CLONING OF ENDOCHITINASE GENE FROM NATIVE ISOLATES OF *Trichoderma* Sp.

AISHWARYA R. ANIGOL

DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES,
DHARWAD – 580 005

SEPTEMBER - 2004

CLONING OF ENDOCHITINASE GENE FROM NATIVE ISOLATES OF *Trichoderma* Sp.

Thesis Submitted to the
University of Agricultural Sciences, Dharwad
in partial fulfillment of the requirements for the
Degree of

MASTER OF SCIENCE (AGRICULTURE)
IN
PLANT BIOTECHNOLOGY

By

AISHWARYA R. ANIGOL

DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES,
DHARWAD – 580 005

SEPTEMBER - 2004

DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES
DHARWAD

CERTIFICATE

This is to certify that thesis entitled **"CLONING OF ENDOCHITINASE GENE FROM NATIVE ISOLATES OF *Trichoderma* Sp."** submitted by **AISHWARYA R. ANIGOL**, for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** of the University of Agricultural Sciences, Dharwad is a record of research work done by her during the period of her study in this university, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

DHARWAD
SEPTEMBER,2004.

(SUMANGALA BHAT)
MAJOR ADVISOR

Approved by :

Chairman ----------
(SUMANGALA BHAT)

Members
1. ---------- 13/12/04
(M. S. KURUVINASHETTI)

2. ----------
(P. U. KRISHNARAJ)

3. ----------
(M. G. PALAKSHAPPA)

4. Shirnalli ----------
(GEETA SHIRNHALLI)

ACKNOWLEDGEMENT

The satisfaction that I feel at the successful completion of my research work would be incomplete if I do not mention the people whose able guidance, suggestion and encouragement crowned my effort with success.

I express my deep sense of gratitude to ***Dr. Sumangala Bhat,*** *Assistant Professor, Department of Biotechnology, University of Agricultural Sciences, Dharwad and esteemed chairman of my advisory committee for selecting me and guiding me throughout the wonderful project.*

I take this opportunity to thank ***Dr. M.S. Kuruvinashetti,*** *Professor and Head, Department of Biotechnology, University of Agricultural Sciences, Dharwad;* ***Dr. P.U. Krishnaraj,*** *Assistant Professor (Sr. Scale), Department of Biotechnology, University of Agricultural Sciences, Dharwad;* ***Dr. Geeta Shirnhalli,*** *Senior Microbiologist, Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad;* ***Dr. M.G. Palakshappa,*** *Senior Scientist, MARS, Dharwad esteemed members of my advisory committee for critically going through the manuscript and making suitable corrections.*

I am even grateful and thankful to ***Dr. Ramesh Bhat,*** *Assistant Professor;* ***Dr. Narayan Moger,*** *Assistant Professor;* ***Dr. B. Fakruddin,*** *Assistant Professor,* ***Dr. Sheshagiri,*** *Department of Biotechnology and all*

the teachers of University of Agricultural Sciences, Dharwad, for their instantaneous help and valuable suggestions during my tenure of studies.

This thesis would be incomplete if I do not reckon the sacrifices, love, affection and support of my family members, mother ***Smt. Malati R. Anigol****, father* ***Shri. Ramling F. Anigol*** *and brothers* ***Praphul*** *and* ***Sharat****.*

I lack in words to express my deep sense of gratitude to my beloved friends, Ruku, Laxmi, Bharati, Shobha, Saru, Sangeetha, Arati, Mamata, Priya, Ashwini, Anjana, Jayapadma, Aseem, Anand and Kumaraswamy for their affection, kindness and best wishes throughout my educational endeavor.

I also extend my loving gratitude to ever loving kids of the neighbourhood, Arpita, Nikita, Poorvi, Aishwarya, Shweta, Komal, Rahul, Ananya and to Rakhee brothers, Kiran, Maltesh, Praveen, Sachin and Chandrakant.

