated following separation by SDS-PAGE, was examined and compared with that of the original *S. aureofaciens* CMUAc130. The results obtained by Western-blot analysis, showed that the specific protein with apparent molecular mass of 40 KDa which is present in the secreted proteins of strain CMUAc130 (Figure 6.12, lane 1), corresponding to the 40 KDa chitinase, was detected in secreted proteins of JM109/pChi40_Sau (Figure 6.12, lane 2). No bands of immunological reactivity from the secreted proteins of *E. coli* JM109 were found (Figure 6.12, lane 3). The specific activity of *E. coli* JM109/pChi40_Sau expressed chitinase in various treatment steps was shown in Table 6.3.

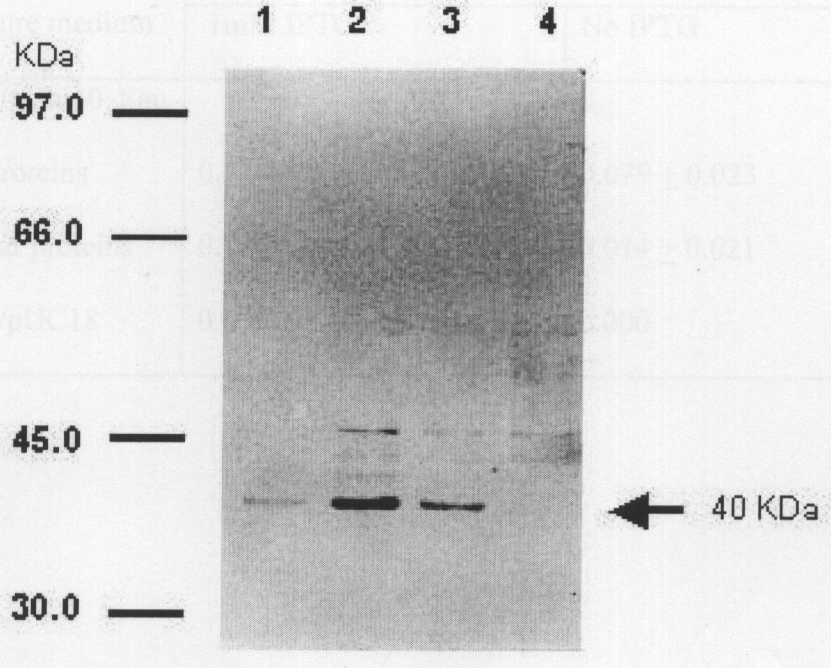


**Figure 6.12** Western-blot analysis showing the expression of the Chia_Strau gene. Lane: 1, crude chitinase from *S. aureofaciens* CMUAc130; 2, secreted protein from *E. coli* JM109/pChi40_Sau with 1mM IPTG induction; 3, secreted protein from *E. coli* JM109/pChi40_Sau without IPTG induction; 4, secreted protein from *E. coli* JM109.

**Table 6.2** Chitinase activity of *E. coli* JM109 carrying pChi40_Sau in LB after overnight growth at 37°C and 3 h adding 1 mM IPTG.

| Source of culture medium | Chitinase activity of heterologous expression (U/ml) | |
|---|---|---|
| | 1mM IPTG | No IPTG |
| *E. coli* JM109/pChi40_Sau | | |
| Secreted proteins | 0.274 ± 0.058 | 0.079 ± 0.023 |
| Intracellular proteins | 0.106 ± 0.072 | 0.014 ± 0.021 |
| *E. coli* JM109/pUC18 | 0.000 | 0.000 |

**Table 6.3** Specific activity of *E. coli* JM109/pChi40_Sau chitinase in various treatment steps.

| Treatment step | Volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (U/ml) | Total activity (U) | Specific activity (U/mg) | Yield (%) |
|---|---|---|---|---|---|---|---|
| Culture filtrate | 1000 | 1.96 | 1960.0 | 0.28 | 280.28 | 0.143 | 100.00 |
| $(NH_4)_2SO_4$ treatment | 20 | 81.25 | 1625.0 | 12.45 | 248.63 | 0.153 | 88.71 |
| Sephadex G-75 | 30 | 1.27 | 38.1 | 4.68 | 140.40 | 3.685 | 50.09 |
| Freez dried | 3 | 10.72 | 32.1 | 43.25 | 129.77 | 4.035 | 46.30 |
| Ultrafiltration | 1.5 | 19.04 | 28.5 | 82.50 | 123.75 | 4.333 | 44.15 |

## 6.6 Antifungal activity of expressed chitinase from *E. coli* JM109/pChi40_Sau

The chitinolytic *E. coli* strain carrying the plasmid pChi40_Sau was tested for antifungal activity against the phytopathogenic fungi *C. musae* and *F. oxysporum* and was found to suppress the latter's growth in plate culture. The original strain CMUAc130 showed stronger suppression of the fungi, whereas almost no suppression was observed with the non transformed *E. coli* strain (Figure 6.13).

The effects of crude preparations of the chitinases secreted by the original *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40_Sau on spore germination of *F. oxysporum* were assayed. The rate of spore germination in the control was 80.6% $\pm$ 8.0% (Figure 6.14a). The *F. oxysporum* spore germination assay showed that in the presence of crude preparations of secreted proteins produced by strain CMUAc130 and JM109/ pChi40_Sau, the rate of spore germination significantly decreased relative to the control (39.0% $\pm$ 7.5% and 56.6% $\pm$ 5.5%, respectively) (Figure 6.14b and 6.14c). When the amount of secreted proteins of the parent strain JM109 equivalent to that in crude preparations of chitinases secreted by stain CMUAc130 and JM109/pChi40_Sau was added to the experimental mixture as a test solution, almost no difference from the control was found (79.0% $\pm$ 4.5%) (Figure 6.14d).

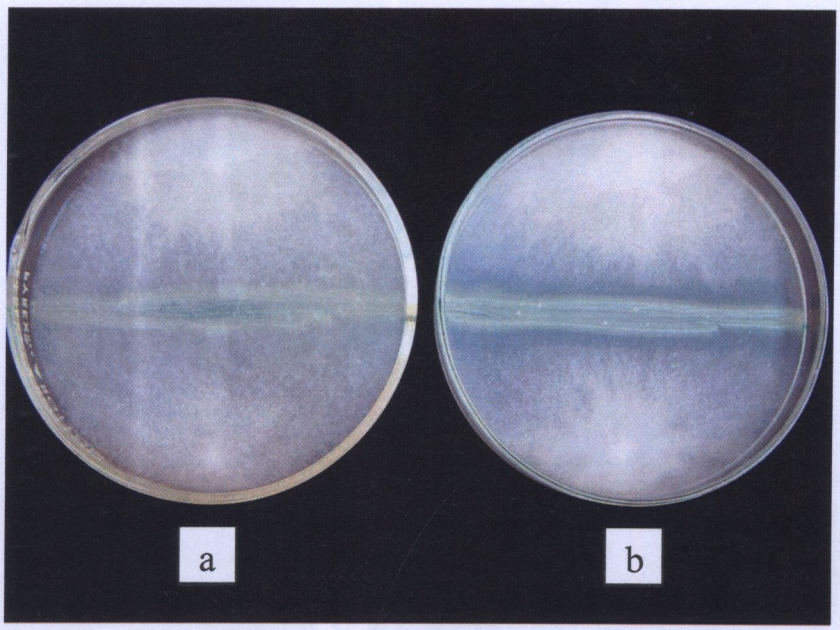


**Figure 6.13** Assay of antifungal activities on plates. A suspension of bacterial cells was seeds in a line through the center of ISP-2 medium plate. Two agar disks from an actively growing culture of *F. oxysporum* were placed on either side of the bacterial growth area, and the plates were incubated at 30°C for 5 days. Plates: a, *E. coli* JM109/pUC18; b, *E. coli* JM109/pChi40_Sau.

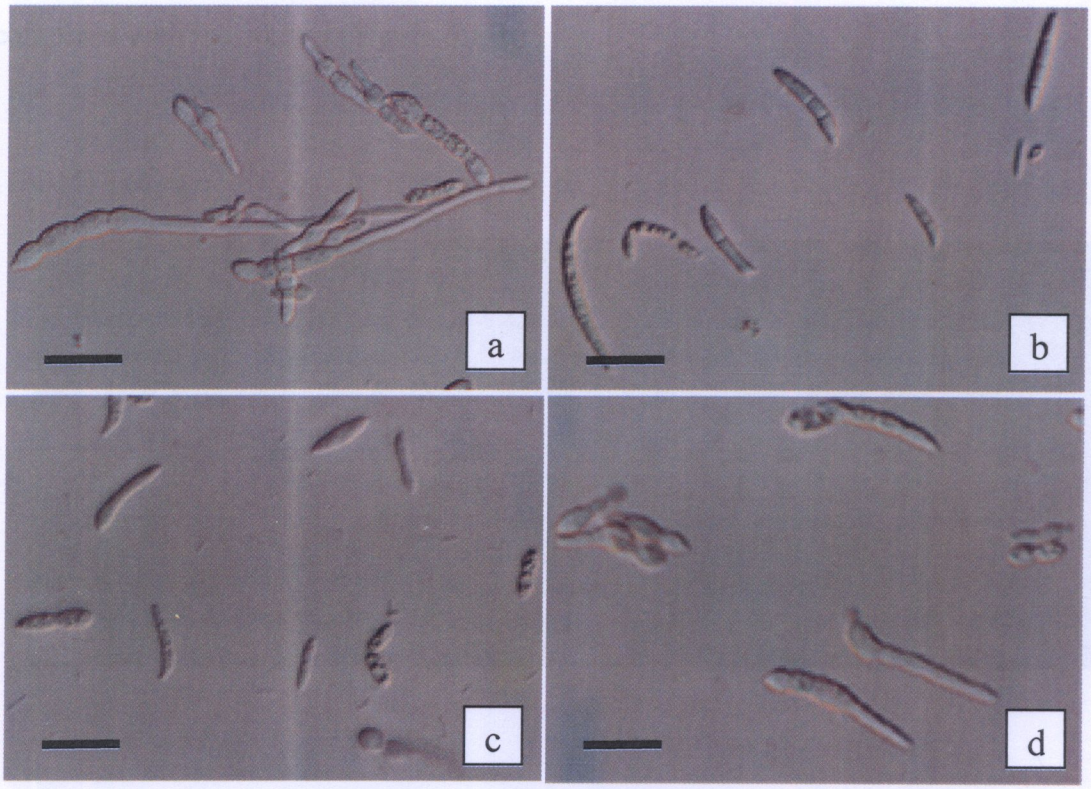


**Figure 6.14** Spore germination inhibition after incubation at 30°C for 24 h with: a) 20 ml sterile water, b) 5 U crude chitinase of *S. aureofaciens* CMUAc130, c) 5 U crude chitinase of JM109/pChi40_Sau, d) 20 ml culture broth of JM109/pUC18.

Bar = 10 μm.

Morphological changes were observed in microslide cultures of *F. oxysporum* treated with crude and purified chitinase from *E. coli* JM109/pChi40_Sau. Inhibition of spore germination and fungal cell wall lysis was noted (Figure 6.15a) when compared with the control (Figure 6.15b).

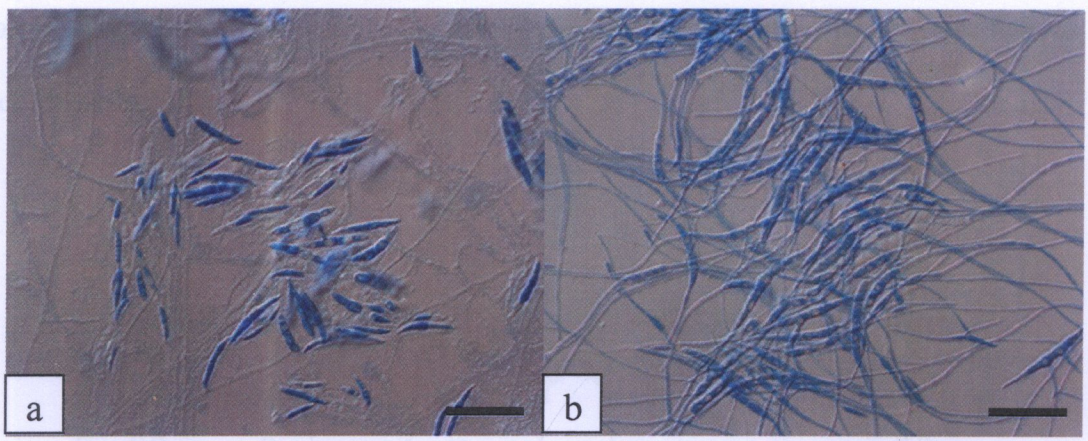


**Figure 6.15** Morphological changes of *F. oxysporum* mycelium after treatment with crude chitinase of *E. coli* JM109/pChi40_Sau (a) and 5 min-boiled crude chitinase of *E. coli* JM109/pChi40_Sau (b) for 12 h at 37°C. Bar = 10 µm.

## 6.7 Purification of expressed chitinase from *E. coli* JM109/pChi40_Sau

Chitinase activity of *E. coli* JM109 carrying pChi40_Sau was measured after overnight growth in LB at 37°C and 3 h adding 1 mM IPTG. The chitinase obtained in LB culture as a crude enzyme was partially purified. The crude enzyme was concentrated by precipitation with 80% $(NH_4)_2SO_4$ and was further subjected to Sephadex G-75 column purification. Four protein peaks were obtained; of these, only peak b showed chitinase activity (Figure 6.16). The other protein peaks were devoid of chitinase activity.

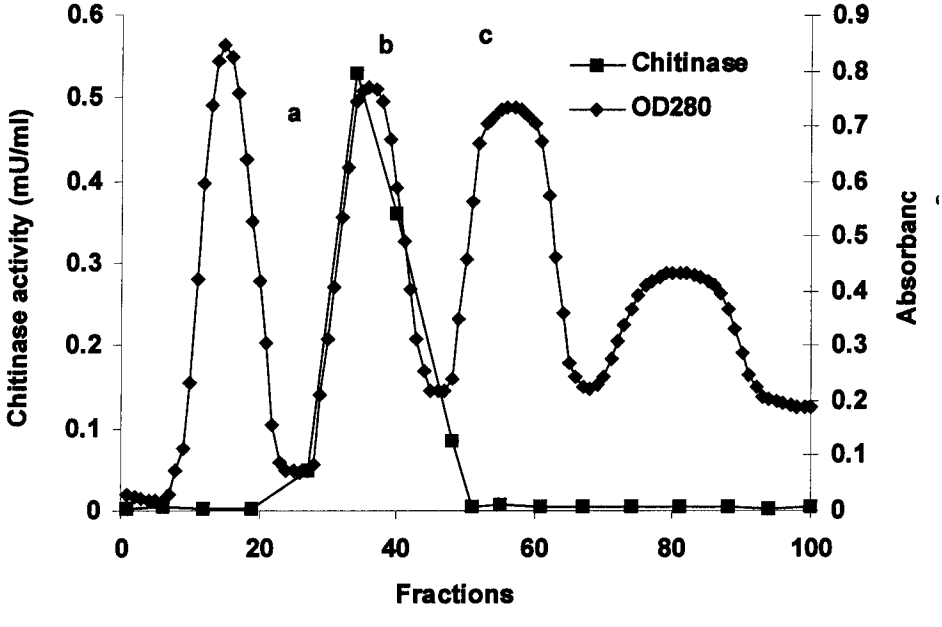


**Figure 6.16** Chitinase activity of different protein fractions obtained on Sephadex G-75 gel chromatography from crude chitinase of *E. coli* JM109/pChi40_Sau.

The crude and purified chitinase of *E. coli* JM109/pChi40_Sau, when applied to a plate diffusion method of *F. oxysporum* culture, caused the inhibition of growth (Figure 6.17I and II, respectively).

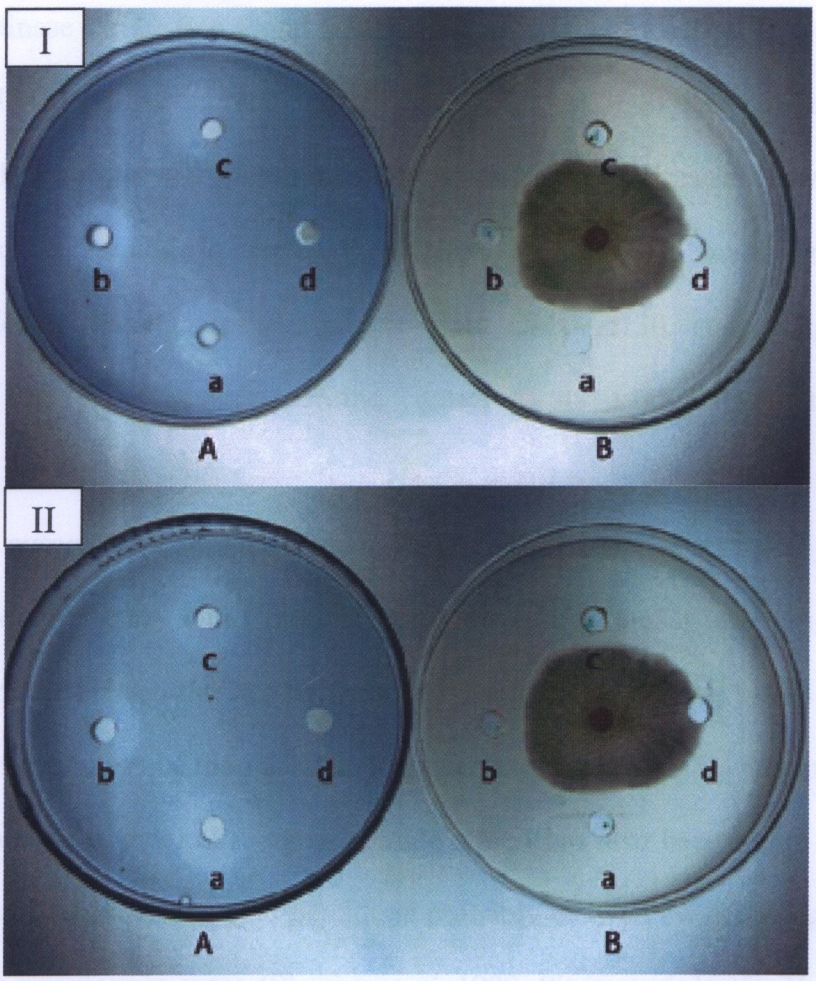


**Figure 6.17** Effects of crude (I) and purified (II) chitinase of *E. coli* JM109/pChi40_Sau on inhibition of *F. oxysporum* growth (B), and chitinolytic activity on colloidal chitin agar (A) with (a) 5 mg $ml^{-1}$, (b) 2.5 mg $ml^{-1}$, (c) 1.25 mg $ml^{-1}$ of chitinase and (d) 5 mg $ml^{-1}$ of 5 min-boiled chitinase.

**Discussion**

The recombinant strain *E. coli* JM109/pChi40_Sau, expressing the *S. aureofaciens* chitinase gene, exhibited chitinolytic activity as determined by the release of *N*-acetylglucosamine from colloidal chitin. The chitinase produced by strain JM109/pChi40_Sau was resolved by SDS-PAGE as a single band corresponding in size to the chitinase Chi40_Sau found in strain *S. aureofaciens* CMUAc130. Contrary to strain CMUAc130, the recombinant *E. coli* JM109/pChi40_Sau strain did not require colloidal chitin in the growth medium to produce the chitinase. However, the chitinolytic activity found in secreted proteins of the recombinant strain was significantly lower than that of the parent strain CMUAc130, suggesting higher expression of the *Chi40* gene under the control of its original promoter *in S. aureofaciens* CMUAc130 cells.

The *S. aureofaciens* chitinase gene characterized in this paper has high DNA sequence homology (95%) with Chi40 encoding the chitinase of *S. thermoviolaceus* OPC-520. Identity between the two chitinases at the amino acid level was 87%. The predicted molecular mass of the 43 KDa precursor included a 3 KDa fragment (30-amino-acid leader sequence) of a signal peptide which has also been found in some other bacteria chitinases. The signal peptide is probably cleaved away during protein transport to the periplasmic space (Shapira *et al.* 1989, Sitrit *et al.* 1995). Hence, the size of the mature Chi40_Sau protein (40 KDa) estimated by amino acid sequence analysis is very close to the apparent molecular mass of 40 KDa estimated by SDS-PAGE.

A database search showed that the similarity between currently known bacterial chitinases correlates with the taxonomic position of *S. aureofaciens*. Thus,

the chitinases of *S. aureofaciens*, *S. plicatus*, *S. lividans* and *S. thermoviolaceus* are closer to those from *B. circulans* than to those of *Alteromonas* spp. (a group of aerobic marine bacteria) or to those of the Gram-negative *S. marcescens* (Figure 6.11). These comparisons suggest that the levels of diversity between various chitinases correlate with the evolutionary distances between the bacteria that produce them. The type-III homology unit of fibronectin found in Chi63 and ChiA does not exist in the amino acid sequence of Chi40_Sau. The fibronectin-like sequence is also not found in *Chi40* from *S. thermoviolaceus* OPC-520 (Tsujibo *et al.* 1993a) and Chitinase gene from *S. erythraeus* (Kamei *et al.* 1989). These results indicate that the fibronectin type-III sequence is not essential for chitinase activity. The amino acid sequence homology could be found between Chi40_Sau and Chi40 from *S. thermoviolaceus* OPC-520 except for a short region which may be involved in the heat stability. Although the amino acid sequence of Chi40_Sau is very similar to that of Chi40 from *S. thermoviolaceus* OPC-520, the heat stability of Chi40_Sau is clearly different from that of Chi40 from *S. thermoviolaceus* OPC-520. It was reported that Chi40 from *S. thermoviolaceus* OPC-520 is stable when heated in 50 mM Tris-HCl (pH 8.0) at 80°C for 30 min (Tsujibo *et al.* 1993a). In contrast, Chi40_Sau showed 25% remaining activity after being heated in the same solution at 55°C for 30 min but was absolutely inactivated when heated at 65°C for 1 min. These results indicate that a short region of amino acid sequence which is different from Chi40 of *S. thermoviolaceus* OPC-520 may be involved in heat stability. One of the regions (amino acid 156-158 of Chi40_Sau) contains an Asp and Glu present in the active site of lysozyme (Metraux *et al.* 1989). These two portions of Chi40_Sau may therefore constitute the catalytic sites of *Chi*, as described in several papers (Kuranda and

Robbins, 1991; Watanabe *et al.* 1992; Tsujibo *et al.* 1993a). Furthermore, the clusters of identical amino acid indicate that the genes probably evolved from a common ancestor.

Most of the cloned chitinase enzyme was not secreted into growth medium, but accumulated in the periplasmic space ( Robbins *et al.*, 1988; Tsujibo *et al.*, 1993b). In contrast the *S. aureofaciens* CMUAc130 chitinase is mostly detected in the culture medium when it was cloned in *E. coli*. This experiment indicated that the signal peptide could be functional in *E. coli*, This has been noted in previous reports by Tsujibo *et al.* (2000) and Li *et al.* (2000). The chitinolytic activity found in the culture medium of the transformant JM109/pChi40_Sau was active in the hydrolysis of the colloidal chitin with 1 mM IPTG but very low in the absence of IPTG. These data indicate that the chitinase was regulated by the *lac*Z promoter.

*E. coli* JM109/pChi40_Sau, expressing the *S. aureofaciens Chi40* gene, acquired the ability to suppress growth of *C. musae* and *F. oxysporum* and their spore gemination *in vitro*. However, the transformant effected less significant fungal suppression in vitro than did the parent strain CMUAc130. This difference can be explained not only by the lower level of chitinolytic activity secreted by the transformant (probably because the *ChiA* gene was cloned under the control of the relatively weak *lac* promoter) but also by the fact that the parent strain in addition to the chitinase has been found to produce other chitinolytic enzymes and other antifungal substances (Taechowisan *et al.* 2003). Because of this activity has been found to be important for the biocontrol of *C. musae* and *F. oxysporum* by strain CMUAc130 (Taechowisan *et al.* 2003).

The results presented in this work confirm the role of chitinases in the antifungal activity of various microbial antagonists (Ordentlich *et al.* 1988; Shapira *et al.* 1989; Lorito *et al.* 1993; Tsujibo *et al.* 2000). The *S. aureofaciens* CMUAc130 chitinase gene could therefore be used to transform other bacteria or plants and provide them with the ability to control fungal phytopathogens. And the application of *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40_Sau to soil or plant infested with *F. oxysporum* should be done elsewhere.

# CHAPTER VII

# GENERAL DISCUSSION AND CONCLUSION

Actinomycetes constitute a significant component of the microbial population in most soils. They produce many extracellular enzymes in soil. By decomposing complex mixtures of polymers in dead plant, animal and fungi material (McCarthy and Williams 1992; Chen *et al.*, 1991; Wang *et al.*, 1989), they are important in soil biodegradation by the recycling of nutrients associated with recalcitrant polymers. Currently, there is no convincing evidence for the existence of endophytic actinomycetes, but it has long been recognized that actinomyctes in root nodules of certain shrubs and trees could colonize their tissues (Backer *et al.*, 1980; Knowlton *et al.*, 1980). The isolation of the endophytic actinomycetes was made according to published reports (Okazaki *et al.*, 1995; Sardi *et al.*, 1992; Shimizu *et al.*, 2000) and stimulated study of various aspects of the biology of these interesting and important endophytic actinomycetes and explore their antimicrobial potential of these actinomycetes against phytopathogenic fungi.

In this study, most isolates of endophytic actinomycetes were obtained from the root tissues. *In vitro* assay they formed a clear growth-inhibition zone against two phytopathogenic fungi, *Colletotrichum musae* and *Fusarium oxysporum*, indicating that these isolates can produce antifungal material(s). However a large number of endophytic actinomycetes isolates had no clear antifungal activity against the phytopathogenic fungi. As stated in several reports, actinomycete activity in plants not only protects against pathogens, but also the influence their metabolic products on

plant growth and physiology (Katznelson and Cole, 1965; Mishra *et al.*, 1987). Further investigations are therefore necessary to understand the other forms of relationship between endophytic actinomycetes and plants tissues and the usefulness of this phenomenon in agriculture. However, it needs more investigation to disclose the possibility that these endophytic actinomycetes could be used for biocontrol of the related plant diseases by introducing them into the crops such as wheat.

Chitinase (EC 3.3.1.14), the enzyme that degrades chitin, are widely distributed in nature and play an important role in the degradation of chitin. They serve a variety of other functions including morphogenesis, defense and have been reported in different microorganisms, including *Streptomyces*. Due to their chitinolytic abilities, they have been shown to play a role in the degradation of chitin from fungal cell wall. Since the endophytic actinomycetes were isolated from the selected plants which are known to have endophytes rich in biodiversity, most of the isolated endophytic actinomyce colonies growth on colloidal chitin agar were apparently chitinolytic since they formed a clearing zone. The endophytic actinomycetes that were able to degrade colloidal chitin on colloidal chitin agar comprised 56.35 % of the endophytic actinomycete isolates. The strain identified as *Streptomyces aureofaciens* CMUAc130 was the most effective in chitinase production and antifungal activity. This isolate was selected for a more detailed study of chitinase production, its effectiveness in fungal cell wall lysis and the potential in growth inhibition of *C. musae* and *F. oxysporum* by ethyl acetate extracts of its fermented broth.

There have been several attempts to explain the antagonistic action of *S. aureofaciens* CMUAc 130 chitinase against phytopathogenic fungi either by fungal

cell wall lysis or by inhibition of hyphal extension inhibition and inhibition of spore germination. These results indicate that in fact all types of these treatments are involved in antagonistic action of phytopathogenic fungi growth.

Chitinase production by *S. aureofaciens* was rather low amount. It is about 0.09 mU/ml from the culture filtrate. The chitinase gene from *S. aureofaciens* CMUAc 130 was cloned and over-expressed in *E. coli* JM109. Its yield was rather high amount. It is about 0.28 U/ml. The PCR cloning of the chi40_Sau gene encoding chitinase was pursued to address its function and to ascertain the antifungal activity, as well as to compare with chitinase from the parent strain for fungal biocontrol testing.

The recombinant chitinase that over-expressed from *E. coli* JM109 has a potential in antagonistic action similar to the purified chitinase from *S. aureofaciens* CMUAc 130. The purified chitinase of *S. aureofaciens* CMUAc130 and the recombinant chitinase were shown to inhibit spore germination by *F. oxysporum*. This chitinase (5 U) caused bursting of the spores and germinating hyphal tips of *F. oxysporum* after 24 h of incubation at 30°C. Gel filtration chromatography on a total extracellular protein sample from *S. aureofaciens* CMUAc 130 and *E. coli* JM109 containing pChi40_Sau showed the presence of a single enzyme. The molecular size of the protein was calculated to be 40 KDa by SDS-PAGE. This result would suggest that this enzyme occurs as a monomer. The chitinolytic enzyme produced by *S. aureofaciens* CMUAc130 is similar size to a similar enzyme from *Streptomyces lividans* (Miyashita and Fujii 1993) and *Streptomyces thermoviolaceus* (Tsujibo et al., 1993a) which have a calculated molecular size of 40 KDa. However, it is smaller than the chitinolytic enzyme isolated from other strain of *Streptomyces plicatus* (Robbins

*et al.*, 1988) and *Streptomyces olivaceoviridis* (Li *et al.*, 2000), which have molecular weights about 63 and 92 KDa, respectively.

However both chitinase (chitinase from the fermented broth of *S. aureofaciens* CMUAc 130 and over-expression of the *S. aureofaciens* CMUAc 130 chitinase from *E. coli* JM109) showed the antagonistic action less than the crude cultured of *S. aureofaciens* CMUAc 130. This results indicated the crude culture may has the other antifungal substances that showed synergism from the biocontrol phytopathogenic fungi. The chitinase activity may affect the target fungus by weakening the cell-wall structure which would facilitate the diffusion of fungitoxic metabolites of crude culture into the cell. Therefore, Endophytic *S. aureofaciens* CMUAc130 with a broad antifungal spectrum both chitinase and other antifungal substances should be considered as a valuable candidate of biocontrol agent of some plant diseases. A unique, effective use of endophytic *S. aureofaciens* CMUAc130 for disease control of *in vivo* tissue-cultured seedlings of some plants would be investigated to confirm the *in vitro* positive antagonism.

## REFERENCES


Abd-Allah, E.F. (2001). *Streptomyces plicatus* as a model biocontrol agent. *Fol. Microbiol.* 46: 309-314.

Altschul, S.F., Madden, T.L. and Schaffer, A.A. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.

Alwadi, H.M. and Baka, Z.A. (2001). Microorganisms associated with *Withania somnifera* leaves. *Microbiol. Res.* 156: 303-309.

Amann, E., Brosius, J. and Ptashne, M. (1983). Vector bearing a hybrid *trp-lac* promoter useful for regulated expression of cloned gene in *Escherichia coli*. *Gene* 25: 167-178.

Armand, S., Tomita, H., Heyraud, A., Gey, C., Watanage, T. and Henrissat, B. (1994). Stereochemical course of the hydrolysis reaction catalyzed by chitinases a1 and D from *Bacillus circulans* WL-12. *FEMS Lett.*, 343: 177-180.

Bade, M.L. and Hickey, K. (1988). Classification of enzymes hydrolyzing chitins. In: 4th International Conference on Chitin and Chitosan (Trundheim, 1988) (eds: S.B. Gudmund, T. Anthonsen and P. Sandford) pp. 835. London: Elseveir Applied Science.

Bai, Y., Aoust, F.D., Smith, D.L. and Driscoll, B.T. (2002). Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Can. J. Microbiol.* 48: 230-238.

Baker, D., Newcomb, W. and Torrey, J.G. (1980). Characterization of an ineffective

actinorhizal microsymbiont, *Frankia* sp. Eu11 (Actinomycetes). *Can. J. Microbiol.* 26: 1072-1089.

Baladaris-Inglis, V., Cheria, M., Peter, I.W. and Peberdy, J.F. (1997). Sequence analysis of the catalytic domain of *Metarhizium anisopiliae* chitinase. *Braz. J. Genet.* 20: 161-164.

Becker, B., Lechevalier, M.P., Gordon, R.E. and Lechevalier, H.A. (1964). Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. *Appl. Microbiol.* 12: 421-423.

Benyagoub, M., Benhamou, N. and Carisse, O. (1998). Cytochemical investigation of antagonistic interaction between a *Microsphaeropsis* sp. (isolate P130A) and *Venturia inaequalis*. *Phytopathol.* 88: 605-613.

Berger, L.R. and Reynolds, D.M. (1958). The chitinase system of a strain of *Streptomyces griseus*. *Biochem. Biophys. Acta* 29: 522-534.

Beyer, M. and Diekmann, H. (1985). The chitinase system of *Streptomyces* sp. ATCC11238 and its significance for fungal cell wall degradation. *Appl. Microbiol. Biotechnol.* 23: 140-146.

Binder, A. Ghose, T.K. (1978) Adsorption of cellulose by *Trichoderma viride*. *Biotechnol. Bioeng.* 19: 1187-1199.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant DNA. *Nucleic Acid Res.* 7: 1513-1523.

Blaak, H., Schellmann, J., Walter, S., Henrissat, B. and Schrempf, H. (1993). Characteristics of an exo-chitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitnases. *Eur. J. Biochem.* 214: 659-669.

Blaak, H. and Schremph, H. (1995). Binding and substrate specificities of a *Streptomyces olivaceoviridis* chitinase in comparison with its proteolytically processed form. *Eur. J. Biochem.* 229: 132-139.

Blaiseau, P.L., Kunz, C., Grison, R., Bertheau, Y. and Brygoo, Y. (1992). Cloning and expression of chitinase gene from fungi *Aphanocladium album. Curr. Genet.* 21: 61-66.

Boone, C.J. and Pine, L. (1968). Rapid method for characterization of actinomycetes by cell wall composition. *Appl. Microbiol.* 16: 279-284.

Bork, P. and Doolittle, R. F. (1992). Proposed acquisition of an animal protein domain by bacteria. *Proc. Natl. Acad. Sci. USA*, 89: 8990-8994.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli. Proc. Natl. Acad. Sci. USA.* 75: 4801-4805.

Burberg, M.B., Haandrikman, A.J., Lecnhouts, K.J., Venema, G. and Nes, I.F. (1994). Expression of chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum. Appl. Microbiol. Biotechnol.* 2: 108-115.

Bussaban, B., Lumyong, S., Lumyong, P., McKenzie, E.H.C. and Hyde, K.D. (2001). Endophytic fungi from *Amomum siamense. Can. J. Microbiol.* 47: 1-6.

Campbell, R. (1983). Ultrastructural studies of *Gaeumannomyces graminis* in the water films on wheat roots and the effect of clay on the interaction between this fungus and abtagonistic bacteria. *Can. J. Microbiol.* 29: 39-45.

Carsolio, C., Gutierrex, A., Jimenez, B., Van, M.M. and Herrera-Estrella, A. (1994). Characterization of ech-42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proc. Natl. Acad. Sci. USA.* 91: 10903-10907.

Clarke, P. (1956). The occurance of chitinase in some bacteria. *J. Gen. Microbiol.* 14: 188-196.

Chen, J.P., Nagayama, F. and Chang, M.C. (1991). Cloning and expression of a chitinase gene from *Aeromonas hydrophila* in *Escherichia coli. Appl. Environ. Microbiol.* 57: 2426-2428.

Chernin, L., Ismailov, Z., Haran, S. and Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ. Microbiol.* 61: 1720-1726.

Cohen, E. (1993). Chitin synthesis and degradation as targets for pesticide action. *Arch. Insect Biochem. Physiol.* 22: 245-261.

Crawford, D.L., Lynch, J.M., Whipps, J.M. and Ousley, M.A. (1993). Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appl. Environ. Microbiol.* 59: 3899-3905.

Cross, T. and Attwell, R.W. (1975). Actinomycete spores. In: Spores VI (eds: P. Gerhardt, R.N. Costilow and H.L. Sagdoff) pp. 3-14. Washington D.C.: American Society for Microbiology.

Cross, T. and Goodfellow, M. (1973). Taxonomy and classification of the actinomycetes. In: Sykes, G., Skinner, F.A. (eds.). Actinomycetales. London: Academic Press. pp. 76.

Csaba, F., Tamas, W. and Laszle, H (1996). Assignment of PCR-amplified chitinase

sequence cloned from *Trichoderma harzianum* to resolved chromosomes of potential biocontrol species of *Trichoderma*. *FEMS Microbiol. Lett.* 145: 385-391.

Davies, G. and Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure* 3: 853-859.

Delic, L., Robbins, P. and Westpheling, J. (1992). Direct repeat sequences are implicated in the regulation of two *Streptomyces* chitinase promoters that are subject to carbon catabolite control. *Proc. Natl. Acad. Sci. USA*. 89: 1885-1889.

Doi, R.H. (1991). Regulation of gene expression. In: Modern Microbial Genetics (eds: U.N. Streips and R.E. Yasbin) pp.15-39. USA: John-Wiley and Sons Inc.

Drautz, H. and Zahner, H. (1986). New microbial metabolites. In: Biological, biochemical and biomedical aspects of actinomycetes (eds: G. Szabo, S. Biro and M. Goodfellow) pp. 227-234. Akademiai Kiado.

Embley, T.M. and Stackebrandt, E. (1994). The molecular phylogeny and systematics of the actinomycetes. *Ann. Rev. Microbiol.* 48: 257-289.

Ensign, J.C. (1978). Formation. properties. and germination of actinomycete spores. *Appl. Environ. Microbiol.* 53: 2793-2799.

Fayad, K.P., Simao-Beaunoir, A.M., Gautheir, A., Leclerc, C., Mamady, H., Beaulieu, C. and Brzezinski, R. (2001). Purification and properties of a β-1,6-glucanase from *Streptomyces* sp. EF-14, an actinomycete antagonistic to *Phytophthora* spp. *Appl. Microbiol. Biotechnol.* 57: 117-123.

Felse, P.A. and Panda, T. (1999). Regulation and cloning of microbial chitinase genes. *Appl. Microbiol. Biotechnol.* 51: 141-151.

Felsenstein, J. (1995). PHYLIP (phylogenetic inference package) version 3.57c. Department of Genentics, University of Washington, Seattle, WA, USA.

Fisher, P.J., Anson, A.E. and Petrini, O. (1984). Antibiotic activity of some endophytic fungi from ericaceous plants. *Bot. Helv.* 94: 249-253.

Fisher, P.J., Anson A.E. and Petrini, O. (1986). Fungal endophytes in *Ulex europaeus* and *Ulex galli. Trans. Brit. Mycol. Soc.* 86: 153-156.

Flach, J., Pilet, P.E. and Jolles, P. (1992). What's new in chitinase research? *Experientia.* 48: 701-716.

Fravel, D.R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annu. Rev. Phytopathol.* 26: 75-91.

Fuchs, R.L., Mepherson, S.A. and Drahos, D.J. (1986). Cloning of *Serratia marcescens* gene encoding chitinase. *Appl. Environ. Microbiol.* 51: 504-509.

Fujii, T. and Miyashita, K. (1993). Multiple domain structure in a chitinase gene (chiC) of *Streptomyces lividans. J. Gen. Microbiol.* 139: 677-686.

Gacto, M., Vicente-Soler, J., Cansado, J. and Villa, T.G. (2000). Characterization of an extracellular enzyme system produced by *Micromonospora chalcea* with lytic activity on yeast cells. *J. Appl. Microbiol.* 88: 961-967.

Garbera, P., Overbeek, L.S., Vuurde, J.W. and Elsas, J.D. (2001). Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16SrDNA based PCR fragments. *Microb. Ecol.* 41: 369-383.

Getha, K. and Vikineswary, S. (2002). Antagonistic effects *of Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f.sp. *cubense* race 4: Indirect evidence for the role of antibiosis in the antagonistic process. *J.*

*Indust. Microbiol. Biotechnol.* 28: 303-310.

Gomes, R.C., Semedo, L.T., Soares, R.M., Alviano, C.S., Linhares, L.F. and Coelho, R.R. (2000). Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. *Lett. Appl. Microbiol.* 30: 146-150.

Gomes, R.C., Semedo, L.T., Soares, R.M., Linhares, L.F., Ulhoa, C.J., Alviano, C.S., and Coelho, R.R. (2001). Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *J. Appl. Microbiol.* 90: 653-661.

Gooday, G.W. (1990). The ecology of chitin decomposition. *Adv. Microb. Ecol.*, 11: 387-430.

Gooday G.W., Wei-yun Z. and O'Donnell R.W. (1992). What are the roles of chitin and chitosan. *Biodegradation* 1: 177-190.

Goodfellow, M. (1989). Suprageneric classification of actinomycetes. In: Bergey's Manual of Systematic Bacteriology, Vol. 4 (ed: S.T. Williams) pp. 2333-2339. Williams and Wikins, Baltimore.

Gopalakrishnan, B., Muthukrishnan, S. and Kramer, K.J. (1995). Baculovirus-mediated expression of a *Mandusa sexta* chitinase gene: Properties of the recombinant protein. *Insect Biochem. Molec. Biol.* 25: 255-265.

Gordon, R.E. (1966). Some strains in search of a genus *Corynebacterium*, *Mycobacterium*, *Nocardia* or what? *J. Gen. Microbiol.* 43: 329-343.

Gunaratna, K.R. and Balasubramanian, R. (1994). Partial purificationand properties of extracellular chitinase produced by *Acremonium obclavatum*, an antagonist to the groundnut rust, *Puccinia arachidis*. *World J. Microbiol. Biotechnol.* 10: 342-345.

Gunji, S., Arima, K. and Beppu, T. (1983). Screening of antifungal antibiotics according to activities inducing morphological abnormalities. *Agric. Biol. Chem.* 47: 2061-2069.

Gupta, R., Saxena, R.K., Chaturvedi, P. and Virdi, J.S. (1995). Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. *J. Appl. Bacteriol.* 78: 378-383.

Gurney, K.A. and Mantle, P.G., (1993). Biosynthesis of 1-*N*-methylalbonoursin by an endophytic *Streptomyces* sp. isolated from perennial ryegrass. *J. Nat. Prod. (Lloydia)* 56: 1194-1198.

Habsah, M., Amran, M. and Mackeen, M.M. (2000). Screening of *Zingiberaceae* extracts for antimicrobial and antioxidant activities. *J. Ethnopharmacol.* 72: 403-410.

Hadar, Y., Harman, G.E., Taylor, A.G. and Norton, J.M. (1983). Effects of pregerminating of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. *Phytopathol.* 73: 1322-1325.

Haima, P., Bron, S. and Venema, G. (1990). Novel plasmid marker rescue transformation system for molecular cloning in *Bacillus subtilis* enabling direct selection of recombinants. *Mol. Gen. Genet.* 223: 185-191.

Hajlaou, M.R., Traquair, W.R., Jarvis, W.R. and Belanger, R.R. (1994). Antifungal activity of extracellular metabolites produced by *Sporothrix flocculosa. Biocontrol Sci. Technol.* 4: 229-237.

Hannig, G. and Makridges, S. (1998). Strategies for optimizing heterologous protein expression in *Escherichia coli. Trends Biotechnol.* 16: 54-60.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press. New York.

Harwood, C.R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnol.* 10: 247-256.

Henrisat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280: 309-316.

Henrissat, B. and Bairoch, Q. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293: 781-788.

Higgins, D.G., Bleasby, A.J. and Fuchs, R., (1992). CLUSTAL V: improved software for multiple sequence alignment. *Comput. Appl. Biosci.* 8: 189-191.

Holmes, D.S. and Quigley, M. (1988). A rapid boiling method for a preparation of bacterial plasmids. *Anal. Biochem.* 76: 141-193.

Hopwood, D.A., Bibb, M.J. and Chater, K.F. (1985). Preparation of Chromosomal, Plasmid and Phage DNA. In: Genetic manipulation of *Streptomyces*: a laboratory manual. (eds: D.A. Hopwood, M.J. Bibb and K.F. Chater) pp. 79-80. Norwich: F. Crowe and Sons.

Huber, M., Cabib, E. and Miller, L.H. (1991). Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proc. Natl. Acad. Sci. USA.* 88: 2807-2817.

Hsu, S.C. and Lockwood, J.L. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Appl. Microbiol.* 29: 422-426.

Irene, G., Jose, M.L., De la Cruz, J., Tahia, B., Antonio, L., Jose, A. and Pintor, T. (1994). Cloning and characterization of chitinase (Chit 42) from mycoparasitic

fungus *Trichoderma harzianum. Curr. Genet.* 27: 83-89.

Iseli, B., Armand, S., Boller, T., Neuhaus, J.M. and Henrissat, B. (1996). Plant chitinases use two different hydrolytic mechanisms. *FEBS Lett.* 383: 186-188.

Jannatipour, M., Soto-Gil, R.W., Childers, L.C. and Zyskind, J.W. (1987). Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli. J Bacteriol.* 169: 3785-3791.

Jeuniaux, C. (1966). Chitinase. *Methods Enzymol.* 8: 645-650.

Jones, J.D.G., Grady, K.L., Suslow, T.V., and Bedbrook, J.R. (1986). Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens. EMBO J.*, 5: 467-473.

Jones, R.K. (1985). Fungicides for bedding plants. *Bedding Plants Inc. News* 16: 3-4.

Joshi, S. and Kozlowski, M. (1986). Cloning of *Serratia liquefaciens* chitinases genes. In: Proceedings of the 3rd International Symposium on Molecular Genetics and Plant Microbial Interaction (eds: D.P.S. Verma, N.N. Brisson) pp 328-330. Dordrecht, Netherlands.

Joshi, S., Kozlowski, M. and Suriovicz, M. (1987). Chitinase and chitobiase overproduction from genetically engineered *Serratia liquefaciens. Prog. Biotechnol.* 3: 95-99.

Joshi, S., Kozlowski, M., Selvaraj, G., Iyer, V.N. and Davies, R.W. (1988). Cloning of genes of chitin utillzation regulation of *Serratia liquefaciens. J. Bacteriol.* 170: 2984-2988.

Joshi, S., Kozlowski, M., Richen, S. and Comberbach, D.M. (1989). Chitinase and chitibiose production during fermentation of genetically improved *Serratia liquefaciens. Enzyme Microb. Technol.* 11: 289-296.

Kamei, K., Yamamura, Y., Hara, S., and Ikenaka, T. (1989). Amino acid sequence of chitinase from *Streptomyces erythraeus*. *J. Biochem.*, 105: 979-985.