I am strongly beholden to my friends Abdur, Rajkumar, Mahender, Sureshbabu, Sangamesh, Sandeep, Saijyoti, Nazneen, Hadapad, Dadapeer, Krishnamurthy, Kassahun; my Seniors, Mangala, Basavanna, Sanjeev, Veena, Ashajyoti, Somashekhar, Padmavati, Kotresh and Junior friends, Upendra, Ziya, Savita, Ashok, Spoorthi and Poornima for their kind co-operation and timely help throughout the study, Marithammavar (Sr. Lab. Assistant), Satish, Yellappa, Santosh, Yellamma and Nagamma, all who helped me in different ways that could not be enumerated.

Finally I thank all my team for bestowing me with essential strength and necessary succor which moulded me into a person of substance amidst several hurdles.

Last but not least, I express my heartfelt thanks to ***Mr. Vinay (Basavanna Graphics)*** *for neat and timely preparation of this manuscript.*

DHARWAD
SEPT. 2004

(AISHWARYA R. ANIGOL)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

I. INTRODUCTION

Worldwide the demand for food is growing as populations expand. According to UN figures, the world's population is expected to increase by 35 per cent to 7.7×10^9 by 2020, mostly in developing countries. Global food security is a major goal for developing and developed nations to feed the ever-growing population. There are chronic shortages of food in many regions of the world.

Biotic stresses take a heavy toll of the 5 billion tonnes of food currently produced every year. Plant diseases alone reduce global food production by more than 10 per cent. Indiscriminate use of chemicals to overcome the pathogens has caused enhancement of overhead costs, accumulation of toxic chemical residues in food chain and soil pollution leading to loss of soil health. Apart from this, the chemicals tend to become less efficient due to the evolution of resistance among the pathogens. Under these circumstances, the use of various eco-friendly bio-control agents is increasingly being stressed as an important component of the integrated pest management.

Among the many biocontrol agents available, *Trichoderma* spp. has proved effective and against most of the diseases. Species belonging to the genus *Trichoderma* are fascinating fungi, producing a number of fungal cell wall degrading enzymes and different antifungal compounds, which are responsible for inhibition of pathogens. Addition of chitinolytic or glucanolytic enzymes from *T. harzianum* strain P1 (Lorito *et al.*, 1994) and *Gliocladium virens* (Di Pietro *et al.*, 1993) to the reaction mixture

synergistically enhanced the antifungal properties of five different fungistatic compounds against *Botrytis cinerea.*

The chitinolytic system of fungi catalyses the breakdown of chitin, an insoluble linear ß-1, 4 linked homopolymer of N-acetylglucosamine (GlcNAc). Numerous investigations on "pathogenesis related proteins" implicated in plant host resistance against pathogenic fungi are identified as chitinases (Huynh *et al.*, 1992; Verburg and Hunynh 1991). In plants, induction of chitinase and other hydrolytic enzymes is one of the co-ordinated, often complex and multifaceted defense mechanism triggered in response to phytopathogen attack.

There are many commercial formulations of these biocontrol agents available for using them for soil amendment to combat the soil borne phytopathogens. Genetic transformation of existing biocontrol fungi that are well adjusted to their environment is likely to enhance their biocontrol capabilities. One attractive approach would be to select the stable and safe transgenic fungi with different genes (Chitinase and Glucanase), which express co-ordinately to combat the phytopathogens.

Despite the interest in these biocontrol fungi, their application is limited due to the low field performance. Fungal chitinases (and the genes encoding them) appear to be more effective in their ability to inhibit pathogenic fungi than enzymes from other sources. The modification of plant lines with new chitinase genes has increased resistance against several fungal pathogens and may point to a generally practiced approach to controlling crop losses due to fungi (Graham and Sticklen 1994).

Therefore the present study was conducted with the following objectives;

1. Screen the available fungal isolates for chitinase activity.

2. Clone an endochitinase gene from one of these fungi.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Agriculture is the backbone of Indian economy. Achievement of higher productivity and production to meet the everlasting demand of food commodities is the need of the hour. Loss due to the pests is one of the major limiting factors for sustained increase in crop productivity and production. On an average, the avoidable crop loss caused by pests such as insects, diseases, weeds etc., in India has been estimated to range from 10-30 per cent of the total production. Plant diseases alone reduce global food production by more than 10 per cent.