Kampfer, P., Kroppenstedt, R.M. and Dott, W. (1991). A numerical classification of the genera *Streptomyces* and *Streptoverticillium* using miniaturised physiological tests. *J. Gen. Microbiol.* 137: 1831-1891.

Karagouni, A.D., Vionis, A.P., Baker, P.W. and Wellington, E.M.H. (1993). The effect of soil moisture content on spore germination, mycelium development and survival of a seeded *Streptomyces* in soil. *Microbiol.* 2: 47-51.

Kataoka, M., Ueda, K., Kudo, T., Seki, T. and Yoshida, T. (1997). Application of the variable region in 16S rDNA to create an index for rapid species identification in the genus *Streptomyces*. *FEMS Microbiol. Lett.* 151: 249-255.

Katznelson, H. and Cole, S.E. (1965). Production of gibberellin-like substances by bacteria and actinomycetes. *Can. J. Microbiol.* 11: 733-741.

Kiyotaku, M., Takeshi, F., Aiko, W. and Hideto, U. (1997). Nucleotide sequence and expression of a gene (*chiB*) for chitinases from *Streptomyces lividans*. *J. Ferment. Bioeng.* 83: 26-31.

Knowlton, S., Berry, A. and Torrey, J.G. (1980). Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. *Can. J. Microbiol.* 26: 970-977.

Kolbe, S., Fischer, S., Becirevic, A., Hinz, P. and Schrempf, H. (1998). The *Streptomyces reticuli* α-chitinbinding protein Chb2 and its gene. *Microbiol.* 144: 1291-1297.

Kuranda, M.J. and Robbins, P.W. (1987). Cloning and heterologous expression of glycosidase gene from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.*

84: 2585-2589.

Kuranda, M.J., and Robbins, P.W. (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 266: 19758-19767.

Kweon, J.P., Takeshi, O., Yukikazu, Y., Katsunori, T., Tsuyoshi, N., Makoto, K. and Hideyuki, M. (1997). Molecular cloning nucleotide sequencing and regulation of *chiA* gene encoding one of chitinases from *Enterobacter* sp. G-1. *J. Ferment. Bioeng.* 84: 493-501.

Labeda, D.P. (1992). DNA-DNA hybridization in the systematics of *Streptomyces*. *Gene* 115: 249-253.

Labeda, D.P. and Lyons, A.J. (1991). Deoxyribonucleic acid relatedness among species of the *Streptomyces cyaneas* cluster. *System. Appl. Microbiol.* 14: 158-161.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277: 680-685.

Leblond, P., Redenbach, M. and Cullum, J. (1993). Physical map of the *Streptomyces lividans* 66 genome and comparison with that of the related strain *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 175: 3422-3429.

Lechevalier, H.A. and Lechevalier, M.P. (1965). Classification desactinomycetes aerobies basees sur leur morphologie et leur composition chimique. *Annales de l'Institut Pasteur* 108: 662 - 673.

Lechevalier, H.A. and Lechevalier, M.P. (1967). Biology of the actinomycetes. *Annual Rev. Microbiol.* 21: 71-100.

Lechevalier, H.A. Lechevalier, M.P. and Gerber, N.N. (1971). Chemical composition

as a criterion in the classification of actinomycetes. *Adv. Appl. Microbiol.* 14: 47-72.

Lechevalier, H.A., Lechevalier, M.P., Handley, D.A., Ghosh, B.K. and Carmichael, J.W. (1977). Strains of fusidia which can be mistaken for actinomycetes. *Mycologia.* 69: 81-95.

Lechevalier, H.A. (1968). Identification of aerobic actinomycetes of clinical importance. *J. Lab. Clin. Med.* 71: 934-944.

Lechevalier, M.P., Lechevalier, H.A. and Horan, A.C. (1973). Chemical characteristics and classification of *Nocardiae. Can. J. Microbiol.* 19: 965-972.

Leonid, S.C., Leonardo, F., Valodinin, S. and Shoshan, H. (1997). Molecular cloning, structural analysis and expression in *E. coli* of a chitinase gene from *Enterobacter agglomerans. Appl. Environ. Microbiol.* 63: 834-839.

Li, H., Plattner, H., Schimz, K.L., Kieb, M., Diekmann and Meens J. (2000). Cloning, sequencing and heterologous expression of a new chitinase gene, *chi92*, from *Streptomyces olivaceoviridis* ATCC11238. *Biotechnol. Lett.* 22: 1203-1209.

Li, X.M., Novotna, J., Vohradsky, J. and Weiser, J. (2001). Major proteins related to chlortetracycline biosynthesis in a *Streptomyces aureofaciens* production strain studied by quantitative proteomics. *Appl. Microbiol. Biotechnol.* 57: 717-724.

Lim, H.S., Kim, Y.S. and Kim, S.D. (1991). *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. *Appl. Environ. Microbiol.* 57: 510-516.

Lima, L.H., De Marco, J.L., Ulhoa, C.J. and Felix, C.R. (1999). Synthesis of a

*Trichoderma* chitinase which affects the *Sclerotium rolfsii* and *Rhizoctonia solani* cell walls. *Folia Microbiol.* 44: 45-49.

Limon, M.C., Lora, J.M., Irene, G., Cruz, J., dela Llobell, A., Benitez, T. and Pintor, J.A. (1995). Primary structure and expression pattern of the 33-KDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. *Curr. Genet.* 28: 478-483.

Lin, C.S., Chen, H.C. and Lin, F.P. (1997). Expression and characterization of the recombinant gene encoding chitinase from *Aeromonas caviae*. *Enzyme Microb. Technol.* 21: 472-478.

Liu, C.H., Zou, W.X., Ren, H.L. and Tan, R.X. (2001). Antifungal activity of *Artemisia annua* endophyte cultures against ohytopathogenic fungi. *J. Biotechnol.* 88: 277-282.

Lloyd, A.B. (1969). Behaviour of *Streptomyces* in soil. *J. Gen. Microbiol.* 56: 165-170.

Lloyd, A.B., Noveroske, R.L. and Lockwood, J.L. (1965). Lysis of fungal mycelium by *Streptomyces* spp. and their chitinase systems. *Phytopathol.* 55: 871-875.

Lockwood, J.L. and Lingappa, B.T. (1963). Fungitoxicity of sterilized soil inoculated with soil microflora. *Phytopathol.* 53: 917-920.

Lorito, M., Di Pietro, A., Hayes, C.K., Woo, S.L., and Harman, G.E. (1993) Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. *Phytopathol.* 83: 721-728.

Lorito, M., Hayes, C.K., Zoina, A., Scala, F., Del Sorbo, G., Woo, S.L. and Haarman, G.E. (1994). Potential of genes and gene products from *Trichoderma* sp. and *Gliocladium* sp. for the development of biological pesticides. *Mol. Biotechnol.*

2:209-217.

Mahadevan, B., and Crawford, D.L. 1997. Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108. *Enzyme Microb. Technol.* 20: 489-493.

Mansour, F.A. and Mohamedin, A.H. (2001). *Candida albicans* cell wall lytic enzyme produced by *Streptomyces thermodiastaticus*. *Microbiol.* 105: 87-101.

Mark, B.L., Wasney, G.A., Saly, T.J.S., Khan, A.R., Cao, Z., Robbins, P.W., James, M.N.G. and Triggs-Raine, B.L. (1998). Structural and functional characterization of *Streptomyces plicatus* β-*N* acetylhexosaminidase by comparative mole-cularmedoling and site-directed mutagenesis. *J. Biol. Chem.* 273: 19618-19624.

Matashita, K., Fujii, T. and Sawada, Y. (1991). Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. *J. Gen. Microbiol.* 137: 2065-2072.

McCarthy, A.J. and Williams, S.T. (1992). Actinomycetes as agents of biodegradation in the environment. *Gene* 15: 189-192.

McGee, P.A., Hincksman, M.A. and White, C.S., (1991). Inhibition of growth of fungi isolated from plants by *Acremonium strictum*. *Aust. J. Agric. Res.* 42: 1187-1194.

Metraux, J.P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., William, S., Payne, G., Carnes, M., and Ryals, J. (1989). Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proc. Natl. Acad. Sci. USA*. 86: 896-900.

Miller, J.W. 1972. Experiments in molecular genetics. Cold Spring Harbor

Laboratory, Cold Spring Harbor, N.Y.

Mishra, S.K., Taft, W.H., Putnam, A,R. and Ries, S.K. (1987). Plant growth regulatory metabolites from novel actinomycetes. *J. Plant Grow. Regulat.* 6: 75-84.

Mitsutomi, M., Hata, T. and Kuwahara, T. (1995). Purificatikon and characterization of novel chitinases from *Streptomyces griseus* HUT6037. *J. Ferment. Bioeng.* 80: 153-158.

Miyashita, K., and Fujii, T. (1993). Nucleotide sequence and analysis of a gene (*ChiA*) for a chitinase from *Streptomyces lividans* 66. *Biosci. Biotechnol. Biochem.*, 57: 1691-1698.

Miyashita, K., Fujii, T., Sawada, Y., (1991). Molecular cloning and characterisation of chitinase genes from *Streptomyces lividans* 66. *J. Gen. Microbiol.* 137: 2065-2072.

Miyashita, K., Fujii, T., Watanabe, A. and Ueno, V. (1997). Nucleotide sequence and expression of a gene (*chiB*) for a chitinase from *Streptomyces lividans. J. Ferment. Bioeng.*, 83: 26-31.

Molly, C. and Burke, B. (1997). Expression and secretion of *Janthinobacterium lividans* chitinase in *Saccharomyces cerevisiae. Biotechnol. Lett.* 19: 1161-1164.

Monreal, J., Reese, E.T. (1969) The chitinase of *Serratia marcescens. Can. J. Microbiol.* 15: 689-696.

Morita, R.Y. (1985). Starvation an miniaturisation of heterotrophs, with special

emphasis on maintenance of the starved viable state. In: Bacteria in their Natural Environments. (eds: M. Fletcher and G.D. Floodgate) pp. 111-130. London: Academic Press.

Mortineer, P.S., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. (1981) The Prokaryotes: A handbook on habitats, isolation and identification of bacteria. Berlin: Springer-Verlag.

Neugebauer, E., Gamache, B., Dery, C.V. and Brzezinski, R. (1991). Chitinolytic properties of *Streptomyces lividans*. *Arch. Microbiol.*, 156: 192-197.

Ni, X. and Westpheling, J. (1997). Direct repeat sequences in the *Streptomyces* chitinase-63 promoter direct both glucose repression and chitin induction. *Proc. Natl. Acad. Sci. USA.* 94: 13116-13121.

Nishioka, M., Furuya, N., Nakashima, N. and Matsuyama, N. (1997). Antibacterial activity of metabolites produced by *Erwinia* spp. against various phytopathogenic bacteria. *Ann. Phytopathol. Soc. Jpn.* 63: 99-102.

O'Brien, M. and Colwell, R.R. (1987). A rapid test for chitinase activity that uses 4-methylumbelliferyl-*N*-Acetyl-β-D-glucosaminide. *Appl. Environ. Microbiol.* 53: 1718-1720.

Ohno, T., Armand, S., Hata, T., Nikaidou, N., Henrissat, B., Mitsutomi, M. and Watanabe, T. (1996). A modular family. 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT6037. *J. Bacteriol.* 178: 5065-5070.

Okazaki, T., Takahashi, K., Kizuka, M. and Enokita, R. (1995). Studies on actinomycetes isolated from plant leaves. *Ann. Rep. Sankyo Res. Lab.* 47: 97-106.

Oppenheim, A.B. and Chet, I. (1992). Cloned chitinase in fungal pathogen control

strategies. *Trends Biotechnol.* 10: 392-394.

Ordentlich, A., Elad, Y., and Chet, I. (1988). The role of chitinase of *Serratia marcescens* in biocontrol *Sclerotium rolfsii*. *Phytopathol.* 78: 84-87.

Otoguro, M. Hayakawa, M., Yamazaki, T. and Iimura, Y. (2001). An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter. *J. Appl. Microbiol.* 91: 118-130.

Peberdy, J.F. (1990). Fungal cell walls. In: A review: Biochemistry of cell walls and membranes in fungi. (eds: P.J. Kuhn, A.P.J. Trici, M.J. Jung, M.W. Goosey and L.G. Copping) pp. 5-30. London: Springer-Verlag.

Pegg, G.F. (1982). Chitinase from *Verticillium alboatrum*. In Sabato, G.D. (ed) Methods in Enzymology. Vol.161, Academic Press. London, pp. 474-479.

Petrolini, B., Quaroni, S. and Saracchi, M. (1986). Scanning electron microscopy investigations on the relationships between bacteria and plant tissues: Comparative techniques for specimen preparation. *Riv. Pathol. Veget.* S. 22, 7-15.

Primrose, S.B. (1991). Molecular Biotechnology. 2nd. Edn., Oxford: Blackwell Scientific Publications. 13-14.

Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H. and Hopwood, D.A. (1996). A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2X) chromosome. *Mol. Microbiol.* 21: 77-96.

Redshow, P.A., McCann, P.A., Sankaran, L. and Pogell, B.M. (1976). Control of differentiation in *Streptomyces*: involvement of extrachromosomal deoxyribonucleic acid and glucose repression in aerial mycelia development.

*J. Bacteriol.* 125: 698-705.

Reissig, J.L., Strominger, J.L., and Leloir, L.F. (1955). A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.*, 217: 959-966.

Richmond, D.V. (1975). Effects of toxicants on the morphology and fine structure of fungi. *Adv. Appl. Microbiol.* 19: 289-319.

Robbins, P.W., Albright, C. and Benfield, B. (1988). Cloning and expression of *Streptomyces plicatus* (Chitinase-63) in *Escherichia coli. J. Biol. Chem.* 263: 443-447.

Robbins, P.W., Overbye, K., Albright, C., Benfield, B. and Pero, J. (1992). Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. *Gene* 111: 69-76.

Robbins, P.W., Trimble, R.B., Wirth, D.F., Hering, C., Maley, F., Maley, G.F., Das, R., Gibson, B.W., Royal, N. and Biemann, K. (1984). Primary structure of the *Streptomyces* enzyme endo -β-*N*-acetylglucosaminidase H. *J. Biol. Chem.* 259: 2577-7583.

Roberts, W.K. and Selitrennikoff, C.P. (1986). Isolation and partial characterization of two antifungal proteins from barley. *Biochem. Biophys. Acta*, 880: 161-170.

Roffey, P.E. and Pemberton, J.M. (1990). Cloning and expression of an *Aeromonas hydrophila* chitinase gene in *Escherichia coli. Curr. Microbiol.* 21: 329-337.

Romaguera, A, Menge, U., Breves, R. and Dickmann, H. (1992). Chitinases of *Streptomyces olivaceoviridis* and significance of processing for multiplicity. *J. Bacteriol.* 174: 3450-3454.

Rothrock, C.S. and Gottlieb, D. (1984). Role of antibiosis in antagonism of

*Streptomyces hygroscopicus* var. *geldanus* to *Rhizoctonia solani* in soil. *Can. J. Microbiol.* 30: 1440-1447.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: A laboratory manual., 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Sahai, A.S. and Monacha, M.S. (1993). Chitinases of fungi and plants: their involvement in host parasite interaction. *FEMS Microbiol. Rev.* 11: 317-338.

Saito, A., Fujii, T., Yoneyama, T., Redenbach, M., Ohno, T., Watanabe, T. and Miyashita, K. (1999). High-multiplicity of chitinase genes in *Streptomyces coelicolor* A3(2). *Biosci. Biotechnol. Biochem.* 63: 710-8.

Sardi, P., Saracchi, M., Ouaroni, S., Petrolini, B., Borgonovoli, G.E. and Merli, S. (1992). Isolation of endophytic *Streptomyces* from surface-sterilized roots. *Appl. Environ. Microbiol.* 58: 2691-2693.

Schippers, B., Bakker, A.W. and Bakker, P.A.H.M. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Ann. Rev. Phytopathol.* 25: 339-358.

Schnellmann, J., Zeltins, A., Blaak, H. and Schrempf, H. (1994). The novel lectin-like protein Chb1 is encoded by a chitin-inducible *Streptomyces olivaceoviridis* gene and binds specifically to crystalline α-chitin of fungi and other organisms. *Mol. Microbiol.* 13: 807-819.

Selitrennikoff, C.P. (2001). Antifungal proteins. *Appl. Environ. Microbiol.* 67: 2883-2894.

Shapairo, M., Preisler, H.R. and Robertson, J.L. (1987). Enhancement of Baculovirus activity on gypsy moth (Lepidoptera: Lymantridae) by chitinase. *J. Econ. Entomol.* 80: 1113-1116.

Shapira, R., Ordentlich, A., Chet, I., and Oppenheim, A. (1989). Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathol.* 79: 1246-1249.

Shimizu, M., Nakagawa, Y., Sato, Y., Furumai, T., Igarashi, Y., Onaka, H., Yoshida, R. and Kunoh, H. (2000). Studies on endophytic actinomycetes (I) *Streptomyces* sp. isolated from Rhododendron and its antifungal activity. *J. Gen. Plant Pathol.* 66: 360-366.

Shirling, E.B. and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Inter. J. Sys.Bacteriol.* 16: 313-340.

Sitrit, Y., Vorgias, C.E., Chet, I., and Oppenheim, A. (1995). Cloning and primary structure of the *chiA* gene from *Aeromonas caviae*. *J. Bacteriol.*, 177: 4187-4189.

Skujins, J., Pukite, V. and McLaren, A.D. (1970). Chitinase of *Streptomyces* sp.: purification and properties. *Enzymologia* 39: 353-370.

Skujins, J.J., Pogieter, H.J., and Alexander, M. (1965). Dissolution of fungal walls by a streptomycete-chitinase and glucannase. *Arch. Biochem. Biophys.* 111: 358-364.

Sottnek, F.O., Brown, J.M., Weaver, R.E. and Carroll, G.F. (1977). Recognition of *Oerskovia* species in the clinical laboratory: Characterization of 35 isolates. *Inter. J. Sys. Bacteriol.* 27: 263-270.

St Leger, R., Copper, R.M., Charnley, A.I.C. (1986) Cuticle degrading enzymes of emptopthagenic fungi: regulation of production of chitinolytic enzymes. *J. Gen. Microbiol.* 132: 1509-1517.

Stackebrandt, E., Witt, D., Kemmerling, C., Kroppenstedt, R. and Liesack, W. (1991).

Designation of *Streptomyces* 16S and 23S rRNA-based target regions for oligonucleotide probes. *Appl. Environ. Microbiol.* 57: 1468-1477.

Stevenson, I.L. (1956). Antibiotic activity of actinomycetes in soil as demonstrated by direct observation techniques. *J. Gen. Microbiol.* 15: 372-380.

Sundheim, L. (1987). Conjugational transfer of chitinase encoding genes. *J. Agric. Sci. Finland* 59: 207-215.

Sundheim, L., Poplawsky, A.R. and Ellingboe, A.H. (1988). Molecular cloning of two chitinase genes from *Serratia marcescens* and their expression in *Pseudomonas* species. *Physiol. Mol. Pathol.* 33: 483-491.

Suzuki, K., Suzuki, M., Taiyoji, M., Nikaidou, N. and Watanabe, T. (1998). Chitin binding protein (Cbp21) in the culture supermatant of *Serratia marcescens* 2170. *Biosci. Biotechnol. Biochem.* 62: 128-135.

Taechowisan, T., Peberdy, J.F., Lumyong, S. (2003). Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World J. Microbiol. Biotechnol.* 19: 381-385.

Tagawa, K. and Okazaki, K. (1991). Isolation and some cultural conditions of *Streptomyces* species which produce enzymes lysing *Aspergillus niger* cell wall. *J. Ferment. Bioeng.* 71: 230-236.

Tahvonen, R. (1982). Preliminary experiments into the use of *Streptomyces* spp. isolated from peat in the biological control of soil and seed-borne diseases in peat culture. *J. Sci. Agricul. Soc. Finland* 54: 357-369.

Takeshi, W., Kiichi, K., Tomoko, S., Naoki, N., Kazushi, D., Megumi, S., Mayumi, T., Santiago, F. and Miguel, R. (1997). Genetic analysis of chitinase system of

*Serratia marcescens. J. Bacteriol.* 179: 7111-7117.

Tantivanich, S., Pantuwatana, S., Bhumiratana, A. and Panbangred, W. (1998). Multiple chitinase enzymes from a single gene of *Bacillus licheniformis* TP-1. *J. Ferment. Bioeng.* 85: 259-265.

Tominaga, Y. and Tsujisaka, Y. (1976). Purifications and some properties of two chitinases from *Streptomyces orientalis* which lyse *Rhizopus* cell wall. *Agr. Biol. Chem.* 40: 2325-2333.

Towbin, H., Staehelin, T., and Gordon, L. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA*, 76: 4350-4354.

Trejo-Estrada, S.R., Paszezynski, A. and Crawford, D.L. (1998). Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. *J. Ind. Microbiol. Biotechnol.* 21: 81-90.

Triger, E.G., Polyanskaya, L.M., Kozhevin, P.A. and Zvyagintsev, D.G. (1991). Autoregulation of spore germination in streptomycetes grown on rich and poor media. *Microbiol.* 60: 461-465.

Trudel, J. and Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* 178: 362-366.

Tsujibo, H., Endo, H., Minoura, K., Miyamoto, K., and Inamori, Y. (1993a). Cloning and sequence analysis of the gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Gene* 134: 113-117.

Tsujibo, H., Hatano, N., Mikami, T., Izumizawa, Y., Miyamoto, K. and Inamori, Y. (1998). Cloning, charac-terization and expression of β-*N*-acetylglucosaminidase gene from *Streptomyces thermoviolaceus* OPC-520.

*Biochem. Biophys. Acta* 1425: 437-440.

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y. (1993b). Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. *J. Bacteriol.* 175: 176-181.

Tsujibo, H., Okamoto, T., Hatano, N., Miyamoto, K., Watanabe, T., Mitsutomi, M., and Inamori, Y. (2000). Family 19 chitinases from *Streptomyces thermoviolaceus* OPC-520: Molecular cloning and characterization. *Biosci. Biotechnol. Biochem.*, 64: 2445-2453.

Ueno, H., Miyashita, K., Swada, Y. and Oba, Y. (1990). Purification and some properties of extracellular chitinase from *Streptomyces* sp. S.84. *J. Gen. Appl. Microbiol.* 36: 377-392.

Ulhoa, C.J. and Peberdy, J.F. (1991). Regulation of chitinase synthesis in *Trichoderma harzianum. J. Gen. Microbiol.* 137: 2163-2169.

Valadaris-Inglis, V., Cheria, M., Peter, I.W., Peberdy, J.F. (1997) Sequence analysis of the catalytic domain of *Metarhizium antisopiliae* chitinase. *Braz. J. Genet.* 20: 161-164.

Vasseur, V., Arigoni, F., Anderson, H., Defago, G., Bompeix, G., and Seng, J.M. (1990). Isolation and characterization of *Aphanocladium album* chitinase overproducing mutants. *J. Gen. Microbiol.* 136: 2561-2567.

Viterbo, A., Haran, J., Friesem, D., Ramot, O. and Chet, I. (2001). Antifungal activity of a novel endochitinase gene (*chit36*) from *Trichoderma harzianum* Rifai TM. 200: 169-174.

Vorgias, C.E., Kingswell, A.J. and Dauter, Z. (1992). Crystallization of recombinant

chitinase from the cloned *ChiA* Gene o *Serratia marcescens*. *J. Mol. Biol.* 226: 897-898.

Waksman, S.A. (1959). The actinomycetes, Vol. I. Nature, occurrence, and activities. Baltimore: Williams & Wilkins.

Waksman, S.A. (1961) The actinomycetes, Vol.II Classification, identification and descriptions of genera and species. Baltimore: Williams and Wilkins.

Walters, L.L., Irons, K.P., Guzman, H. and Tesh, R.B. (1993). Formation and composition of the peritrophic membrane in the sand Fly, *Phlebotomus perniciosus* (Diptera: *Psychodidae*). *J. Med. Entomol.* 30: 179-198.

Wan, G.S., Young, C.Y., Young, K.C., Hwa, C.Y., Ju, C.Y., Dong, B.J., Yeol, L.S. and Moo, J.C. (1997). Isolation and characterisation of 54 KDa and 22 KDa chitinase genes of *Serratia marcescens* KCTC2172. *FEMS. Microbiol. Lett.* 151: 197-204.

Wang, Z., Crawford, D.L., Pometto, A.L. and Rafii, F. (1989). Survival and effects of wild-type, mutant, and recombinant *Streptomyces* in a soil ecosystem. *Can. J. Microbiol.* 35: 535-543.

Watanabe, T., Kobori, K., Miyashita, K., Fuji, T., Sakai, H., Uchida, M., and Tanaka, H. (1993). Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* 268: 18567-18572.

Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K., and Tanaka, H. (1992). Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. *J. Bacteriol.*, 174: 408-414.

Watanabe, T., Suzuki, K., Oyanagi, W., Ohniski, K. and Tanaka, H. (1990). Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. *J. Biol. Chem.* 265: 15659-15665.

Watson, E.T. and Williams, S.T. (1974). Studies on the ecology of actinomycetes in soil – VI. Actinomycetes in a coastal sand belt. *Soil. Biol. Biochem.* 6: 43-52.

Wellington, E.M.H., Cresswell, N. and Herron, P.R. (1992). Gene transfer between *Streptomyces* in soil. *Gene* 115: 193-198.

White, J.D., Hanselmann, R., Jackson, R.W., Porter, W.J., Chla, Y., Tiller, T. and Wang, S. (2001). Total synthesis of rutamycin B, a macrolide antibiotic from *Streptomyces aureofaciens*. *J. Org. Chem.* 66: 5217-5231.

Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A. and Sakin, M.J. (1983). Numerical classification of *Streptomyces* and related genera. *J. Gen. Microbiol.* 129: 1743-1813.

Williams, S.T., Lanning, S. and Wellington, E.M.H. (1984). Ecology of Actinomycetes. In: The biology of the Actinomycetes. (eds: M. Goodfellow, M. Mordarski, and S.T. Williams) pp. 481-528. London: Academic Press.

Williams, S.T. and Robison, C.S. (1981). The role of *Streptomycetes* in decomposition of chitin in acidic soils. *J. Gen. Microbiol.* 127: 55-63.

Williams, S.T., Sharpe, M.E. and Holt, J.G. (1989). Bergey'S Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, Maryland.

Wiwat, C., Lertcanawanichakul, M., Siwayaprahm, P., Pantuwatana, S. and Bhumiratana, A. (1997). Expression of chitinase encoding genes from *Aeromonas hydrophilia* and *Pseudomonas maltophilia* in *Bacillus*

*thuringiensis* sub sp. Israelensis. *Gene* 179: 119-126.

Wiwat, C., Siwayaprahm, P. and Bhumiratana, A. (1999). Purification and characterization of chitinase from *Bacillus circulans* No.4.1 *Curr. Microbiol.* 39: 134-140.

Wood, T.M. and Bhat, K.M. (1988). Method for measuring cellulase activities. In: Methods in Enzymology. (eds: W.A. Wood and S.T. Kellogg) pp. 87-112. New York: Academic Press.

Wortman, A.T., Somerville, C.C. and Colwell, R.R. (1986). Chitinase determinants of *Vibro vulnificus*: gene cloning and applications of a chitinase probe. *Appl. Environ. Microbiol.* 52: 142-145.

Wynne, E.C. and Pemberton, J.M. (1986). Cloning of a gene cluster from *Cellvibrio mixtus* which codes for cellulose, chitinase, amylase, and pectinase. *Appl. Environ. Microbiol.* 52: 1362-1367.

Young, M.E., Carroad, P.A. (1981) Dependence of extracellular chitinase activity of *Serratia marcescens* QMB 1466 on continuous culture dilution rate. *Can. J. Microbiol.* 27: 142-144.

Yuan, W.M. and Crawford, D.L. (1995). Characterization of *Streptomyces lydicus* WYEC108 as a potential biological agent against fungal root and seed rots. *Appl. Environ. Microbiol.* 61: 3119-3129.

Zhang, A., Wang, Y. and Ruan, J. (1997). Aproposal to revive the genus Kitasatospora (Qmura, Takahashi, Iwai, and Tanaka 1982). *Int. J. Sys. Bact.* 47: 1048-1054.

# APPENDIX

## Appendix A : medium

1. Colloidal chitin broth (per liter) (Hsu and Lockwood 1975)

| | |
|---|---|
| Colloidal chitin | 10 g. |
| Calcium carbonate | 0.02 g. |
| Ferrous sulphate | 0.01 g. |
| Magnesium sulphate | 0.05 g. |
| Potassium chloride | 1.71 g. |
| Disodium hydrogen phosphate | 1.63 g. |

pH 7.2

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

2. Colloidal chitin basal medium (per liter) (Hsu and Lockwood 1975)

| | |
|---|---|
| Colloidal chitin | 15 g. |
| Yeast extract | 0.5 g. |
| Ammonium sulphate | 1 g. |
| Magnesium sulphate | 0.3 g. |
| Potassium dihydrogen phosphate | 1.36 g. |
| Agar | 15 g. |

pH 7.2

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

3. Hickey-Tresner medium (HT) (per liter) (Redshow *et al.* 1976)

| | |
|---|---|
| Dextrin | 10 g. |
| Yeast extract | 1 g. |
| Beef extract | 1 g. |
| N-Z Amine type A | 2 g. |
| Cobalt chloride | 0.02 g. |
| Agar | 15 g. |

pH 7.0

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

4. International Streptomyces project medium 2 (per liter) (Shirling and Gottlieb 1966)

| | |
|---|---|
| Yeast extract | 4 g. |
| Malt extract | 10 g. |
| Dextrose | 4 g. |
| Agar | 20 g. |

pH 7.3

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

5. Luria-Bertani medium (LB) (per liter) (Miller 1972)

| | |
|---|---|
| Tryptone | 10 g. |
| Yeast extract | 5 g. |
| Sodium chloride | 5 g. |
| Agar | 15 g. |

pH 7.2

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

6. Potato Dextrose Agar (PDA) (per liter)

| | |
|---|---|
| Potato | 200 g. |
| Glucose | 20 g. |
| Agar | 15 g. |

pH 5.5

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

7. SOB medium (per liter) (Miller 1972)

| | |
|---|---|
| Tryptone | 10 g. |
| Yeast extract | 5 g. |
| Sodium chloride | 0.5 g. |
| Potassium chloride | 0.18 g. |
| Magnesium chloride | 0.8 g. |

pH 7.2

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

8. Humic acid-salts-vitamin agar (HV) (per liter) (Otoguro *et al.* 2001)

Humic acid-salt agar

| | |
|---|---|
| Humic acid | 1 g. |
| Disodium hydrogen phosphate | 0.5 g. |
| Potassium chloride | 1.71 g. |
| Calcium carbonate | 0.02 g. |
| Ferrous sulphate | 0.01 g. |
| Magnesium sulphate | 0.05 g. |
| Agar | 15 g. |

pH 7.2

Resolved humic acid in 2.5 ml of 1N NaOH and sterilized 990 ml humic acid-salts in the autoclaved at 121°C for 15 minutes. Let it cool to 55-60°C. Added 10 ml of 100X vitamin sterile solution and mixed well.

100X vitamins

| | |
|---|---|
| Thiamine hydrochloride | 0.5 g. |
| Riboflavin | 0.5 g. |
| Niacin | 0.5 g. |
| Pyridoxin | 0.5 g. |
| Inositol | 0.5 g. |
| Calcium Pantothenate | 0.5 g. |
| p-Aminobenzoic acid | 0.5 g. |
| Biotin | 0.25 g. |

Suspended the vitamins in 10 ml of distilled water and sterilized by 0.22 μm filtration and kept at 4°C.

## Appendix B: Preparation of colloidal chitin (Berger and Reynolds 1958)

Fifteen grams of crude chitin (from crab shells, Sigma) was washed alternately in 200 ml of 1N NaOH for 24 h periods each, for five times. Then, it was washed four times with 100 ml of 95% (v/v) ethanol. Fifteen grams of the purified white chitin was dissolved in 100 ml conc. HCl and stirred in an ice bath for 20 min. The mixture was filtered through glass wool, and the solution was poured into cold distilled water to precipitate the chitin. The insoluble chitin on the glass wool was treated again with HCl, and the process was repeated until no more precipitate was obtained when the filtrate was added to cold water. The colloidal chitin was allowed to settle overnight and the supernatant was decanted. The remaining suspension was neutralized to pH 7.0 with NaOH. The precipitated chitin was centrifuged, washed, and stored as a paste at 4°C.

## Preparation of ball milled chitin (Berger and Reynolds 1958)

Ball milled chitin was obtained by milling 100 g of crude crab shell chitin (Sigma) overnight in a Pascall ball mill at 60 rev $min^{-1}$. The resulting powder was stored in an airtight container at room temperature.

## Appendix C: Determination of protein concentration by Bradford's method (Bradford 1976)

The concentration of protein in the sample was measured by the method described by Bradford (1976). The reagent kit (Bio-Rad) was prepared as recommended by the manufacturer. A 100 µl of protein sample was added to 1 ml of the diluted dye solution (concentrated dye reagent 1 part : distilled water 4 parts), and mixed thoroughly, left at room temperature for 5 min. The absorbance at 595 nm was measured after 2 min against reagent blank (100 µl of buffer mixed with 1 ml of the diluted dye solution). The colour of the reaction is stable and can be kept for 3 h. The concentration of protein was plotted against the standard curve (bovine serum albumin (BSA)).

### Determination of protein concentration by spectrophotometer at 280 nm

Proteins actively absorb light in the ultraviolet region with two maxima, 280 and 200 nm. Absorption spectroscopy involves the absorption of a photon by an electron. Only those photons with a certain energy level can be absorbed as defined by the difference in energy between the orbital of the unexcited electron and a higher energy orbital. This is why there are absorption maxima. Photons with higher energy have shorter wavelengths. Thus, electrons that are excited at 280 nm have absorbed less energy than those at 200 nm. Less energy is required for the electrons which absorb at 280 nm because these electrons lie within aromatic rings which stabilize the excited state due to resonance. Amino acids which have aromatic rings are phenylalanine, tryptophan, histidine and tyrosine. It should be noted that those proteins with few of these amino acid residues would be expected to have little

absorbance at 280 nm. In addition to secondary structure, the tertiary structure of a protein can also play a role in its absorbance spectrum because interactions between different amino acids can further stabilize electron excited states. Consequently, conditions such as buffer pH, polarity and ionic strength that alter tertiary structure can alter the absorbance spectrum of a protein. The amount of protein should be 20 to 3000 μg in range.

Procedure

1. Turn on the UV lamp of the spectrophotometer and warm up the machine (usually 15 min). Adjust the wavelength to 280 nm.
2. Zero the spectrophotometer using the buffer in which the protein is dissolved as a blank.
3. Measure the absorbance of the protein solution.
4. For unknown or protein mixture, use the following formula for a rough estimate (when using a cuvette with a path length 1 cm, divide the absorbance reading at 280 nm by the path length in centimeters):

Concentration (mg/ml) = absorbance of protein at 280 nm

**Table C**. Absorbance at 595 nm of various concentrations of BSA by Bradford's method.

| BSA concentration (μg/ml) | Absorbance 595 nm |
|---|---|
| 0.5 | 0.123 |
| 1.0 | 0.201 |
| 2.0 | 0.304 |
| 3.0 | 0.402 |
| 4.0 | 0.511 |
| 5.0 | 0.601 |

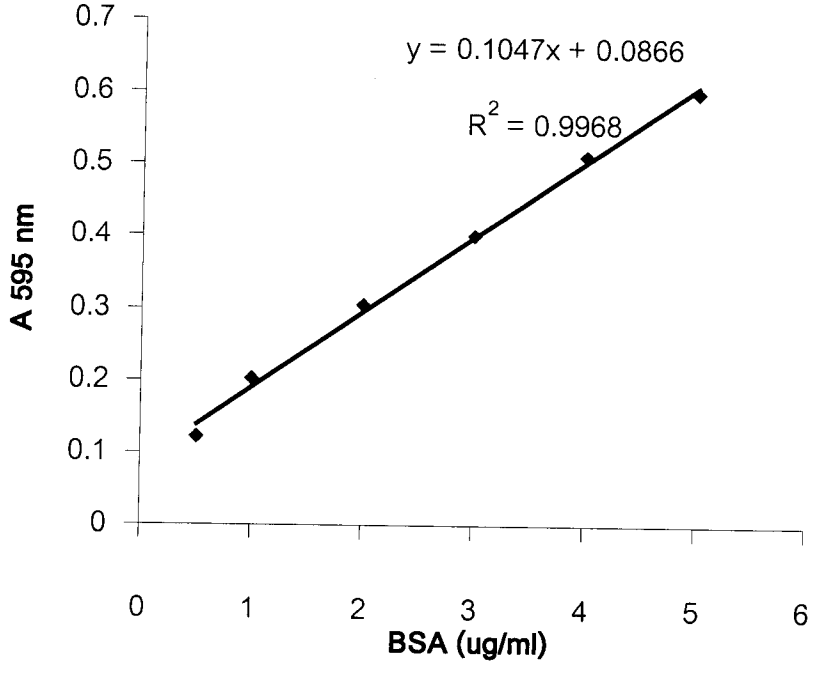


**Figure C** Standard curve for protein concentration determination using bovine serum albumin as standard

## Appendix D: Determination of reducing sugar concentration by Somogyi Nelson's method (Wood and Bhat 1988)

### Reagent

1. Copper reagent: the mixture of 100 ml of 10% $CuSO_4.5H_2O$ and 900 ml of Phosphate-Tartrate solution.

   Phosphate-Tartrate solution: dissolved 28 g of $Na_2HPO_4$, 40 g of sodium potassium tartrate and 120 g of $Na_2SO_4$ in 700 ml of distilled water and added 100 ml of 1N NaOH, then adjusted to a final volume of 900 ml.

2. Nelson's Arsenomolybdate colour reagent: the mixture of 25 ml of 12% $Na_2HAsO_4.7H_2O$ and 475 ml of Ammonium molybdate solution.

   Ammonium molybdate solution: dissolved 25g of $(NH_4)_6Mo_7O_{24}.4H_2O$ in 450 ml of distilled water and 21 ml of conc. $H_2SO_4$

### Procedure

1. Added 2 ml of reducing sugar sample and 2 ml of Copper reagent to the test tube and using distilled water as a control.
2. Mixed thoroughly and boiled for 15 min and placed in cold water.
3. Added 2 ml of Nelson's Arsenomolybdate colour reagent to the test tube and mixed thoroughly.
4. Recorded the absorbance at 520 nm using the distilled water as a blank.

**Table D** Absorbance at 520 nm of various concentrations of NAG by Somogyi Nelson's method.

| NAG concentration (mg/ml) | Absorbance 520 nm |
|---|---|
| 0 | 0 |
| 0.02 | 0.0618 |
| 0.04 | 0.1303 |
| 0.06 | 0.1956 |
| 0.08 | 0.2619 |
| 0.10 | 0.3523 |
| 0.12 | 0.4271 |
| 0.14 | 0.5036 |
| 0.16 | 0.5933 |
| 0.18 | 0.6624 |
| 0.20 | 0.7280 |

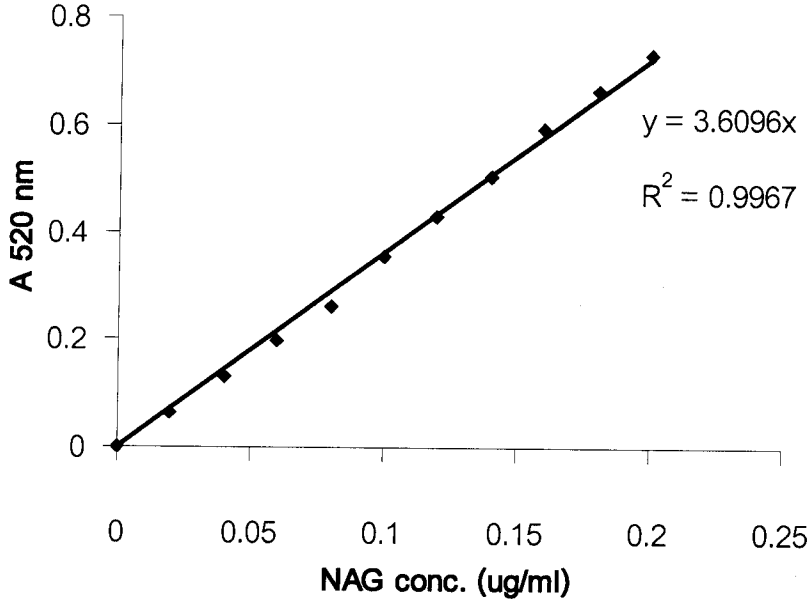


**Figure D** Standard curve of NAG concentration against absorbance at 520 nm by Somogyi Nelson's method

## Appendix E: Determination of NAG concentration by Reissig's method (Reissig *et al.* 1955)

### Reagent

1. 0.8 M Potassium tetraborate, the pH was adjusted to 9.1 with 1N KOH.
2. 10X p-Dimethylaminobenzaldehyde (DMAB) reagent: Dissolved 10 g of DMAB in 2.1 ml of distilled water, 10.4 ml of conc. HCl and 87.5 ml of glacial acetic acid.

### Procedure

1. Added 200 µl of NAG sample and 40 µl of 0.8 M Potassium tetraborate to the Eppendorf tube and using distilled water as a control.
2. Mixed thoroughly and boiled for 5 min and placed in cold water.
3. Added 1.2 ml of 1X DMAB reagent to the test tube and mixed thoroughly.
4. Recorded the absorbance at 585 nm using the distilled water as a blank.

**Table E** Absorbance at 585 nm of various concentrations of NAG by Reissig's method.

| NAG concentration (mg/ml) | Absorbance 585 nm |
|---|---|
| 0 | 0.0748 |
| 0.02 | 0.1366 |
| 0.04 | 0.2051 |
| 0.06 | 0.2704 |
| 0.08 | 0.3367 |
| 0.10 | 0.4271 |
| 0.12 | 0.5019 |
| 0.14 | 0.5784 |
| 0.16 | 0.6681 |
| 0.18 | 0.7372 |
| 0.20 | 0.8028 |

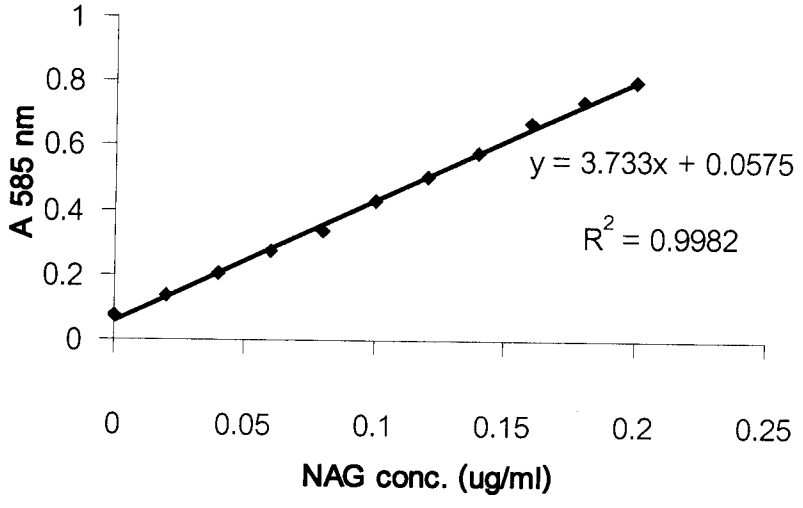


**Figure E** Standard curve of NAG concentration against absorbance at 585 nm by Reissig's method

## Appendix F: SDS-PAGE

### Reagent

1. Acrylamide stock solution (30% (w/v) acrylamide-bisacrylamide): Dissolved 29.2 g of acrylamide and 0.8 g of bisacrylamide in 100 ml of distilled water. Filter the homogeneous solution through a 0.45-μm filter membrane.
2. 1.0 M Tris-HCl (pH 8.8): dissolved 12.2 g of Tris base in distilled water and adjusted to pH 8.8 with HCl and added distilled water to a final volume of 100 ml.
3. 0.5 M Tris –HCl (pH 6.8): dissolved 6.1 g of Tris base in distilled water and adjusted to pH 6.8 with HCl and added distilled water to a final volume of 100 ml.
4. 10% (w/v) Sodium dodecyl sulfate (SDS): Dissolved 10 g of SDS in 100 ml of distilled water.
5. 1.5% (w/v) Ammonium persulfate (APS): Dissolved 15 μg of APS in 1 ml of distilled water. Prepared the APS solution fresh daily.
6. TEMED (*N,N,N',N'*-tetramethylenediamine)
7. 5X sample buffer: containing 250 mM Tris-HCl pH 6.8, 9.2% SDS, 40% glycerol, 20% β-mercaptoethanol and 0.8% bromphenol blue.
8. 5X electrophoresis buffer: containing 25 mM Tris-HCl pH 6.8, 192 mM glycine and 0.1% SDS.
9. Coomassie gel stain: containing 0.1% (w/v) Coomassie brilliant blue R-25 (w/v) in 40% (v/v) methanol and 10% (v/v) acetic acid.

10. Coomassie gel destain: containing 40% (v/v) methanol and 10% (v/v) acetic acid.

Preparation of slab gels

The clean glass plates (7 x 10 cm and 8 x 10 cm) were assembled with 0.75 mm spacers in the gel casting stand. The separating gel solution for 2 gels was prepared. The solution contained 10% SDS and 375 mM Tris-HCl (pH8.8). It was poured into the gel cassette, using a Pasteur pipette, to about 0.7 cm above the level which will be occupied by the well-forming combs. Isopropanol was immediately overlaid and the gels were allowed to polymerized for 1-2 h at room temperature. After pouring off the alcohol, the area above the polymerized gel was dried with filter papers. Five mililiters of the stacking gel monomer solution containing 4% acrylamide, 0.1% SDS and 125 mM Tris-HCl (pH 6.8) was laid over the separating gel and the combs were inserted carefully. The gels were allowed to polymerize at room temperature for at least 30 min. Thereafter, the combs were removed gently. All wells were blotted carefully with filter papers to remove resting fluid and air bubbles. The gels were ready for sample application.

Preparation of the samples

Samples were solubilized in equal volume of 2X sample buffer, containing 62.5 mM Tris-HCl (pH 6.8), 5% 2-β-mercaptoethanol and 0.01% bromphenol blue, and denatured at 100°C for 3 min. The volume loaded per well was 10 μl (for 10 lane comb). The concentration of proteins was about 7.5 $\mu g\ well^{-1}$.