Most of the agricultural and horticultural crop species suffer severe yield losses due to fungal attack. In the Indian context, fungal diseases are rated either the most important or the second most important factor contributing to yield losses of major cereals, pulses and oilseed crops.

2.1 STRATEGIES TO CONTROL PLANT DISEASES

Different strategies of disease control include resistant crop varieties, changing cultural practices or storage conditions less favorable for pathogen attack and survival, employing biological controls, applying chemical pesticides or a combination of these in integrated pest management.

Currently, major method of control is the use of chemical pesticides. However, greater use of these pesticides may contaminate the food or accumulate in the soil and ground water and be introduced into the food chain. However, non-chemical approaches do exist. They are

less effective, or are too costly. Therefore biological control shows great potential for disease control.

2.2 *Trichoderma* SPECIES AS BIOCONTROL AGENTS

Different biocontrol agents can be used for the control of diseases. These include many of bacteria, fungi and actinomycetes. Frequently studied fungi in relation to biological control are mostly species of *Trichoderma.* Gaffer (1968) reported that *T. viride* Pers. inhibited the growth of *Macrophomina phaseolina.* Elad *et al.* (1986) reported inhibition of linear growth and sclerotial production of *M. phaseolina* by four isolates of *Trichoderma harzianum* Rifai. Anon (1999) reported the effective control of charcoal rot in sorghum when *T. viride* followed by *T. harzianum* were used. Similarly, Reddy and Hindumathi (1997) reported, 80 percent reduction of sorghum charcoal rot in susceptible variety CSV-8R through use of vesicular arbuscular mycorrhizal fungi. Several reports are available on antagonistic potential of *Trichoderma, Gliocladium* etc. against *M. phaseolina* and related soil borne sclerotial fungal pathogens (El-Katatny *et al.*, 2000). The mode of action of *Trichoderma* is suggested to be either by mycoparasitism, competition, antibiosis or a combination of all these (Elad, 1996). A commercial biocontrol agent, isolate T39 of *Trichoderma harzianum*, can be regarded as a model to demonstrate biocontrol and the mechanisms involved. This biocontrol agent controls the foliar pathogens, *Botrytis cineria, Pseudoperonospora cubensis, Sclerotinia sclerotiorum and Sphaerothica fusca* in cucumber under commercial greenhouse conditions. Involvement of locally and systematically induced resistance has been demonstrated. Cells of the *Trichoderma* applied to the roots, and dead

cells applied to the leaves of cucumber plants induced control of powdery mildew. *Trichoderma* suppressed the enzymes of *B. cinerea*, such as pectinases, cutinases, glucanases and chitinases through the action of proteases secreted on plant surfaces. A combination of several modes of action is responsible for biocontrol (Elad, 2000).

Many factors have to be considered while deciding whether a biological system is feasible for control of a particular pathogen. Availability of a suitable microorganism capable of maintaining itself on the plant host in case of leaf or stem diseases, persistence in the ecosystem in desirable numbers where the crop is grown and competitive ability against other soil or plant microflora, are some of the essential factors for the biological control agent to be effective against any disease. Mere ability to control pathogen *in vitro* is not enough. It has to be effective against pathogens *in vivo* (Sharma and Sankaran, 1988). Even the biocontrol agents having all the above characters may not perform evenly through all the crop stages. Evidence for this is given in the study of *T. virens* in cotton by Howell (2004). The pre-emergence phase of cotton seedling disease was effectively controlled by strains of *T. virens* but they were much less efficient at post-emergence phase of the disease caused by *Rhizoctonia solani* (Howell, 2004).