Electrophoresis

After loading the samples and standard protein markers, The gel cassettes were installed in the electrophoretic apparatus. The electrode reservoir buffer solution

(0.1% SDS, 192 mM glycine and 25 mM Tris-HCl) was added into the inner and outer chambers. Electrophoresis was carried out with a constant voltage of 100 V (per 2 gels) for approximately 1 h 45 min. The bromphenol blue tracking dye was observed until it ran from the bottom of the gels into the buffer for about 25-30 min, the electrophoresis was then stopped. The gels were removed from the glass plates for staining or electro-transferring to nitrocellulose membranes.

Coomassie brilliant blue staining

The gel was placed in container and covered with 5ml of fixing solution (50% methanol, 10% acetic acid and distilled water), agitated slowly for 1 h at room temperature. Protein bands were stained by soaking for overnight with Coomassie brilliant blue R-250 solution (0.1% Coomassie brilliant blue R-250 in 40% methanol, 10% acetic acid and distilled water). Excess stain and background were removed from the gels by agitating in acid methanol solution (40% methanol, 10% acetic acid and distilled water) for overnight.

## Appendix G: Molecular weight determination

SDS-PAGE is used to determine the Molecular weight of protein by comparison of protein mobilities with those of several marker proteins of known molecular weight. Protein mobilities are calculated as the $R_f$ value.

Procedure

1. After gel electrophoresis and staining. Measured the distance of protein migration and that of tracking dye.
2. Calculated $R_f$ values.

   $R_f$ = distance of protein migration / distance of tracking dye migration

3. Ploted the logarithms of the molecular weight of the protein standard as a function of the $R_f$ values.
4. Read molecular weight of the unknown protein from the graph based on its $R_f$ value.

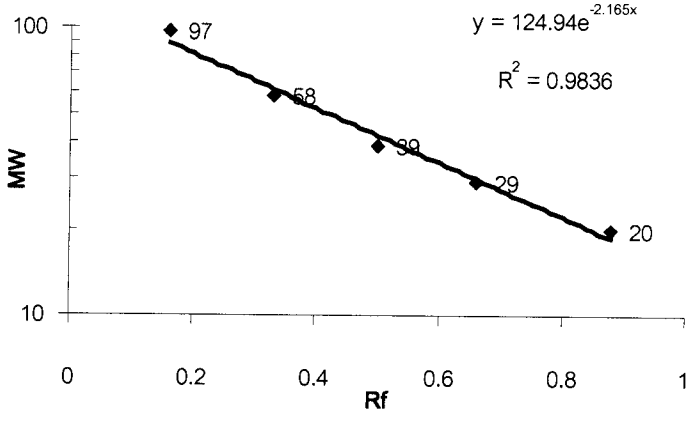


**Figure G** Semilogarithmic graph of molecular weight against relative mobility.

## Appendix H: Storage media and bacterial cultures

### Storage media

Bacteria can be stored for up to 2 years in stab cultures or indefinitely in cultures containing glycerol*.

### *Cultures containing Glycerol

### Bacterial cultures growing in liquid medium

To 2 ml of bacterial culture, add 1 ml of 45% sterile glycerol (sterilized by autoclaving for 20 minutes at 15 lb/sq.in. on liquid cycle). Vortex the culture to ensure that the glycerol is evenly dispersed. Transfer the culture to a labeled storage tube equipped with a screw cap and an air-right gasket. Freeze the culture rapidly, and then transfer the tube -70°C for long term storage.

To recover the bacteria, scrape the frozen surface of the culture with a sterile inoculation needle, and then immediately streak the bacteria that adhere to the needle onto the surface of an LB agar plate containing the appropriate antibiotics. Return the frozen culture the storage at -70°C. Incubate the plate overnight at 37°C.

Bacterial cultures growing on agar plates

Scrape the bacteria growing on the surface of an agar plate into 2 ml of LB medium in a sterile tube. Add an equal volume of LB medium containing 30% sterile glycerol. Vortex the mixture to ensure that the glycerol in completely dispersed. Dispense aliquots of the bacteria glycerine mixture into sterile tubes equipped with screw caps and airtight baskets. Freeze the cultures as described above.

## Appendix I: Purification of nucleic acids, standard markers for electrophoresis and concentrating nucleic acids

### 1. Preparation of organic reagents

#### Phenol

Most batches of commercial liquified phenol are clear and colorless and can be used in molecular cloning without redistillation. Occasionally, batches of liquified phenol are pink or yellow, and these should be rejected and returned to the manufacturer. Crystalline phenol is not recommended because it must be redistilled at 160°C to remove oxidation products, such as quinones, that cause the breakdown of phosphodiester bonds or cause cross-linking of RNA and DNA.

**Caution :** Phenol is highly corrosive and can cause severe burns. Wear gloves, protective clothing, and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Any areas of skin that come into contact with phenol should be rinsed with a large volume of water and washed with soap and water. Do not use ethanol.

#### Equilibration of phenol

Before use, phenol must be equilibrated to pH > 7.8 because DNA will partition into the organic phase at acid pH.

1. Liquified phenol should be stored at -20°C. As needed, remove the phenol from the freezer, allow it to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1% this compound is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ion. In addition, its yellow color provides a convenient way to identify the organic phase.

2. To the melted phenol, add an equal volume of buffer (usually 0.5M Tris-HCl [pH 8.0] at room temperature). Stir the mixture on a magnetic stirrer for 15 minutes, and then turn off the stirrer. When the two phases have separated, aspirate as much as possible of the upper (aqueous) phase using a glass pipette attached to a vacuum lined equipped with traps.

3. Add an equal volume of 0.1M Tris-HCl (pH 8.0) to the phenol. Stir the mixer on a magnetic stirrer for 15 minutes, and then turn off the stirrer. Remove the upper aqueous phase as described in step 2. Repeat the extractions until the pH of phenolic phase is > 7.8 (as measured with pH paper).

4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of 0.1 M Tris-HCl (pH 8.0) containing 0.2% β-mercaptoethanol. The phenol solution may be stored in this form under 100 mM Tris-HCl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month

**Phenol : Chloroform : Isoamyl Alcohol (25 : 24 : 1)**

A mixture consisting of equal parts of equilibrated phenol and chloroform : isoamyl alcohol (24:1) is frequently used to remove proteins from preparations of nucleic acids. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction.

Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol : chloroform : isoamyl alcohol mixture my be stored under 100 mM Tris-HCl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month.

**Purification of nucleic acids**

Perhaps the most basic of all procedures in molecular cloning is the

purification of nucleic acids. The key step, the removal of proteins, can often be carried out simply by extracting aqueous solutions of nucleic acids with phenol : chloroform and chloroform. Such extractions are used whenever it is necessary to inactivate and remove enzymes that are used in one step of a cloning operation before proceeding to the next. However, additional measures are required when nucleic acids are purified from complex mixtures of molecules such as cell lysates. In these cases, it is usual to remove most of the protein by digestion with proteolytic enzymes such as pronase or proteinase K, which are active against a board spectrum of native protein, before extracting with organic solvents.

**Extraction of Phenol : Chloroform**

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol : chloroform and then with chloroform. This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Furthermore, although phenol denatures proteins efficiently, it dose not completely inhibit RNAase activity, and it a solvent for RNA molecules that contain long tracts of poly(A). Both of these problem can be circumvented by using a mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The subsequent extraction with chloroform removes any lingering traces of phenol from the nucleic acid preparation, Extraction with ether, which was widely used for these purpose for many years, is no longer required for routine purification of DNA.

**Standard markers for get electrophoresis**

Sizes of marker fragments in base pairs

| λ/*Hind*III | λ/*Hind*II-*Eco*RI | λ/*Eco*RI | pUC18 /*Sau3A*I | ∅X174/*Hae*III |
|---|---|---|---|---|
| 23,130 | 21,226 | 21,226 | 955 | 1,353 |
| 9,416 | 5,148 | 7,421 | 585 | 1,078 |
| 9,557 | 4,973 | 5,804 | 341 | 872 |
| 4,361 | 4,268 | 5,643 | 258 | 603 |
| 2,022 | 3,530 | 4,878 | 141 | 310 |
| 2,027 | 2,027 | 3,530 | 105 | 281 |
| 564 | 1,904 | | 78 | 271 |
| 125 | 1,584 | | 75 | 234 |
| | 1,375 | | 46 | 194 |
| | 947 | | 36 | 118 |
| | 831 | | | 72 |
| | 564 | | | |
| | 125 | | | |

**Concentrating nucleic acids**

**Preciptitation with Ethanol or Isopropanol**

The most widely used method for concentrating nucleic acids is precipitation with ethanol. The precipitate of nucleic acid, which is allowed to form in the presence of moderate concentrations of monovalent cations, is recover by centrifugation and redissolved in an appropriate buffer at the desired concentration. The technique is rapid and is quantitative even with picogram amounts of DNA and RNA.

The three major variables are:

- The temperature at which the precipitate is allowed to form Until a few years ago, ethanol precipitation was routinely carried out at low temperature (e.g., in a dry-ice/methanol bath). This is now known to be unnecessary: At 0°C in the absence carrier, DNA at concentrations as low as 20 ng $ml^{-1}$ will form a precipitate that can be quantitatively recovered by centrifugation in microfuge.

- The type and concentration of monovalent cations used in the precipitation mixture. The choice among these salts is largely a matter of personal preferance.

Ammonium acetate (2.0 – 2.5 M) is frequently used to reduce the co-precipitation of dNTPs. For example, two sequential precipitations of DNA in the presence of 2 M ammonium acetate result in the removal of over 99% of the dNTPs from preparations of DNA. However, ammonium acetate should not be used when the precipitated nucleic acid is to be phosphorelated. Since bacteriophage T4 polynucleotide kinase is inhibited by ammonium ions.

Sodium chloride (0.2 M) should be used if the DNA sample contain SDS. The detergent then remains soluble in 70% ethanol.

Sodium acetate (0.3 M ; pH 5.2) is used for most routine precipitations of DNA and RNA.

| Salt Soutions | Stock solution (M) | Final concentration (M) |
|---|---|---|
| Ammonium acetate | 10.0 | 2.0-2.5 |
| Lithium chloride | 8.0 | 0.8 |
| Sodium chloride | 5.0 | 0.2 |
| Sodium acetate | 3.0 (pH 5.2) | 0.3 |

The time and speed of centrifugation as little as 20 g of nucleic acid in a volume of 1 ml can be quantitatively recovered in the absence of carrier by centrifugation at 12,000g for 15 minutes at 0-4°C in a microfuge. However, when lower concentrations of DNA or very small fragments (<100 nucleotides) are processed, more extensive centrifugation is required to cause the pellet of nucleic acid to adhere tightly to the centrifuge tube. A number of ultracentrifuge heads are now sold that hold very small volumes of fluid. If one of these is not available, centrifugation can be carried out by floating the sealed microfuge tube containing the ethanolic solution of nucleic acid in and ultracentrifuge tube (e.g., Beckman SW28 or equivalent) that has been three-quarters filled with water. Centrifugation is then carried out at 27,000 rpm for 1-2 hours at 4°C. After ultracentrifugation, the microfuge is removed and supernatant is carefully removed and discarded using an automatic micropipettor. The pellet of nucleic acid may then be washed with 70% ethanol and recovered by centrifugation at 12,000g for 10 minutes at 0 - 4°C in a

microfuge. This method allows the recovery of picogram quantities of nucleic acid and obviates the need for carriers.

# Appendix J: Nucleotide sequence and GenBank accession number of 16S rRNA of *Streptomyces aureofaciens* CMUAc130

```
LOCUS       AB105068              1525 bp    DNA     linear   BCT 07-MAR-2003
DEFINITION  Streptomyces aureofaciens gene for 16S rRNA, partial sequence,
            strain:CMUAc130.
ACCESSION   AB105068
VERSION     AB105068.1  GI:28875542
SOURCE      Streptomyces aureofaciens
  ORGANISM  Streptomyces aureofaciens
            Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
            Streptomycineae; Streptomycetaceae; Streptomyces.
REFERENCE   1
  AUTHORS   Taechowisan,T., Peberdy,J.F. and Lumyong,S.
  TITLE     Streptomyces aureofaciens gene for 16S rRNA, partial sequence,
            strain CMUAc130
  JOURNAL   Published Only in Database (2003)
REFERENCE   2  (bases 1 to 1525)
  AUTHORS   Taechowisan,T., Peberdy,J.F. and Lumyong,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAR-2003) Thongchai Taechowisan, Chiang Mai
            University, Biology; Hua Kaew Road, Chiang Mai 50200, Thailand
            (E-mail:tewson84@hotmail.com, Tel:66-053-943346, Fax:66-053-
892259)
FEATURES             Location/Qualifiers
     source          1..1525
                     /organism="Streptomyces aureofaciens"
                     /mol_type="genomic DNA"
                     /strain="CMUAc130"
                     /db_xref="taxon:1894"
                     /note="type culture"
     rRNA            <1..>1525
                     /product="16S ribosomal RNA"
BASE COUNT      334 a    385 c    521 g    285 t
ORIGIN
        1 ccgtcgacga gctcagagtt tgatcctggc tcaggacgaa cgctggcggc gtgcttaaca
       61 catgcaagtc gaacggtgaa gcccttcggg gtggatcagt ggcgaacggg tgagtaacac
      121 gtgggcaatc tgccctgcac tctgggacaa gccctggaaa cggggtctaa taccggatat
      181 gaccttcctc cgcatggggg ttggtggaaa gctccggcgg tgcaggatga gcccgcggcc
      241 tatcagcttg ttggtggggt aatagcctac caaggcggcg acgggtagcc ggcctgagag
      301 ggcgaccggc cacactgcga ctgagacacg gcccagactc ctacgggagg cagcagtggg
      361 gaatattgca caatgagcga aagcctgatg cagcgacgcc gcgtgaggga tgacggcctt
      421 cgggttgtaa acctctttca gcagggaaga agcgcaagtg acggtacctg tagaagaagc
      481 accggctaac tacgtgccag cagccgcggt aatacgtagg gtgcgagcgt tgtccggaat
      541 tattgggcgt aaagctcgta ggcggcctgt cgcgtcggat gtgaaagccc ggggcttaac
      601 ctcgggtctg cattcgatac gggcaggcta gagtgtagta ggggagatcg gaattcctgg
      661 tgtagcggtg agatgcgcag atatcaggag gaacaccggc ggcgaaggcg gatctctggg
      721 ccattactga cgctgaggag cggaagcgtg gggagcgaac aggactagat accctggtag
      781 tccacgccgt aaacgttggg aactaggtgt tggcgacact ccacgtcgtc ggtgccgcag
      841 ctcgcttgag ttccccgcct ggggagtacg gccgcaaggc taagactcaa aggaattgac
      901 gggggcccgc acaagcagcg gagcatgtgg cttaattcga cgcaacgcga agaaccttac
      961 caaggcttga catatgccgg aaacatccag agatgggtgc ctccttgtgg tcggtataca
     1021 ggtggtgcat ggttgtcgtc agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag
     1081 cgcaacccct gttctgtgtt gccaacgagt aatgtcgggg actcacagga gactgccggg
     1141 gtcaactcgg aggaaggtgg ggacgacgtc aagtcatcat gccccttatg tcttgagctg
     1201 cacacgtgct acaatggtcg gtacaaaggg ctgcgatgcc gtgaggcgga gcgaatccca
     1261 aagggccggc ctcagtccgg attggggtct gcaactcgac cccatgaagt tggagttgct
     1321 agtaatcgca gatcagcatg ctgcggtgaa tacgttcccg ggccatgtac acaccgctcg
     1381 tcgcgtcacg aaagtcggta acacccgaag ccggtggcct aacccgtaag ggatggagcc
     1441 gtcggaggtg ggaccagcga ttgggacgga gtcgtaacga ggtagccgtt gcggctggat
     1501 cacctcctta agcttggtac ccggg
//
```

# Appendix K: Nucleotide sequence and GenBank accession number of chitinase gene from *Streptomyces aureofaciens* CMUAc130

```
LOCUS       AB106648              1438 bp    DNA     linear   BCT 25-MAR-2003
DEFINITION  Streptomyces aureofaciens chiA gene for chitinase precursor,
            complete cds.
ACCESSION   AB106648
VERSION     AB106648.1  GI:29170576
SOURCE      Streptomyces aureofaciens
  ORGANISM  Streptomyces aureofaciens
            Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
            Streptomycineae; Streptomycetaceae; Streptomyces.
REFERENCE   1
  AUTHORS   Taechowisan,T., Peberdy,J.F. and Lumyong,S.
  TITLE     Cloning and sequence analysis of the gene encoding a chitinase
from
            endophytic Streptomyces aureofaciens CMUAc130
  JOURNAL   Published Only in Database (2003)
REFERENCE   2  (bases 1 to 1438)
  AUTHORS   Taechowisan,T., Peberdy,J.F. and Lumyong,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAR-2003) Thongchai Taechowisan, Chiang Mai
            University, Department of Biology; Hua Kaew Road, Chiang Mai
50200,
            Thailand (E-mail:tewson84@hotmail.com, Tel:66-53-943346,
            Fax:66-53-892259)
FEATURES             Location/Qualifiers
     source          1..1438
                     /organism="Streptomyces aureofaciens"
                     /mol_type="genomic DNA"
                     /strain="CMUAc130"
                     /db_xref="taxon:1894"
                     /sub_clone="pChi40"
                     /note="type culture"
     -35 signal      51..56
     -10 signal      77..82
     RBS             133..138
     gene            145..1386
                     /gene="chiA"
     CDS             145..1386
                     /gene="chiA"
                     /EC_number="3.2.1.14"
                     /note="similar to Streptomyces thermoviolaceus,
                     Streptomyces plicatus and Streptomyces lividans deposited
                     at DDBJ/EMBL/GenBank Accession Numbers D14536, M82804
                     and D12647, respectively"
                     /codon_start=1
                     /transl_table=11
                     /product="chitinase precursor"
                     /protein_id="BAC66179.1"
                     /db_xref="GI:29170577"

/translation="MRFRHKAAALAATLALPLAGLVGLASPAQAATSATATFQKTSDW
GTGTVKLGYFTNWGVYGRNYHVKNLVTSGSADKITHINYAFGNVQGGKCTIGDSYADY
DKAYTADQSVDGVADTWDQPLRANFNQLRKLKAKYPHIKVLYSFGGWTWSGGFPDAVK
NPAAFAQSCYDLVEDPRWADVFDGIDWEYPNACGLSCDTSGPNAFSNMMKAVRAEFGD
NLVTAAVTADGSDGGKIDATDYGEASKYIDWYNVMTYDFFGAWAKNGPTAPHSPLTSY
DGIPQQGFTSADAIAKFKAKGVPADKLLIGIGFYGRGWTGVTQSAPGGTATGPAAGTY
EAGIEDYKVLKNSCPATGTVAGTAYAHCGSNWWIYDTPDTIKSKMDWANEQGLGGAFV
WDFSGDTANGELVSAINSGLA"
     sig peptide     145..234
                     /gene="chiA"
```

```
     mat_peptide     235..1383
                     /gene="chiA"
                     /product="chitinase 63"
                     /EC_number="3.2.1.14"
BASE COUNT      274 a    532 c    427 g    205 t
ORIGIN
        1 cggccagtgc caagcttgca tgcctgcagg tcgactctag aggatcccca ttgacccagt
       61 ggtccagacc tttctatatt cgcgccacgg gcgtgctgac cgtcatgccc ctgacatccc
      121 ccccgcacac agaggaggcg cttcatgcgc ttcagacaca aagccgcggc actcgcagcg
      181 accctggcgc ttcccctcgc cggcctggtc ggcctcgcga gcccggccca ggcggccacc
      241 agcgcgacgg ccaccttcca gaagacctcg gactggggca ccggcacggt caagctgggc
      301 tacttcacca actggggcgt ctacgggcgc aactaccacg tgaagaacct ggtcacctcc
      361 ggctccgccg acaagatcac gcacatcaac tatgccttcg gcaacgtcca gggcggcaag
      421 tgcaccatcg gcgactccta cgccgactac gacaaggcgt acaccgccga ccagtccgtc
      481 gacggcgtcg cggacacctg ggaccagccg ctgcgcgcaa acttcaacca gctgcgcaag
      541 ttgaaggcca agtacccgca catcaaggtc ctctactcct tcggcggctg gacctggtcc
      601 ggcggcttcc ccgacgccgt gaagaacccg gccgcgttcg cgcagtcctg ctacgacctg
      661 gtcgaggacc cgcgctgggc cgacgtcttc gacggcatcg actgggagta ccccaacgcc
      721 tgcggtctca gctgtgacac cagcggcccc aacgccttca gcaacatgat gaaggccgtg
      781 cgcgccgagt tcggcgacaa cctggtcacc gcggccgtca ccgccgacgg ctcggacggc
      841 ggcaagatcg acgccaccga ctacggcgag gcctcgaagt acatcgactg gtacaacgtg
      901 atgacgtacg acttcttcgg cgcctgggcg aagaacggcc cgaccgcccc gcactcgccg
      961 ctcacctcgt acgacggcat cccgcagcag ggcttcacct ccgccgacgc gatagcgaag
     1021 ttcaaggcca agggcgtccc ggccgacaag ctcctgatcg gcatcggctt ctacggccgc
     1081 ggctggaccg gcgtcacgca gtccgcgccc ggcggcaccg ccaccggccc ggcggccggc
     1141 acctacgagg ccggcatcga ggactacaag gtcctcaaga acagctgccc ggccaccggc
     1201 accgtcgccg gcaccgcgta cgcccactgc ggctccaact ggtggatcta cgacaccccg
     1261 gacaccatca agtcgaagat ggactgggcg aacgagcagg gtctcggcgg cgccttcgtc
     1321 tgggacttca gcggcgacac cgcgaacggc gaactggtga gcgccatcaa cagcggcctg
     1381 gcgtgagcag cacacacggg taccgagctc gaattcgtaa tcatggtcat agctgttt
//
```

## Appendix L : Making a *Streptomyces* spore suspension (Hopwood *et al.* 1985)

Most *Streptomyces* produce copious, haploid, unigenomic spores under suitable culture conditions. Although *Streptomyces* spores arise in chains in the aerial mycelium, individual spores can usually be readily separated by suspending and vortexing in water; for some strains, however, a wetting agent such as 0.1% Tween 80 or 0.0001% Trition X100 is needed. The resulting suspensions are used for many purposes, such as inoculating liquid medium to produce mycelium for isolating plasmid or chromosomal DNA, RNA or enzymes, or for preparing protoplasts; for the isolation of mutants; and for the analysis of recombination or plasmid transfer in crosses. Concentrated spore suspensions ($10^9$ spore $ml^{-1}$) are crucial for purposes like starting reproducible cultures for physiological or fermentation studies, so it is worth experimenting with different media to find one that is good for a particular strain. MS (mannitol soya flour) agar is particularly good for *S. coelicolor* and *S. lividans*.

Suspensions of spores in 20% glycerol, kept frozen at -20°, will usually remain viable for years, even if they are repeatedly thawed and re-frozen for sampling purposes (but not if they start to germinate). Non-sporulating strains, such as whi and some bld strains, die rapidly on freezing in glycerol (though bldA strains retain viability). Spore suspensions frozen in water usually lose about 50% of their viability on freezing and thawing. Suspensions can also be kept frozen in DMSO at -20° (useful for glycerol-sensitive mutants).

Fresh plate culture (or slant) of the strain

Sterile water, 20% glycerol in water (sterilized by autoclaving), Rigid inoculation loop; pipettes; filter tubes containing non-absorbent cotton wool; centrifuge tubes; screw cap containers, e.g. 7.5 ml and 20 ml. The following should be

carried out in a laminar flow hood or other sterile environment. Vortex mixer ; bench centrifuge; compressed are supply.

1. Add 9 ml of sterile water to the plate (or slant). It is convenient to keep a supply of 20 ml screw cap bottles containing 9 ml amounts of sterile water ready for making spore suspensions. Plate cultures usually yield more spores than slants and are routinely used, provided there is access to a laminar flow hood for inoculating them. Slants are less prone to contamination, if this is a special problem.

2. Scrape the surface of culture with an inoculating loop, first with gentle pressure and then gradually more vigorously, so as to suspend the spores. Gentle scraping is important for agarase-producing species such as *S. coelicolor*, otherwise lumps of agar medium will clog the filter. Some use a small piece of cotton wool, held in sterilized forceps, to rub the surface of the culture gently to suspend the spores. Others collect the spores dry by rolling glass beads over the culture surface and then suspend the spores in water when needed.

3. Pour the crude suspension back into the container that held the sterile water, or use a sterile syringe or pipette, and agitate the liquid as violently as possible on a vortex mixer for about a minute to break up spore chains.

4. Filter the suspension through non absorbent cotton wool, using a filter tube. If a piece of cotton wool was used to suspend the spores the suspension may be free enough of mycelial fragments and pieces of agar medium that filtration is superfluous.

5. Pour the filtered suspension into a centrifuge tube and spin for 5-10 min at 2000 rpm to pellet the spores. The centrifugation step is to remove compounds dissolved from the growth medium. These may include growth factors that could interfere with

the selective use of auxotrophic markers, or "staling" compounds that might reduce the longevity of the spores, or inhibit germination.

6. As soon as the centrifuge stops, pour off the supernatant. If the spore pellet is left in the tube, even for a few minutes, after the centrifuge has stopped, it will often become detached from the wall of the tube.

7. Agitate the tube on the vortex mixer for a few seconds to disperse the pellet in the drop of water remaining in the tube. It is easier to disperse the spores in a minimum volume of liquid than in the final volume.

8. Add sterile 20% glycerol (usually 1-2 ml for the spores from a well-sporulating slant or plate) and briefly agitate again. Transfer the suspension to a screw cap bottle for freezing at -20° ; 7.5 ml bottles are convenient for this. If the spores are just for immediate use, they can be suspended in water ; if you then decide to keep them, add a roughly equal volume of 40% glycerol and freeze

## Appendix M: Plating out a *Streptomyces* spore suspension (Hopwood *et al.* 1985)

*Streptomyces* spores (and even mycelial fragments) are quite resistant to osmotic damage and so dilutions can be made directly into distilled water, even from 20% glycerol or concentrated sucrose solutions. When using frozen spore suspensions, take care to keep them on ice on the bench if you plan to refreeze them, because they rapidly lose viability on refreezing if spore germination has begun.

We usually transfer 0.5 ml or 1 ml of suspension using conventional glass pipettes to 4.5 ml or 9 ml of water, respectively, for each successive tenfold dilution step, but smaller volumes can of course be handled accurately using automatic micro-pitettes (such as the Pipetman manufactured by Gilson). A fresh pipette or tip must be used for each successive dilution step.

For plating, we usually spread 0.1 ml of spore suspension on each standard Petri disk using a glass spreader. The same spreader can be used for a series of plates at the same dilution or when moving from more dilute to less dilute suspensions, because any carry-over of spores is then negligible. However, use a different spreader for selective plates containing different growth factors since the carry-over of traces of these compounds can cause background growth. Note that, in plating undiluted spore stocks in 20% glycerol, significant amounts of this potential carbon source are added to the plate; the spores may need to be spun down and resuspended in water if this may be a problem.

When preparing dense lawns, for example when looking for pocks or for making "plate-crosses", we normally dry the plates in a laminar flow cabinet after spreading; drying them before-hand, so that the suspension rapidly soaks in, may lead to patchy growth. When plating for isolated colonies, for example of recombinants

from a cross, there is usually no need to dry the plates; just incubate them with the agar surface facing upwards for the first day or so.

***Streptomyces* cultures on agar**

To obtain confluent cultures on agar, the organisms have to inoculated over the entire surface of the medium. This is because *Streptomyces* colonies, in contrast to those of most moulds, will spread only over a very limited distance within a reasonable time so point inoculation of *Streptomyces* will not yield a confluent culture. It is best of use a suspension of inoculum in liquid as starting material. This need not be a carefully prepared spore suspension, although this is very suitable.

It is undesirable to propagate cultures by successive rounds of mass culture. Instead they should be plated, or streaked out, and a single colony taken to start the next culture. This precaution reduces the accumulation of revertrants and the gradual loss of unselected plasmids, which is otherwise an ever-present possibility when the variant forms grow or sporulate letter than the original genotype; as a visual manifestation of this, mass subculture very often yields obvious morphological heterogeneity.

It is not uncommon to observe heterogeneity even in confluently grown lawns. Two features in particular have been noted. One is the appearance of tiny plaque-like holes in the aerial mycelial mat, often reminiscent of plasmid-induced pocks. It has usually proved difficult to establish the cause of such spontaneous "self-pock", although there are reports of material resembling defective phages being associated with some such pocks. The other feature probably results from *in situ* germination of spores produced on a culture that has sporulated, so that white tufts may appear, or the whole culture surface may even become white again. Such germination is perhaps

stimulated by the gradual diffusion of nutrients up the aerial mycelium, causing a second developmental cycle.

## Appendix N: Table of chitinase activity

Table N1 Optimization of chitinase production from *S. aureofaciens* CMUAc130.

| Chitinase activity (mU ml$^{-1}$) (Mean±SD) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature (°C) of incubation | | | | | | pH of culture medium | | | | | | |
| 25 | 30 | 37 | 40 | 45 | 50 | 4.0 | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 | 8.0 | 9.0 |
| 0.0386±0.0070 | 0.0720±0.0026 | 0.0770±0.0079 | 0.0773±0.0020 | 0.0653±0.0075 | 0.0410±0.0065 | 0.0080±0.0020 | 0.0216±0.0080 | 0.0423±0.0055 | 0.0650±0.0060 | 0.0770±0.0062 | 0.0760±0.0025 | 0.0590±0.0072 | 0.0350±0.0081 |

Table N2 Optimization of chitinase production from *S. aureofaciens* CMUAc130.

| Chitinase activity (mU ml$^{-1}$) (Mean±SD) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Shaking speed (rev min$^{-1}$) | | | | Colloidal chitin concentration (1%) | | | | | | |
| 100 | 120 | 150 | 180 | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| 0.0770±0.0030 | 0.0760±0.0026 | 0.0755±0.0045 | 0.0710±0.0034 | 0.0436±0.0086 | 0.0616±0.0032 | 0.0820±0.0036 | 0.0796±0.0025 | 0.0806±0.0037 | 0.0806±0.0035 | 0.0840±0.0020 |

Table N3 Time course of changes in chitinase activity and the concentration of reducing sugar during the culture of *S. aureofaciens* CMUAc130 in 1% colloidal chitin medium at 30°C.

| Day of incubation | Chitinase activity (mU $ml^{-1}$) (Mean+SD) | Concentration of reducing sugar (mg $ml^{-1}$) (Mean+SD) |
|---|---|---|
| 1 | 0.0066+0.0015 | 0.0076+0.0032 |
| 2 | 0.0120+0.0043 | 0.0103+0.0057 |
| 3 | 0.0170+0.0026 | 0.0173+0.0015 |
| 4 | 0.0206+0.0035 | 0.0217+0.0025 |
| 5 | 0.0323+0.0049 | 0.0323+0.0063 |
| 6 | 0.0483+0.0035 | 0.0397+0.0040 |
| 7 | 0.0776+0.0035 | 0.0523+0.0041 |
| 8 | 0.0800+0.0040 | 0.0657+0.0058 |
| 9 | 0.0803+0.0040 | 0.0803+0.0085 |
| 10 | 0.0813+0.0055 | 0.0947+0.0015 |
| 11 | 0.0783+0.0060 | 0.0973+0.0020 |
| 12 | 0.0756+0.0041 | 0.1013+0.0020 |
| 13 | 0.0710+0.0026 | 0.1066+0.0015 |
| 14 | 0.0620+0.0020 | 0.1093+0.0025 |

Table N4 Effect of different chitin substrates (1%) on chitinase production after incubation at 30°C for 7 days.

| Chitin substrates (1%) | Chitinase activity (mU $ml^{-1}$) (Mean±SD) |
|---|---|
| Ball milled chitin | 0.0363±0.0096 |
| Crude chitin | 0.0360±0.0060 |
| Colloidal chitin | 0.0803±0.0050 |
| *Schizosaccharomyces* sp. cell walls | 0.0183±0.0035 |

Table N5 Effect of 0.5% sugar additives and 0.3% other carbon source additives with 1% colloidal chitin on chitinase production after incubation at 37°C for 7 days.

| Sugar/other carbon source | Chitinase activity (mU $ml^{-1}$) (Mean±SD) |
|---|---|
| *N*-acetylglucosamine | 0.0923±0.0045 |
| Arabinose | 0.0313±0.0070 |
| Cellobiose | 0.0340±0.0075 |
| Fructose | 0.0273±0.0060 |
| Glucose (0.5%) | 0.0363±0.0055 |
| Lactose | 0.0287±0.0061 |
| Mannose | 0.0563±0.0051 |
| Raffinose | 0.0747±0.0070 |
| Sucrose | 0.0293±0.0066 |
| Xylose | 0.0470±0.0040 |
| Control | 0.0820±0.0036 |
| Glucose (0.3%) | 0.0363±0.0055 |
| CM- cellulose | 0.1176±0.0224 |
| Starch | 0.1150±0.0180 |
| Pectin | 0.0920±0.0026 |

Table N6 Effect of different concentrations of *N*-acetylglucosamine in 1% colloidal chitin medium on chitinase production after incubation at 30°C for 7 days.

| *N*-acetylglucosamine concentrations (%) | Chitinase activity (mU $ml^{-1}$) (Mean±SD) |
|---|---|
| 0.0 | 0.0880+0.0030 |
| 0.1 | 0.0893+0.0002 |
| 0.5 | 0.0917+0.0035 |
| 1.0 | 0.0353+0.0045 |
| 1.5 | 0.0200+0.0020 |

Table N7 Effect of different temperature on chitinase activity. The chitinase activity was measured after the enzyme was added to a substrate solution (pH 7.0) and incubated for 1 h at different temperature.

| Temperature (°C) of incubation | Chitinase activity (mU $ml^{-1}$) (Mean±SD) |
|---|---|
| 25 | 0.0657+0.0080 |
| 30 | 0.1453+0.0196 |
| 37 | 0.1560+0.0157 |
| 40 | 0.1523+0.0146 |
| 45 | 0.1546+0.0107 |
| 50 | 0.1126+0.0090 |
| 55 | 0.0497+0.0077 |

Table N8 Effect of different pH on chitinase activity. The chitinase activity was measured after the enzyme was added to a substrate solution at different pH and incubated for 1 h at 37°C.

| pH of substrate solution | Chitinase activity (mU $ml^{-1}$) (Mean±SD) |
|---|---|
| 4.0 | 0.0510+0.0090 |
| 5.0 | 0.0620+0.0095 |
| 5.5 | 0.1140+0.0098 |
| 6.0 | 0.1276+0.0129 |
| 6.5 | 0.1263+0.0080 |
| 7.0 | 0.1326+0.0083 |
| 8.0 | 0.1200+0.0182 |
| 9.0 | 0.0643+0.0070 |

Table N9 Substrate specificity of chitinase activity. The chitinase activity was measured after the enzyme was added to a substrate solution in phosphate buffer (pH 7.0) for 1 h at 37°C.

| Substrate | Chitinase activity (mU $ml^{-1}$) (Mean±SD) |
|---|---|
| Crude chitin | 0.0102+0.0034 |
| Ball milled chitin | 0.01864+0.0042 |
| Colloidal chitin | 0.0234+0.0056 |
| *Schizosaccharomyces* sp. cell walls | 0.0124+0.0035 |
| Chitotriose | 0.0264+0.0027 |
| Chitotetraose | 0.0301+0.0038 |
| Chitobiose | 0.0009+0.0003 |
| CM-cellulose | 0.0009+0.0002 |

Table N10 Production of *S. aureofaciens* CMUAc130 chitinase on mixed substrate containing colloidal chitin and chitin derived from different fungal cell walls.

| Fungal cell walls | Chitinase activity on mixed substrate ratio (%fungal cell walls : % colloidal chitin) | | |
|---|---|---|---|
| | 0:1 (mU $ml^{-1}$)(Mean±SD) | 3:0 (mU $ml^{-1}$)(Mean±SD) | 3:1 (mU $ml^{-1}$)(Mean±SD) |
| *Bipolaris* sp. | 0.0200+0.0035 | 0.0110+0.0036 | 0.0310+0.0043 |
| *Colletotrichum musae* | 0.0200+0.0035 | 0.0193+0.0055 | 0.0573+0.0055 |
| *Candida albicans* | 0.0200+0.0035 | 0.0187+0.0037 | 0.0547+0.0070 |
| *Drechslera* sp. | 0.0200+0.0035 | 0.0180+0.0026 | 0.0493+0.0061 |
| *Fusarium oxysporum* | 0.0200+0.0035 | 0.0163+0.0055 | 0.0433+0.0065 |
| *Rhizoctonia* sp. | 0.0200+0.0035 | 0.0110+0.0020 | 0.0330+0.0072 |
| *Sclerotium* sp. | 0.0200+0.0035 | 0.0933+0.0015 | 0.0273+0.0060 |

# CURRICULUM VITAE

| | |
|---|---|
| **Name** | Mr. Thongchai Taechowisan |
| **Date of birth** | February 4, 1969 |
| **Address** | 54 Intra-arsa Road, Panatnikhom, Chonburi 20140, Thailand |
| **Academic background** | Finish high school from Panatpithayakarn School, Panatnikhom, Chonburi in 1988<br>Bachelor of Science (Microbiology) cum laude Burapha University, Chonburi in 1991<br>Master of Science (Microbiology) Mahidol University, Bangkok in 1995 |
| **Scholarship** | University Development Commission (U.D.C.) Scholarship |
| **Position held & office** | Department of Biology, Faculty of Science, Silpakorn University, Thailand<br>Position: Assistance Professor |

**Publication**

Taechowisan, T., Peberdy, J.F., Lumyong, S. (2003). Isolation of endophytic Actinomycetes from selected plants and their antifungal activity. *World J. Microbiol. Biotechnol.* **19**: 381-385.

Taechowisan, T., Lumyong, S. (2003). Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galanga* against phytopathogenic fungi. *Ann. Microbiol.* **53**: 25-36.

Taechowisan, T., Peberdy, J.F., Lumyong, S. (2003). Chitinase production by endophytic *Streptomyces aureofaciens* CMUAc130 and its antagonism against phytopathogenic fungi. *Ann. Microbiol.* (In press).

Taechowisan, T., Peberdy, J.F., Lumyong, S. (2003). PCR cloning and heterologous expression of chitinase gene of endophytic *Streptomyces aureofaciens* CMUAc130. *J. Gen. Appl. Microbiol.* (In Press).

Oral presentation in the topic "Chitinase production by endophytic *Streptomyces* sp. CMUAc130 and its potential in fungal cell wall lysis": in The 14$^{th}$ Annual Meeting of the Thai Society for Biotechnology at Sofitel Raja Orchid Hotel, Khon Kaen, Thailand during November 12-15, 2002.

Poster presentation in the topic "Isolation of endophytic actinomycetes and their antifungal activity": in The 28$^{th}$ Congress on Science and Technology of Thailand at Queen Sirikit National Convention Center, Bangkok, Thailand during October 24-26, 2002.

Poster presentation in the topic "Antifungal activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galanga* against

phytopathogenic fungi": in The 3rd Asia-Pacific Mycological Congress on Biodiversity and Biotechnology (AMC2002) at Yunan University, Kunming, Yunan, China during November 4-8, 2002.

Poster presentation in the topic "Cloning, sequencing and heterologous expression of family 19 chitinase gene from endophytic *Streptomyces aureofaciens* CMUAc130": in The 1st FEMS Congress of European Microbiologists at Cutural and Congress Centre, Ljubljana, Slovenia during June 29 to July 3, 2003.

Poster presentation in the topic "Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galanga* against phytopathogenic fungi": in The 5th National Seminar on Pharmaceutical Biotechnology at Holiday Garden Hotel, Chiang Mai, Thailand during September 3-5, 2003.

Poster presentation in the topic "Isolation of endophytic actinomycetes and their antifungal activity": in The 7th Biodiversity Research and Training Program Annual Conference at Lotus Pangsuankaew Hotel, Chiang Mai, Thailand during October 13-16, 2003.

Poster presentation in the topic "Cloning, sequencing and heterologous expression of chitinase gene from endophytic *Streptomyces aureofaciens* CMUAc130 for antifungal activity improvement": in The 3rd Federation of Asia Pacific Microbiology Societies (FAPMS) Conference at Nikko Hotel, Kuala Lumpur, Malaysia during October 15-18, 2003.

Poster presentation in the topic "Cloning, sequencing and heterologous expression of family 19 chitinase gene from endophytic *Streptomyces aureofaciens* CMUAc130": in The 29th Congress on Science and Technology of Thailand at Khon Kaen University, Khon Kaen, Thailand during October 20-22, 2003.

World Journal of Microbiology & Biotechnology 19: 381–385, 2003.



# Isolation of endophytic actinomycetes from selected plants and their antifungal activity

Thongchai Taechowisan[1], John F. Peberdy[2] and Saisamorn Lumyong[1,*]
[1]*Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
[2]*School of Life and Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK*
**Author for correspondence: Tel.: +66-53-943346 Ext: 1503, Fax: +66-53-892259,*
*E-mail: scboi009@chiangmai.ac.th*

Received 21 August 2002; accepted 20 December 2002

*Keywords:* Actinomycetes, antifungal activity, endophytes, *Micromonospora*, *Nocardia*, *Streptomyces*

## Summary

The isolation of endophytic actinomycetes from surface-sterilized tissues of 36 plant species was made using humic acid–vitamin (HV) agar as a selection medium. Of the 330 isolates recovered, 212 were from roots, 97 from leaves and 21 isolates from stems with a prevalence of 3.9, 1.7 and 0.3%, respectively. Identification of endophytic actinomycetes was based on their morphology and the amino acid composition of the whole-cell extract. Most isolates were classified as *Streptomyces* sp. (n = 277); with the remainder belonging to *Microbispora* sp. (n = 14), *Nocardia* sp. (n = 8) and *Micromonospora* sp. (n = 4). Four isolates were unclassified and 23 were lost during subculture. The most prevalent group of isolates were the *Streptomyces* sp. occurring in 6.4% of the tissue samples of *Zingiber officinale*. Scanning electron microscopy investigation of this plant revealed that 7.5% of the root and 5% of the leaf samples contained endophytes. Three of the *Streptomyces* sp. isolates strongly inhibited *Colletotrichum musae*, five were very active against *Fusarium oxysporum* and two strongly inhibited growth of both test fungi.

## Introduction

Actinomycetes are prokaryotes which have a hyphal (hence fungal-like) morphology. Most of the actinomycetes described are soil microorganisms and are active in the decomposition of plant tissues, and thereby in the recycling of carbon and nitrogen. Members of the genus *Frankia* are an exception, as these bacteria exist both as free-living forms and as endophytes forming nodules on host plants (Baker *et al.* 1980; Knowlton *et al.* 1980). However, these reports give no indication of prevalence of different species growing as endophytes in the different tissues of their host plants. Several reports refer to actinomycete activity in the protection of the plant host against pathogens and the influence of their metabolic products on plant growth and physiology (Katznelson & Cole 1965; Tahvonen 1982; Williams *et al.* 1984; Drautz & Zahner 1986; Schippers *et al.* 1987). Other reports refer to pathological interactions of endophytic actinomycetes with plants (Alwadi & Baka 2000).

We are interested in the antifungal activity of endophytic actinomycetes which has been a focus in the exploitation of these organisms as excellent biocontrol agents against phytopathogenic fungi. To date, however, much less is known about the antifungal antibiotics produced by endophytic actinomycetes (Sardi *et al.* 1992). The present study involved the isolation of actinomycetes from the tissues of healthy plants, an initial identification of them and an evaluation of the antifungal activity of their secondary metabolites.

## Materials and methods

### *Sample collection*

Leaf, stem and root tissues were recovered from healthy representatives of herbaceous and woody plants from the environs of Chiang Mai, Thailand during the period September, 2001–February, 2002.

### *Isolation of actinomycetes*

The samples were dissected into leaves, stems and roots, washed in running tap water and cut into small pieces of ca. $4 \times 4$ mm$^2$. Tissue pieces were rinsed in 0.1% Tween20 for 30 s, then in 1% sodium hypochlorite for 5 min, and then washed in sterile distilled water for 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of

humic acid–vitamin (HV) agar (Otoguro *et al.* 2001) containing 100 μg nystatin and cycloheximide/ml, and incubation at 30 °C was continued for about 1 month. The colonies were inoculated onto to ISP-2 medium (Shirling & Gottlieb 1966) for purification. The isolated colonies were subcultured onto a Hickey–Tresner (HT) medium (Redshow *et al.* 1976) slants to establish stock cultures. Isolate prevalence was calculated as follows (Bussaban *et al.* 2001)

$$\text{Isolate prevalence} = \frac{\text{Number of samples yielding one isolate} \times 100}{\text{Number of samples in that trial}}$$

*Morphological observations*

Leaf, stem and root materials from *Zingiber officinale* L. (Zingiberaceae) and *Alpinia galanga* L. (Zingiberaceae) were selected for microscopic observation by scanning electron microscopy (SEM) (JEOL-JSM840A SEM, Tokyo, Japan). Specimens were washed several times using distilled water and fixed overnight in 2.5% glutaraldehyde at 4 °C. They were then dehydrated in a graded alcohol series (30–95%) followed by treatment in acetone and critical-point drying (Petrolini *et al.* 1986). The specimens from each process were mounted on stubs, splutter-coated with gold, and viewed on the SEM at an accelerating voltage of 20 kV. Photomicrographs were recorded on Kodak VP200 film (New York, USA).

*Taxonomic properties*

Methods and media described by the International Streptomyces Project (Shirling & Gottlieb 1966) were used to determine most of the cultural and physiological characteristics. For morphological characteristics, the presence of aerial mycelium, spore mass colour, distinctive reverse colony colour, diffusible pigment, and sporophore and spore chain morphology were recorded after 10 days incubation on ISP-2 medium. Diaminopimelic acid isomers and sugars from whole-cell extract were analysed for chemotaxonomic studies (Becker *et al.* 1964; Boone & Pine 1968).