2.3 HOST PLANT RESISTANCE

2.3.1 Breeding for Resistance by Conventional Method

The most desirable of all the disease control strategies is host plant resistance because it can be highly effective and is environmentally safe. The breeding for resistance to crop diseases by conventional methods is

time consuming and cumbersome. It has to be a continuous process as new races of pathogens evolve and crops lose resistance. Breeders have been successful in protecting some of the major crops grown around the world from fungal diseases. Although it is shown to be possible, wide hybridization programmes face numerous difficulties. The sexual crosses are difficult to make and genetic exchange in the hybrids is poor due to low frequency of pairing between chromosomes of crop species and alien species. Problems can also arise due to undesirable linkage which can lower the yield and quality of the crop variety. For instance, *Lr9 R* gene from *Aegilops umbellulata*, which confers resistance to brown rust of wheat is shown to be linked with yield depression (Ortelli *et al.*, 1996), *Wsm1* conferring resistance against wheat streak has been shown to cause upto 21 per cent yield reduction (Sharp *et al.*, 2002). There is a need to balance the disadvantages of such genes to achieve resistance so as to escape diseases without reduction in yield.

2.3.2 Development of Transgenics for Disease Resistance

Alternative strategies are to be developed that can avoid the problems faced by conventional methods. Such strategies are required particularly when the source of effective resistance is available in taxonomically unrelated species (Grover and Gowthaman, 2003). The most significant development in this area is the varietal development for disease resistance through the use of techniques of gene isolation and genetic transformation to develop transgenics resistant to fungal diseases. The basic steps in this approach is to identify a target genes, isolation and cloning, of these genes and attach it to appropriate promoter and transfer to the desired host for optimal expression.

2.3.2.1 Resistance Genes Encoding Antifungal molecules

Till date, genes encoding many antifungal molecules, which can inhibit fungal growth *in vitro*, have been exploited to make fungus-resistant transgenic plants. Some of these molecules are: pathogenesis related (PR) proteins, ribosome-inactivating proteins, small cystein-rich proteins, lipid transfer proteins, storage albumins, polygalactourodinase inhibitor proteins (PGIPs), antiviral proteins, phytoalexins and non-plant antifungal proteins including the cell wall hydrolyzing enzymes like chitinases and glucanases of biocontrol fungi like *Trichoderma* (Grover and Gowthaman, 2003).

PR proteins are induced during hypersensitive response (HR) and also during systemic acquired resistance (SAR) and therefore are thought to have a role in natural defense or resistance of plants against pathogens. PR proteins have been grouped into five families based on primary structure, serological relatedness and enzymatic and biological activities. PR2 and PR3 are the fungal cell wall hydrolyzing enzymes, glucanases and chitinase, respectively (Kauffmann *et al.*, 1987 and Legrand *et al.*, 1987).

2.4 CHITINASES

2.4.1 Definition and Nomenclature

The complete enzymatic hydrolysis of chitin to free N-acetyl-D-glucosamine is performed by a chitinolytic system. The chitinases: poly-ß-1, 4-(2-acetomido-2-deoxy)-D-glucoside glycanohydrolase: EN 3.2.1.14) hydrolyses polymers of N-acetyl-D-glucosamine, the chitin, including tetramers and, to a lesser extent, trimers. Chitin is the structural

component of the cell wall of fungi as well as of shells or cuticles of arthropods, crustaceans, insects and molluses. Chitobiase (chitobiose acetamido-deoxyglucohydrolase: EN 3.2.1.29) hydrolyses chitobiose (the dimer of N-acetyl-D-glucosamine) and chitotriose.

2.4.2 Occurrence of Chitinases

The chitinase enzyme is found in bacteria, plants, insects, humans and fungi (Flach *et al.*, 1992; Collinge *et al.*, 1993; Boot *et al.*, 1995) and is thought to function in assimilation of chitin, defense against fungal pathogens and separation of dividing cells, although the actual roles of most chitinases remain to be elucidated. In filamentous fungi, chitinases are thought to be involved in processes of requiring cell wall digestion (Gooday, 1990), viz. germination of spores, tip growth of hyphae (Bartnicki-Garcia, 1973), branching of hyphae, hyphal autolysis and differentiation into spores, as well as in assimilation of chitin and mycoparasitism. Their physiological function in plants is induced in response to attack by phytopathogenic fungi and they are reported to combat fungal infection in concert with other antifungal *polypeptides* (Schumbaum *et al.*, 1986 and Roby *et al.*, 1990). Plant chitinases can be induced by various abiotic factors as well as by some bacteria and viruses.