*Antifungal activity of actinomycetes isolates against phytopathogenic fungi*

The endophytic isolates were cultured on plates on ISP-2. Two fungal pathogens *Colletotrichum musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively, were used for screening antifungal activity. They were grown on potato dextrose agar (PDA). Mycelial disks of 6 mm diameter were cut from the plates with the two pathogens and transferred to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony. The plates were incubated at 30 °C for 5–7 days. The width of inhibition zones between the pathogen and the actinomycete isolates was measured and evaluated as follows: +++, 20 mm<; ++, 11–19 mm; +, 2–10 mm; ±, ≤1 mm; –, 0 mm.

## Results and discussion

After 3–4 weeks incubation, the surface of some tissue samples showed hyphal growth which subsequently grew out onto the surface of the HV agar (Figure 1a). This process of growth of the actinomycetes through the surfaces of the tissues was observed by SEM (Figure 1b). Growth of bacteria and fungi from the tissues was almost completely inhibited by the antibiotics included in HV agar leaving the actinomycetes clearly visible. The low level of bacterial contamination ob-

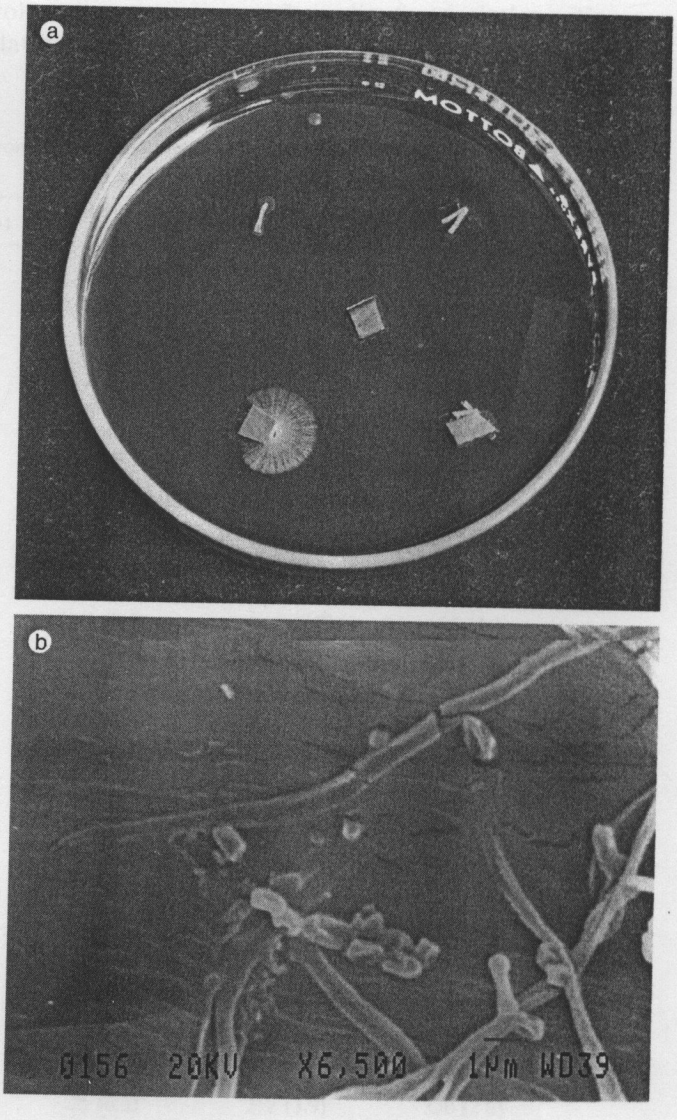


*Figure 1.* Growth of actinomycete colonies from sterilized blocks of plant tissue on HV agar. (a) This plate was photographed after 3 weeks of incubation. (b) Scanning electron micrograph of aerial hyphae of actinomycetes which have grown through the epidermis of a leaf of *A. galanga*. Magnification: 6500×.

served was due to *Bacillus* spp. This contamination may have arisen from spores on the surface of these tissues that were resistant to chemical surface sterilization or may be due to an endophytic *Bacillus* sp. (Garbeva *et al.* 2001; Bai *et al.* 2002). Incubation of surface-sterilized plant parts in a moist chamber and plating of plant tissues on agar media are techniques usually employed in plant pathology, and not often used in microbial ecology. However, they may be extremely useful in the isolation of microorganisms from unusual habitats. Using these techniques, we were able to confirm the presence of endophytic actinomycetes in plant tissues, especially roots, where a large number of these organisms are most probably found. The actinomycete isolates took at least 3 weeks to grow out from the tissues. If the tissue sterilization procedure used in this study was not sufficient to kill surface microbes, they would be expected to grow from specimens within a few days.

Some 36 plant species from the families Acanthaceae, Amaranthaceae, Cruciferae, Cyperaceae, Gramineae, Iridaceae, Labiatiae, Rubiaceae, Rutaceae, Taccaceae, Umbelliferae and Zingiberaceae (Table 1), were examined using a total of 5400 each of root, stem and leaf tissues. Streptomycetes were the most common isolates recovered, being most prevalent from roots (3.9%), leaves (1.8%) and less from stems (0.3%). The mycelia of the actinomycete isolates grew out of the tissue blocks onto the surfaces. Thus, these isolates are considered endophytic rather than ectophytic microbes, as discussed by Okazaki *et al.* (1995). With SEM, hyphae of these organisms could be recognized in the leaf and root tissues of both *Zingiber officinale* and *Ailpinia galanga*. Observations on 40 samples of each tissue type confirmed the high incidence in roots (7.5%) and leaves (5%), but hyphae were not seen in stems. It is clear therefore that roots present a good habitat for these endophytic actinomycetes. However, the frequency reports from the SEM observations were higher suggesting that the visual observation revealed non-viable hyphae or organisms that could not grow on HV agar, which like all media will be selective according to its nutrient availability. Future studies should therefore involve the use of several media for isolation. The isolates were obtained most frequently from roots and

*Table 1.* Numbers of isolates of endophytic actinomycete per tissue block from leaves, stems and roots or a range of herbaceous and woody plants.

| Family | Host plant | Leaves (%) | Stems (%) | Roots (%) | Total (%) |
|---|---|---|---|---|---|
| Acanthaceae | *Rhinacanthus communis*[a] | 1 (0.6) | 0 (0.0) | 0 (0.0) | 1 (0.2) |
| Amaranthaceae | *Amaranthus gracilis*[a] | 0 (0.0) | 0 (0.0) | 4 (2.6) | 4 (0.8) |
| Cruciferae | *Brassica juncea*[a] | 0 (0.0) | 0 (0.0) | 1 (0.6) | 1 (0.2) |
| Cruciferae | *Brassica oleracea*[a] | 0 (0.0) | 0 (0.0) | 20 (13.3) | 20 (4.4) |
| Cyperaceae | *Cyperus difformis*[a] | 6 (4.0) | 3 (2.0) | 4 (2.6) | 13 (2.8) |
| Cyperaceae | *Cyperus iria*[a] | 3 (2.0) | 1 (0.6) | 5 (3.3) | 9 (2.0) |
| Cyperaceae | *Cyperus kyllingia*[a] | 5 (3.3) | 3 (2.0) | 5 (3.3) | 13 (2.8) |
| Cyperaceae | *Cyperus malaccensis*[a] | 3 (2.0) | 5 (3.3) | 5 (3.3) | 13 (2.8) |
| Cyperaceae | *Cyperus rotundus*[a] | 5 (3.3) | 1 (0.6) | 4 (2.6) | 10 (2.2) |
| Gramineae | *Chloris barbata*[a] | 0 (0.0) | 2 (1.3) | 0 (0.0) | 2 (0.4) |
| Gramineae | *Cymbopogon citratus*[a] | 3 (2.0) | 0 (0.0) | 3 (2.0) | 6 (1.3) |
| Gramineae | *Cymbopogon nardus*[a] | 3 (2.0) | 0 (0.0) | 9 (6.0) | 12 (2.6) |
| Gramineae | *Echinochloa colona*[a] | 3 (2.0) | 0 (0.0) | 1 (0.6) | 4 (0.8) |
| Gramineae | *Echinochloa crusgalli*[a] | 1 (0.6) | 1 (0.6) | 3 (2.0) | 5 (1.1) |
| Gramineae | *Imperata cylindrica*[a] | 0 (0.0) | 0 (0.0) | 8 (5.3) | 8 (1.7) |
| Iridaceae | *Eleutherine palmifolia*[a] | 0 (0.0) | 0 (0.0) | 6 (4.0) | 6 (1.3) |
| Labiatae | *Ocimum tenuiflorum*[a] | 5 (3.3) | 4 (2.6) | 0 (0.0) | 9 (2.0) |
| Rubiaceae | *Coffea arabica*[b] | 2 (1.3) | 0 (0.0) | 2 (1.3) | 4 (0.8) |
| Rutaceae | *Citrus hystrix*[b] | 1 (0.6) | 0 (0.0) | 0 (0.0) | 1 (0.2) |
| Taccaceae | *Tacca chantrieri*[a] | 0 (0.0) | 0 (0.0) | 2 (1.3) | 2 (0.4) |
| Umbelliferae | *Apium graveolens*[a] | 9 (6.0) | 0 (0.0) | 0 (0.0) | 9 (2.0) |
| Umbelliferae | *Coriandrum sativum*[a] | 0 (0.0) | 0 (0.0) | 2 (1.3) | 2 (0.4) |
| Zingiberaceae | *Alpinia blepharocalyx*[a] | 2 (1.3) | 1 (0.6) | 3 (2.0) | 6 (1.3) |
| Zingiberaceae | *Alpinia galanga*[a] | 9 (6.0) | 0 (0.0) | 32 (21.3) | 41 (9.1) |
| Zingiberaceae | *Amomum siamense*[a] | 10 (6.6) | 0 (0.0) | 28 (18.6) | 38 (8.4) |
| Zingiberaceae | *Boesenbergia pandurata*[a] | 4 (2.6) | 0 (0.0) | 19 (12.6) | 23 (5.1) |
| Zingiberaceae | *Curcuma domestica*[a] | 3 (2.0) | 0 (0.0) | 10 (6.6) | 13 (2.8) |
| Zingiberaceae | *Curcuma longa*[a] | 8 (5.3) | 0 (0.0) | 0 (0.0) | 8 (1.7) |
| Zingiberaceae | *Etlingera elatior*[a] | 0 (0.0) | 0 (0.0) | 2 (1.3) | 2 (0.4) |
| Zingiberaceae | *Zingiber cassumunar*[a] | 3 (2.0) | 0 (0.0) | 2 (1.3) | 5 (1.1) |
| Zingiberaceae | *Zingiber officinale*[a] | 8 (5.3) | 0 (0.0) | 33 (22.0) | 40 (8.8) |
| | Totals | 97 (1.7) | 21 (0.3) | 212 (3.9) | 330 (2.0) |

Sterilized tissue blocks were placed on HV agar and incubated for up to a month at 30 °C.
No isolates were recovered from the tree species, *Citrus aurantifolia*[b] (Rutaceae), *Streblus asper*[b] (Moraceae), *Tamarindus indica*[b] (Leguminosae), *Mangifera indica*[b] (Anacardiaceae) and *Dimocarpus longan*[b] (Sapindaceae).
[a] herbaceous plants; [b] woody plants.

less so from other parts. This may relate to the presence of actinomycetes as a large part of the rhizosphere microbial flora (Sardi *et al.* 1992) thus enabling easier infection of a host. However, the presence of endophytic actinomycetes in leaves and stems support previous reports (Okazaki *et al.* 1995; Shimizu *et al.* 2000).

The presence of endophytic actinomycetes, as shown by their isolation from healthy plants, and the SEM investigations on internal tissues, leads to the conclusion that there is a close relationship between these micro-organisms and plant tissues, in which growth of the former could have a favourable effect on plant growth and development. Their biological activities can affect plant growth either through affecting the nutrient supply (Katznelson & Cole 1965; Tahvonen 1982; Williams *et al.* 1984; Drautz & Zahner 1986; Schippers *et al.* 1987) or the *in situ* production of secondary metabolites which stimulate or depress vegetative development (Mishra *et al.* 1987) and may also protect against phytopathogenic microorganisms (Abd-Allah 2001; Getha & Vikineswary 2002).

In this study most of the actinomycetes were obtained from herbaceous plants and very few from woody plants. Similar observations can be drawn from other workers. Okazaki *et al.* (1995) obtained 246 isolates from 172 samples of healthy leaves of monocotyledons such as *Cyperus* sp. and *Carex* sp. and in comparison Shimizu *et al.* (2000) obtained 10 isolates from *Rhodo-dendron* sp. In our experiment the use of only one medium for isolation of actinomycetes may be a factor, however, there are also intrinsic differences between woody and herbaceous species and it maybe to these that we have to look for an explanation. Many tree species have mycorrhizal fungi associated with their roots which may form a barrier to infection of these tissue by other endophytic species.

In total 330 isolates were recovered, the majority of which were *Streptomyces* spp., with the remainder identified as *Microbispora* sp., *Nocardia* sp. and *Micro-monospora* sp. (Figure 2). Four isolates did not develop sporing structures, although meso-diaminopimelic acid was detected in whole cell extracts, confirming an actinomycete status. Correspondingly the prevalence of *Streptomyces* sp. was the highest, ranging from 6.4% for

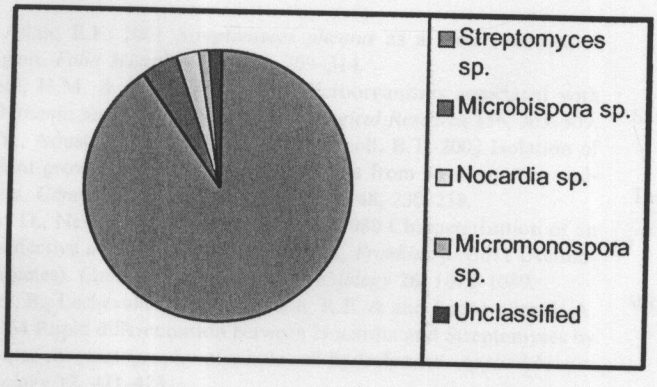


*Figure 2.* The frequency of different actinomycete types isolated from all the plant types investigated.

*Table 2.* The highest prevalence of actinomycete isolates and the specific plants, based on isolations made on HV agar.

| Isolates[a] | Host plant | Highest prevalence |
|---|---|---|
| *Streptomyces* sp. | *Zingiber officinale* | 6.44% |
| *Microbispora* sp. | *Alpinia galanga* | 0.66% |
| *Nocardia* sp. | *Cyperus malaccensis* | 0.44% |
| *Micromonospora* sp. | *Alpinia galanga*, *Boesenbergia pandurata*, *Curcuma domestica* and *Echinochloa colona* | 0.22% |

[a] The most frequently isolated actinomycetes from the specific plants.

*Z. officinale* to 0.2% for *Brassica juncea* (Cruciferae) and for *Citrus hystrix* (Rutaceae). Values of *Microbispora* sp., *Nocardia* sp. and *Micromonospora* sp. were much lower (Table 2). These results indicate that herbaceous plants are the major host for endophytic actinomycetes, with *Streptomyces* spp. being dominant. In contrast, this study has shown that actinomycetes are found only rarely in tree species such as *Citrus aurantifolia* (Rutaceae), *Dimocarpus longana* (Sapindaceae), *Streblus asper* (Moraceae), *Tamarindus indica* (Leguminosae) and *Mangifera indica* (Anacardiaceae).

The antifungal activity of endophytic actinomycete isolates is shown in Table 3. The majority of the isolates (>200) appeared not to produce secondary metabolites which displayed antifungal activity against the two test fungi. The remaining isolates could be divided into five categories according to the size of the growth-inhibition zones produced. This survey revealed that only a small number were strongly inhibitory to *C. musae* and *F. oxysporum* (Figure 3). In a similar study, Sardi *et al.* (1992) obtained ca. 500 isolates from the roots of 13 plant species and most of these were *Streptomyces* sp. They classified these isolates into 72 groups based on their characteristics. After testing antimicrobial activity

*Table 3.* Antifungal activity of endophytic actinomycetes isolates against *C. musae* and *F. oxysporum*.

| Potential antifungal activity | Number of endophytic actinomycetes isolates (%) against tested fungi | | |
|---|---|---|---|
| | *Colletotrichum musae* | *Fusarium oxysporum* | *Colletotrichum musae* and *Fusarium oxysporum* |
| ++++[a] | 3 (0.9%)[b] | 5 (1.5%)[b] | 2 (0.6%)[b] |
| +++ | 10 (3.0%) | 18 (5.4%) | 8 (2.4%) |
| ++ | 44 (13.3%) | 53 (16.0%) | 36 (10.9%) |
| + | 10 (3.0%) | 16 (4.8%) | 8 (2.4%) |
| Not active | 240 (72.7%) | 215 (65.1%) | 253 (76.6%) |

The potential of antifungal activity was evaluated by the zone of fungal growth inhibition on ISP-2 medium after incubation at 30 °C for 7 days.

[a] ++++: Width of growth inhibition zone >20 mm.
+++: 10–20 mm.
++: 1–10 mm.
+: <1 mm.

[b] These isolates were identified to be *Streptomyces* sp.

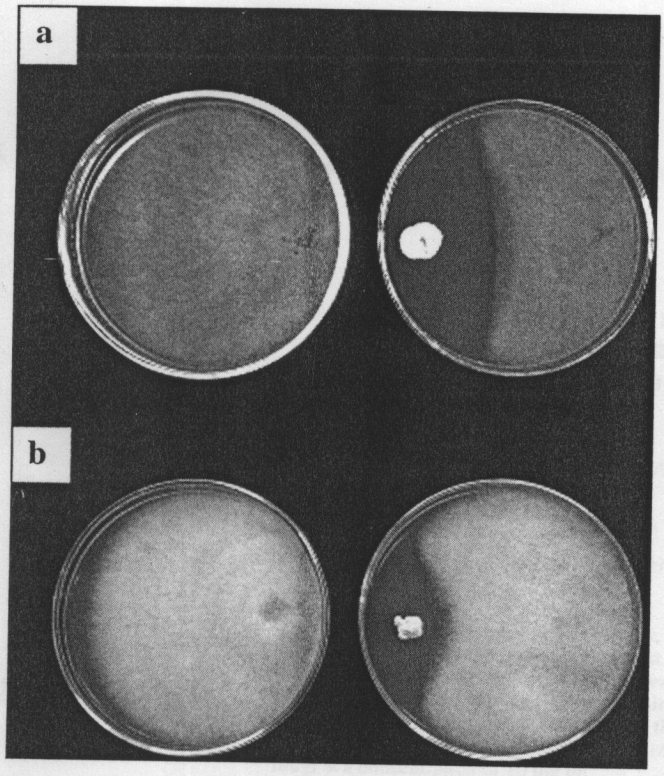


*Figure 3.* Zones of growth inhibition caused by metabolites from isolate CMUAc130, grown on ISP-2 medium for 7 days, against (a) *Colletotrichum musae* and (b) *Fusarium oxysporum*.

of 10 groups against *Micrococcus luteus* and *F. oxysporum*, then found that all groups had antimicrobial activity against one or the other organisms, but not to both. Thus most of their isolates had a narrow antimicrobial spectrum. From the present study results of *in vitro* antifungal activity (Table 3), only two endophytic actinomycetes isolates had a strong potential of antifungal activity to *Colletotrichum musae* and *Fusarium oxysporum*. These results demonstrated that some of endophytic actinomycetes have the potential for inhibiting the growth of tested phytopathogenic fungi. However, more detailed investigation is required to demonstrate the potential of these organisms in the biocontrol of plant diseases.

## References


Abd-Allah, E.F. 2001 *Streptomyces plicatus* as a model biocontrol agent. *Folia Microbiologica* **46**, 309–314.

Alwadi, H.M. & Baka, Z.A. 2001 Microorganisms associated with *Withania somnifera* leaves. *Microbiological Research* **156**, 303–309.

Bai, Y., Aoust, F.D., Smith, D.L. & Driscoll, B.T. 2002 Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Canadian Journal of Microbiology* **48**, 230–238.

Baker, D., Newcomb, W. & Torrey, J.G. 1980 Characterization of an ineffective actinorhizal microsymbiont, *Frankia* sp. Eu11 (Actinomycetes). *Canadian Journal of Microbiology* **26**, 1072–1089.

Becker, B., Lechevalier, M.P., Gordon, R.E. & and Lechevalier, H.A. 1964 Rapid differentiation between Nocardia and Streptomyces by paper chromatography of whole-cell hydrolysates. *Applied Microbiology* **12**, 421–423.

Boone, C.J. & Pine, L. 1968 Rapid method for characterization of actinomycetes by cell wall composition. *Applied Microbiology* **16**, 279–284.

Bussaban, B., Lumyong, S., Lumyong, P., McKenzie, E.H.C. & Hyde, K.D. 2001 Endophytic fungi from *Amomum siamense*. *Canadian Journal of Microbiology* **47**, 1–6.

Drautz, H. & Zahner, H. 1986 New microbial metabolites. In *Biological, Biochemical and Biomedical Aspects of Actinomycetes*, eds. Szabo, G., Biro, S. & Goodfellow, M. pp. 227–234. Budapest: Akademiai Kiado. ISBN 96-3054395-8.

Garbeva, P., van Overbeek, L.S., van Vuurde, J.W. & Elsas, J.D. 2001 Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16SrDNA based PCR fragments. *Microbial Ecology* **41**, 369–383.

Getha, K. & Vikineswary, S. 2002 Antagonistic effects *of Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f.sp. *cubense* race 4: Indirect evidence for the role of antibiosis in the antagonistic process. *Journal of Industrial Microbiology and Biotechnology* **28**, 303–310.

Katznelson, H. & Cole, S.E. 1965 Production of gibberellin-like substances by bacteria and actinomycetes. *Canadian Journal of Microbiology* **11**, 733–741.

Knowlton, S., Berry, A. & and Torrey, J.G. 1980 Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. *Canadian Journal of Microbiology* **26**, 970–977.

Mishra, S.K., Taft, W.H., Putnam, A.R. & Ries, S.K. 1987 Plant growth regulatory metabolites from novel actinomycetes. *Journal of Plant Growth Regulation* **6**, 75–84.

Okazaki, T., Takahashi, K., Kizuka, M. & Enokita, R. 1995 Studies on actinomycetes isolated from plant leaves. *Annual Report of the Sankyo Research Laboratory* **47**, 97–106.

Otoguro, M., Hayakawa, M., Yamazaki, T. & Iimura, Y. 2001 An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter. *Journal of Applied Microbiology* **91**, 118–130.

Petrolini, B., Quaroni, S. & Saracchi, M. 1986 Scanning electron microscopy investigations on the relationships between bacteria and plant tissues. I. Comparative techniques for specimen preparation. *Rivue Patologie Vegetale* S. **22**, 7–15.

Redshow, P.A., McCann, P.A., Sankaran, L. & Pogell, B.M. 1976 Control of differentiation in streptomyces: involvement of extrachromosomal deoxyribonucleic acid and glucose repression in aerial mycelia development. *Journal of Bacteriology* **125**, 698–705.

Sardi, P., Saracchi, M., Ouaroni, S., Petrolini, B., Borgonovoli, G.E. & Merli, S. 1992 Isolation of endophytic *Streptomyces* from surface-sterilized roots. *Applied and Environmental Microbiology* **58**, 2691–2693.