2.4.3 Significance of Fungal Chitinases over Plant Chitinases

Plants have been transformed with plant chitinase encoding genes as a means to alter plant resistance to fungal pathogens, but no single plant chitinase gene has produced an adequate level of resistance (Punja and Raharjo, 1996 and Jach *et al.*, 1995). Reasons for this may be that

(i) plant chitinases usually affect only the hyphal tip and are unable to effectively degrade harder chitin structures, (ii) have weak antifungal activity alone, (iii) are inhibitory only to a limited number of fungal species and (iv) have no effect on several important pathogens (Lorito, *et al.*, 1994 and Lorito *et al.*, 1996). Enzymes of *Trichoderma* spp. are strong inhibitors of many important plant pathogens and the chitinases are able to lyse the "hard" chitin wall of the mature hyphae, conidia, chlamydospores, and sclerotia. *Trichoderma* chitinases are substantially more antifungal than any other chitinases purified thus far from any other source when assayed under the same conditions. They are more active than corresponding plant enzymes, effective over a much wide range of pathogens and are nontoxic to plants at high concentrations. (Neuhaus *et al.*, 1991; Mauch *et al.*, 1988).

The genome of mycoparasitic fungi such as *Trichoderma spp.* has evolved specifically to be capable of using other fungi but not plants as carbon sources and as such represent a potential source of powerful antifungal genes. In terms of antifungal activity, chitinase genes from *Trichoderma* have a clear edge over the plant chitinases. The *Trichoderma* chitinase genes are capable of producing chitinolytic enzymes, which have the antifungal activity level close to that of some chemical fungicides. Extensive testing *in vitro* has shown that there are virtually no chitinous pathogens resistant to *Trichoderma* chitinases and hence they have become excellent candidates for reinforcing plant defense hypersensitive reactions (Neuhaus *et al.*, 1991; Mauch *et al.*, 1988).

2.4.3.1 Properties of fungal chitinases

Based on amino acid sequence similarities, Henrissat and Bairoch (1993) have grouped all chitinases into two families, 18 and 19, under the main class of glycosyl hydrolases. Most of the microbial chitinases belong to family 18. Among fungal chitinases, three types of enzymes are recognized based on their action on chitin substrates (Sahai and Manocha, 1993). These chitinolytic enzymes are exochitinase, endochitinase and N-acetylglucosaminidase. The combined action of all these chitinolytic enzymes can degrade chitin to its monomers, N-acetylglucosamine. Fungal chitinases with their molecular masses differing considerably (Mw 27-130 KDa) in various fungi are generally active at slightly acidic pH, have high temperature optima, and high degrees of stability (which may be due to glycosylation). They are inhibited by divalent cations especially copper and mercury, and are competitively inhibited by chitobinolactone oxime (Rast *et al.*, 1991) and allosamidin (Milewski *et al.*, 1992).

Chitinase activity is stimulated by partial proteolysis of microsomal fractions with commercial proteases such as trypsin or by *partially* purified proteases of fungus itself (Manocha and Bala*subramanian* 1988). Inactivation of chitinase after treatment with commercial phospholipases suggests the requirement of a phospholipid environment for activity, a result reported in diverse fungi (Humphreys and Gooday 1984). A comprehensive survey of the properties and functions of fungal chitinases is lacking, despite the importance of these enzymes in various aspects of fungal physiology.

2.4.3.2 Components of the chitinolytic system of *Trichoderma*

Haran *et al.* (1995) in their study reported that *Trichoderma* species utilize chitinolytic enzymes along with the β-1, 3-glucanase to degrade the pathogen's cell walls and thus reduce disease level. The chitinolytic system of *T. harzianum* is complex consisting of six distinct enzymes (Haran *et al.*, 1995). The system is composed of two β-1, 4 N-acetylglucosaminidase (*chit 102* and *chit 73*) and four endochitinases (*chit 52, chit 42, chit 33 and chit 31*). The newly described enzymes are *chit 73* and *chit 52*. All the chitinolytic enzymes were *induced and* excreted during growth of *Trichoderma* on chitin as the sole carbon source. Only *chit 102* was expressed intracellularly at a low level, when *Trichoderma* was grown on glucose. Polyclonal antibodies raised against a purified 41kDa endochitinase produced by *T. harzianum* strain P1, reacted only with *chit 42*, suggesting that serologically all the other chitinolytic enzymes are not closely related to the 41kDa endochitinase. The complexity and diversity of the chitinolytic system of *T. harzianum* involves the complementary modes of action of six enzymes, all of which are apparently required for maximum efficacy against a broad spectrum of chitin-containing plant pathogenic fungi (Haran *et al.*, 1995).