Schippers, B., Bakker, A.W. & Bakker, P.A.H.M. 1987 Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual Review of Phytopathology* **25**, 339–358.

Shimizu, M., Nakagawa, Y., Sato, Y., Furumai, T., Igarashi, Y., Onaka, H., Yoshida, R. & Kunoh, H. 2000 Studies on endophytic actinomycetes (I) *Streptomyces* sp. isolated from Rhododendron and its antifungal activity. *Journal of General Plant Pathology* **66**, 360–366.

Shirling, E.B. & Gottlieb, D. 1966 Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology* **16**, 313–340.

Tahvonen, R. 1982 Preliminary experiments into the use of *Streptomyces* spp. isolated from peat in the biological control of soil and seed-borne diseases in peat culture. *Journal of the Scientific Agricultural Society of Finland* **54**, 357–369.

Williams, S.T., Lanning, S. & Wellington, E.M.H. 1984 Ecology of actinomycetes In *The Biology of the Actinomycetes*, eds. Goodfellow, M., Mordarski, M. & Williams, S.T. pp. 481–528. London: Academic Press, Inc. ISBN 0-12-289670-X.

Annals of Microbiology, 53 (3), 25-36 (2003)

# Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galanga* against phytopathogenic fungi

T. TAECHOWISAN*, S. LUMYONG

Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

*Received 18 April 2003 / Accepted 20 June 2003*

**Abstract** – Of 59 endophytic actinomycetes, were isolated from the roots of *Zingiber officinale* and *Alpinia galanga*, and tested against *Candida albicans* and phytopathogenic fungi, *Colletotrichum musae* and *Fusarium oxysporum*, ten produced substances that inhibited both phytopathogens and nine had activity against *Candida albicans*. The strain identified as *Streptomyces aureofaciens* CMUAc130 was the most effective in antifungal activity amongst those investigated. Moreover, the extracts of its ferment broth had activity against tested fungi at a concentration of 10 mg $ml^{-1}$.

**Key words**: *Alpinia galanga*, antifungal activity, endophytic actinomycetes, *Zingiber officinale*.

## INTRODUCTION

Inside the tissue of nearly all the healthy plants, there are many endophytic microorganisms. Endophytes are synergistic to their host, at least some of them are thought to be making returns for the nutrition from the plant by producing special substances such as secondary metabolites to prevent the host from successfully attacking fungi and pests. The metabolites of endophytes inhibit a number of microorganisms (Gurney and Mantle 1993). Endophytic actinomycetes from the various plant tissues have antifungal activity (Sardi *et al.*, 1992; Shimizu *et al.*, 2000). To the best of our knowledge, *Zingiber officinale* Rosc. (Zingiberaceae) and *Alpinia galanga* Swartz. (Zingiberaceae), well known for their root extract substances, found these substances to be resistant to bacteria and fungi (Habsah *et al.*, 2000). Are there the endophytic actinomycetes in the root of these plants? Is the resistance associated with the presence of endophytes? Are the endophytes capable of producing antifungal compounds that could be used for controlling the phytopathogenic fungi? We therefore undertook the present study to provide answers to these questions.

* Corresponding author. E-mail: tewson84@hotmail.com

## MATERIALS AND METHODS

**Sample collection.** Two hundred samples of the root tissues of *Zingiber officinale* and *Alpinia galanga* were collected from the environs of Chiang Mai, Thailand during the period of September, 2001 – February, 2002. The most of them were healthy roots.

**Isolation of actinomycetes.** The samples were washed in running tap water and cut into small pieces of ca. 4 x 4 $mm^2$. Tissue pieces were rinsed in 0.1% Tween 20 for 30 s, then in 1% sodium hypochlorite for 5 min, and then washed in sterile distilled water 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of humic acid-vitamin (HV) agar (Otoguro *et al.*, 2001) containing 100 $\mu g\ ml^{-1}$ nystatin and cycloheximide, and incubated at 30 °C for 1 month. The colonies were inoculated onto an International Streptomyces Project-2 (ISP-2) medium (Shirling and Gottlieb, 1966) for purification. The isolated colonies were subcultured onto Hickey-Tresner (HT) medium (Redshow *et al.*, 1976) slant to establish stock cultures.

**Morphological observations.** The actinomycetes isolates were cultured on ISP-2 agar plates at 30 °C for 3 days then the cover slide was fixed down the actinomycetes colony and incubated at 30 °C for further 5 days. The actinomycetes isolates grew on the cover slide was stained with crystal violet for 1 min. The morphology of actinomycetes was observed under light microscope. Root materials from *Z. officinale* and *A. galanga* were selected for microscopic observation by scanning electron microscopy (SEM) (JEOL-JSM840A SEM, Tokyo, Japan). Specimens were washed several times using distilled water and fixed overnight in 2.5% glutaraldehyde at 4 °C. They were then dehydrated in a graded alcohol series (30-95%) followed by treatment in acetone and critical-point drying (Petrolini *et al.*, 1986). The specimens from each process were mounted on stubs, splutter-coated with gold, and viewed on the SEM at an accelerating voltage of 20 KV. Photomicrographs were recorded on Kodak VP200 film (New York, USA).

**Antifungal activity of endophytic actinomycetes isolates against phytopathogenic fungi and yeast.** The endophytic isolates were cultured on plates on ISP-2. Two fungal pathogens *Colletotrichium musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively, were used for screening antifungal activity. They were grown on potato dextrose agar (PDA). Mycelial disks of 6 mm diameter were cut from the plates with the two pathogens and transferred to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony. For antagonistic action to *Candida albicans* ATCC90028, *C. albicans* were cultured in ISP-2 broth at 30 °C for 24 h, the cells were diluted to $10^5$ cells $ml^{-1}$ in soft agar, then were overlayed on pre-grown actinomycete colony on ISP-2 plate. The plates were incubated at 30 °C for 5-7 days. The width of inhibition zones between the pathogen and the actinomycete isolates was measured and evaluated as follows: +++, ≥20 mm; ++, 11-19 mm; +, 2-10 mm; ±, ≤1 mm; -, 0 mm.

**Identification of selected strain.** Endophytic actinomycetes CMUAc130 was selected for identification. For morphological characteristics, the presence of aerial mycelium, spore mass color, distinctive reverse colony color, diffusible pigment, sporophore and spore chain morphology were recorded after 10 days incubation on ISP-2 medium. Diaminopimelic acid isomers and sugars from whole-cell extract were analyzed for chemotaxonomic studies (Becker *et al.*, 1964; Boone and Pine, 1968).

**16S rDNA gene sequencing.** Genomic DNA was isolated from the endophytic actinomycetes CMUAc130 by using a procedure (Hopwood *et al.*, 1985). 16S rDNA was amplified by PCR using *Pfu* DNA polymerase (Promega, USA) and primers A 7-26f (5'-CCGTCGACGAGCTCAGAGTTTGATCCTGGCTCAG-3') and primers B 1523-1504r (5'-CCCGGGTACCAAGCTTAAGGAGGTGATCCAGCCGCA-3'). The conditions used for thermal cycling were as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C 10 min. At the end of the cycles, the reaction mixture was kept at 72 °C for 10 min and then cooled to 4 °C. The 1.5 Kb amplified 16S rDNA fragment was separated by agarose gel electrophoresis and purified by using a QiAquick gel extraction kit (QIAGEN, Germany). The purified fragments were directly sequenced by using dRhodamine dye terminator cycle sequencing kit (Applied Biosystems, USA). The sequencing primers were primer A, primer B, primer C 704-685r (5'-TCTGCGCATTTCACCGCTAC-3') and primer D 1115-1100r (5'-AGGGTTGCGCTCGTTG-3'). Sequencing was performed with a model 310 automatic sequencer (Applied Biosystems).

**Sequencing alignment and phylogenetic analysis.** Reference strains were chosen from BLAST (Altschul *et al.*, 1997) search results. Multiple alignments of sequence determined in this study together with reference sequences obtained from databases and calculations of levels of sequence similarity were carried out using CLUSTAL W 1.74 (Higgins *et al.*, 1992). A phylogenetic tree was reconstructed by using treeing algorithms contained in the PHYLIP package (Felsenstein, 1995). The topology of the neighbour-joining phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (Felsenstein, 1995) with 1000 replicates.

**Antifungal activity of endophytic actinomyces fermented broth.** Among the 59 isolates of endophytic actinomycetes, the isolate CMUAc130 was found to be the best producer of antifungal substances. This isolate was selected for an antifungal activity assay by fermented broth. The inoculum was prepared by introducing the 7-day-old Petri dish culture into 500 ml flasks containing 200 ml of ISP-2 broth, followed by shaking (100 rev $min^{-1}$) continuously for 3 days at 30 °C. The follow-up fermentation was accomplished by adding the inoculum (50 ml) into a modified 1000 ml glass container containing 550 ml of ISP-2 broth, and then reciprocal shaking for 5 days in the same condition. The broth was filtered by Whatman paper No.1 and the filtrate was extracted with ethyl acetate by the evaporator (R-124 BUCHI Rotavapor R-124, Switzerland). The obtained extract was tested at a concentration of 5, 10, 30 mg $ml^{-1}$ (dry extract per volume) by dimethylsulfoxide for

the antifungal activity against phytopathogenic fungi and yeast with the whole plate diffusion method (Nishioka *et al.*, 1997) using 100 µg $ml^{-1}$ nystatin as a positive control. The plates were then incubated at 30 °C for 5 days for fungi and for 24 h for yeast. Each inhibition experiment was replicated thrice.

## RESULTS AND DISCUSSION

The endophytic actinomycetes on the root of host plant were examined by direct scanning electron microscopy (Fig. 1) and by isolation on agar plates. The large number of *Streptomyces* strains isolated from healthy plants and the direct scanning electron microscopy investigations on internal tissues show that there is a close relationship between these microorganisms and roots, in which actinomycete hyphal growth could have a favorable effect. Fifty nine endophytic actinomycetes, isolated from root tissues of *Z. officinale* and *A. galanga*, antagonized the tested phytopathogens and yeast in strikingly different manner. Among them, ten isolates could produce the substances antagonistic against *Colletotrichum musae* and *Fusarium oxysporum* and 9 isolates against *Candida albicans* (the width of growth inhibition zone: 35.7 + 5.4, 28.4 + 7.3 and 21.5 + 3.8 mm, respectively). The prevalence of actinomycetes isolation was 29.5%. Shimizu *et al.* (2000) obtained 10 isolates from the root tissues of *Rhododendron* sp., and in comparison Sardi *et al.* (1992) obtained 499 isolates from 640 root tissues of 13 plant species. Antimicrobial activities of the chemical biosynthesized by the plant endophytes have been reported. Most of them were produced by endophytic fungi (Nishioka *et al.*, 1997; Fisher *et al.*, 1984, 1986; McGee *et al.*, 1991; Liu *et al.*, 2001). The present results indicated that the metabolites of endophytic actinomycetes from root tissues of *Z. officinale* and *A. galanga* have extensive inhibition to the growth of phytopatho-

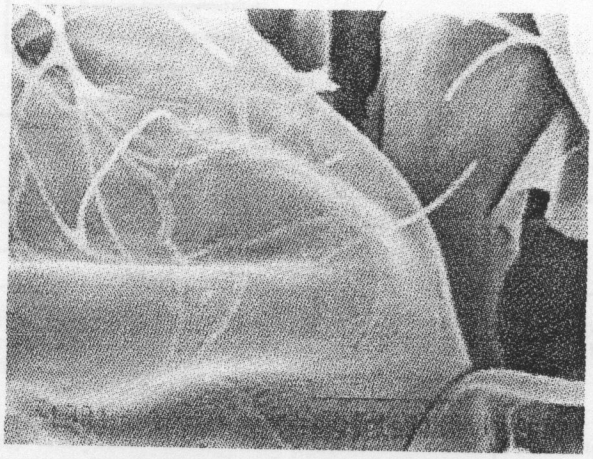

FIG. 1 – Scanning electron micrograph of aerial hyphae of actinomycetes which have grown through the epidermis of a root of *Zingiber officinale*. Magnification: 3,000X. Bar = 10 µm.

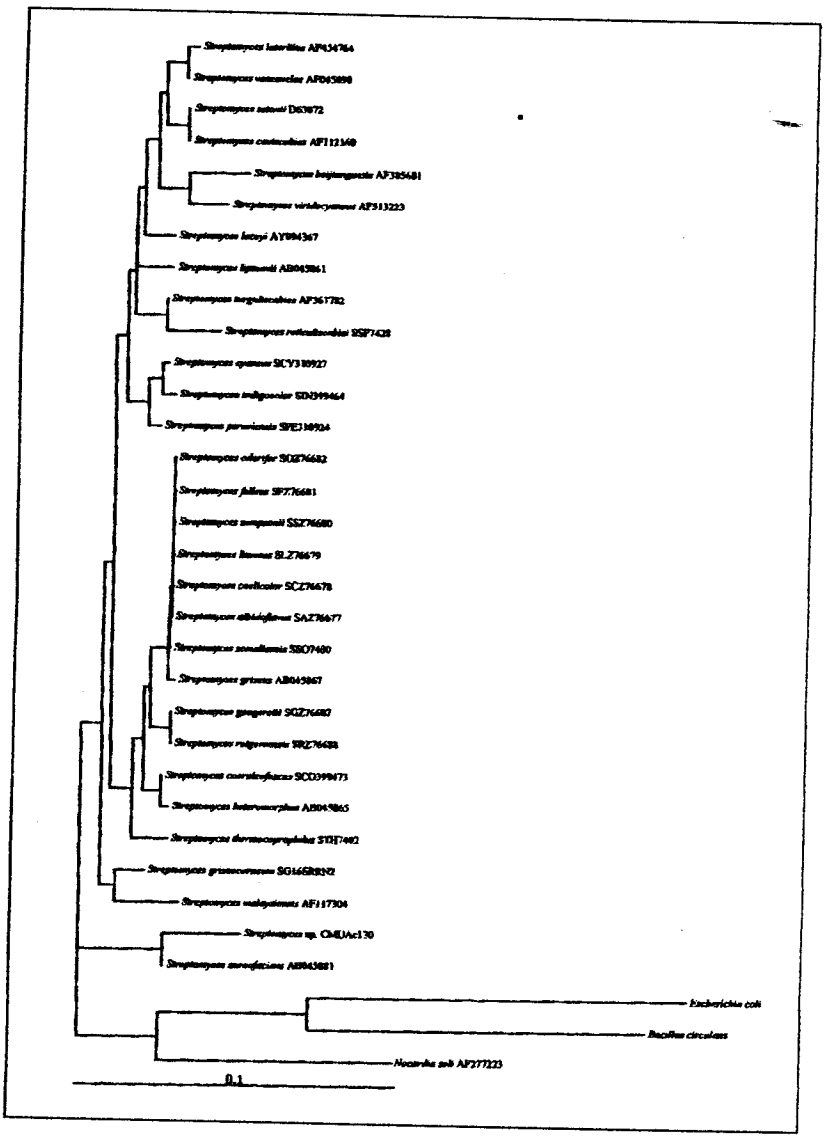


FIG. 2 – Phylogenetic tree showing the relationships of endophytic *Streptomyces aureofaciens* CMUAc130, related species of the same genus and other taxa based on 16S rDNA genes sequences.

genic fungi and yeast. The isolate CMUAc130 showed promissing activities against *C. musae*, *F. oxysporum* and *C. albicans*. It is highly desired to characterize from the ethyl acetate extract of the ferment broth new antifungal principle(s) which could be used as lead compound(s) for the development of new fungicide(s)

necessitated for the control of plant diseases. However the large amount of endophytic actinomycetes isolates had no potential of antifungal activity to the tested phytopathogenic fungi. As stated in several reports, actinomycetes activity in plants not only protect against pathogens but also influence the metabolic products of actinomycetes on plant growth and physiology (Katznelson and Cole, 1965; Mishra *et al.*, 1987). Further investigations are therefore necessary to understand the other forms of relationship between endophytic actinomycetes and plants tissues and the usefulness of this phenomenon in agriculture. However, it needs more investigation to disclose the possibility that those endophytic actinomycetes would be used for biocontrol of the related plant diseases by introducing them into the crops such as wheat.

Based on results in morphological observation as well as on the presence of LL-type diaminopimelic acid in the whole-cell extracts, endophytic actinomycetes CMUAc130 was identified as belonging to the genus *Streptomyces*. Almost the complete 16S rDNA sequence was determined for the endophytic *Streptomyces* sp. CMUAc130 (>95% of the *Escherichia coli* sequence) from position 8 to position 1523 (*E. coli* numbering system; Brosius *et al.*, 1978). BLAST search results for strain CMUAc130 came from non-redundant GenBank + EMBL + DDBJ; when reference sequences were chosen, unidentified and unpublished sequences were excluded. The BLAST search results and the phylogenetic tree (Fig. 2) generated from representative strains of the related genera showed that strain CMUAc130 had high levels of sequence similarity to species of *S. aureofaciens* (accession number: AB045881). 16S rDNA analysis revealed that strain CMUAc130 is phylogenetically closely related to *S. aureofaciens* (the sequence similarity levels were 97%). The nucleotide sequence data reported in this paper appeared in the GenBank, EMBL and DDBJ databases with the accession number AB105068. This isolate producing the most active substance, was fermented to get more broth, the ethyl acetate extracts of which exhibited a broad antifungal spectrum. Even at a

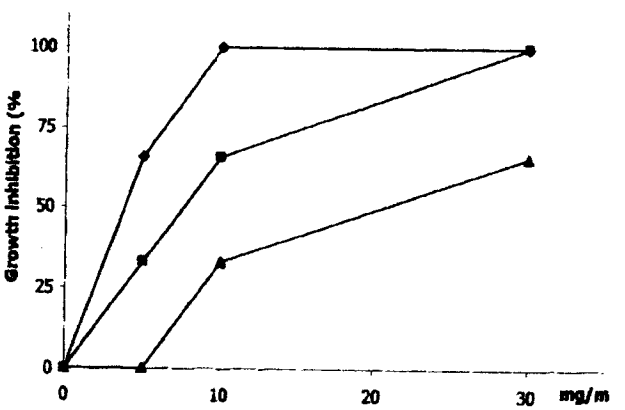


FIG. 3 – Percentage of growth inhibition of phytopathogenic fungi and yeast by ethyl acetate extract of the isolate CMUAc130 at different concentrations, *Colletotrichium musae* (◆), *Fusarium oxysporum* (■) and *Candida albicans* (▲).

concentration of 10 mg $ml^{-1}$ (dried extract/volume), the extract showed discernible growth inhibitions to *C. musae* and *F. oxysporum*. Furthermore, the MICs of ethyl acetate extract of CMUAc130 culture against these fungi was determined to be 5, 10 and 30 mg $ml^{-1}$, respectively (Fig. 3). Endophytic *S. aureofaciens* CMUAc130 with a broad antifungal spectrum is considered as a valuable candidate of biocontrol agent of some plant diseases. A unique, effective use of endophytic *S. aureofaciens* CMUAc130 for disease control of tissue-cultured seedlings of some plants will be investigated elsewhere.

**Acknowledgements**
Funds for this research were provided by Biodiversity and Training Program (BRT T_646003/0295), Thailand and the graduate school of Chiang Mai University, Chiang Mai, Thailand.

## REFERENCES

Altschul S.F., Madden T.L., Schaffer A.A., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., 25: 3389-3402.

Becker B., Lechevalier M.P., Gordon R.E., Lechevalier H.A. (1964). Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol., 12: 421-423.

Boone C.J. and Pine L., (1968). Rapid method for characterization of actinomycetes by cell wall composition. Appl. Microbiol., 16: 279-284.

Brosius J., Palmer M.L., Kennedy P.J., Noller H.F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA., 75: 4801-4805.

Felsenstein J. (1995). PHYLIP (phylogenetic inference package) version 3.57c. Department of Genentics, University of Washington, Seattle, WA, USA.

Fisher P.J., Anson A.E., Petrini O. (1984). Antibiotic activity of some endophytic fungi from ericaceous plants. Bot. Helv., 94: 249-253.

Fisher P.J., Anson A.E., Petrini O. (1986). Fungal endophytes in *Ulex europaeus* and *Ulex galli*. Trans. Brit. Mycol. Soc., 86: 153-156.

Gurney K.A. and Mantle P.G. (1993). Biosynthesis of 1-N-methylalbonoursin by an endophytic *Streptomyces* sp. isolated from perennial ryegrass. J. Nat. Prod. (Lloydia), 56: 1194-1198.

Habsah M., Amran M., Mackeen M.M., et al. (2000). Screening of *Zingiberaceae* extracts for antimicrobial and antioxidant activities. J. Ethnopharmacol., 72: 403-410.

Higgins D.G., Bleasby A.J., Fuchs R. (1992). CLUSTAL V: improved software for multiple sequence alignment. Comput. Appl. Biosci., 8: 189-191.

Hopwood D.A., Bibb M.J., Chater K.F., Kieser T., Bruton C.J., Kieser H.M., Lydiate D.J., Smith C.P., Ward J.M. (1985). Genetic manipulation of *Streptomyces* - a laboratory manual. In Preparation of Chromosomal, Plasmid and Phage DNA, Norwich: F. Crowe & Sons, pp. 79-80.

Katznelson H., Cole S.E. (1965). Production of gibberellin-like substances by bacteria and actinomycetes. Can. J. Microbiol., 11: 733-741.

Liu C.H., Zou W.X., Ren H.L., Tan R.X. (2001). Antifungal activity of *Artemisia annua* endophyte cultures against phytopathogenic fungi. J. Biotechnol., 88: 277-282.

McGee P.A., Hincksman M.A., White C.S. (1991). Inhibition of growth of fungi isolated from plants by *Acremonium strictum*. Aust. J. Agric. Res., 42: 1187-1194.

Mishra S.K., Taft W.H., Putnam A.R., Ries S.K. (1987). Plant growth regulatory metabolites from novel actinomycetes. J. Plant Growth Regul., 6: 75-84.

Nishioka M., Furuya N., Nakashima N., Matsuyama N. (1997). Antibacterial activity of metabolites produced by *Erwinia* spp. against various phytopathogenic bacteria. Ann. Phytopathol. Soc. Jpn., 63: 99-102.

Otoguro M., Hayakawa M., Yamazaki T., Iimura Y., (2001). An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter. J. Appl. Microbiol., 91: 118-130.

Petrolini B., Quaroni S., Saracchi M. (1986). Scanning electron microscopy investigations on the relationships between bacteria and plant tissues. I. Comparative techniques for specimen preparation. Riv. Pat. Veg., SIV., 22: 7-15.

Redshow P.A., McCann P.A., Sankaran L., Pogell B.M. (1976). Control of differentiation in *Streptomyces*: involvement of extrachromosomal deoxyribonucleic acid and glucose repression in aerial mycelia development. J. Bacteriol., 125: 698-705.

Sardi P., Saracchi M., Quaroni S., Petrolini B., Borgonovi G.E., Merli S. (1992). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. Appl. Environ. Microbiol., 58: 2691-2693.

Shimizu M., Nakagawa Y., Sato Y., Furumai T., Igarashi, Y., Onaka H., Yoshida R., Kunoh H. (2000). Studies on endophytic actinomycetes (I) *Streptomyces* sp. Isolated from *Rhododendron* and its antifungal activity. J. Gen. Plant Pathol., 66: 360-366.

Shirling E.B., Gottlieb D. (1966). Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol., 16: 313-340.