2.5 SCREENING MICROBES FOR CHITINOLYTIC ACTIVITY

Chitinolytic microbes have been classified on the basis of substrate hydrolysis, biochemical estimates and DNA based techniques.

2.5.1 Substrate Hydrolysis in Media

Soil and aquatic systems harbor chitin degraders. Most fungi and bacteria produce chitinases only when grown on a chitin-containing substrate i.e., chitinase is an inducible enzyme (Monreal and Reese, 1969). The growth substrates usually used for enumeration of chitin degraders are mushroom chitin (containing glucan) and shrimp chitin. These chitins are used directly or processed to different forms such as swollen chitin, wiley milled chitin, colloidal chitin etc. On colloidal chitin media, *Serratia marcescens* QMb1466 and a related bacterium, *Enterobacter liquefaciens* produced 10 times more enzymes than on swollen chitin or native (wiley milled) chitin (Monreal and Reese 1969).

Serratia marcescens was found to be the most active of the 100 organisms tested for the production of chitinase. *Enterobacter liquefaciens* produced nearly as much enzyme. Under optimal conditions of pH and temperature, yields of chitinase were obtained in 4-6 days (Monreal and Reese, 1969). The influence of pH on chitin hydrolysis by streptomycetes from a range of acidic and neutral soils was studied *in vitro* in an acid soil. A total of 24 streptomycetes isolates of soil were tested for their ability to hydrolyze chitin and diameter of hydrolysis zones were measured. The best 10 strains, which produced the largest hydrolysis zones, were used to study the effect of pH on chitin hydrolysis. The pH and temperature optima of the chitinase complex of *T. longibrachiatum* IMI 92027 were 4.5 and 55°C, respectively (Kovacs *et al.*, 2004)

According to Hsu and Lockwood (1975) agar media made with 0.4 per cent colloidal chitin plus mineral salts and adjusted to pH 8.0 was superior to four other commonly used media for the isolation and enumeration of actinomycetes from water samples. More actinomycetes developed on chitin agar and the development of bacteria and other fungi was suppressed. Frozen and vacuum-dried chitin from aqueous colloidal suspensions was finely divided and gave results comparable to those obtained with media prepared from colloidal suspensions (Hsu and Lockwood, 1975). Thirty *Trichoderma* strains representing 15 species within the genus were screened for extracellular production of chitinolytic enzymes in solid substrate fermentation. In the study by Kovacs *et al.* (2004), *Trichoderma longibrachiatum* IMI 92027 (ATCC 36838) gave the highest yield (5.0 IU/g of dry matter of substrate) after 3

days of fermentation on wheat bran-crude chitin (9:1 mixture) medium. The optimal moisture content (66.7%), chitin content (20%), initial pH of the medium (2.0-5.0), and time course (5 d) of solid substrate fermentation were determined for strain IMI 92027. Cellulase, xylanase, alpha-amylase, and beta-xylosidase activities were also detected (Kovacs *et al.*, 2004).

2.5.2 Inhibition of Growth of Pathogens

Trichoderma isolates were obtained from 31 different soil samples of Eskiserhir. The biocontrol and antifungal effects of these isolates against various plant pathogenic fungi were determined. The culture filtrates of *Trichoderma harzianum* T9, T10, T15 & T19 were effective against plant pathogens and dried/fresh mycelia of plant pathogens such as *Fusarium culmarum, F. oxysporum, F. moniliformae, Rhizoctonia solani, Sclerotium rolfsii, Gaeumannomyces graminis var tritici* and *Drechslera sorokiniana* (Kivanc, 2003; El-Katatny *et al.*, 2000). *Trichoderma harzianum* T19 showed a wide range of inhibitory effects on plant pathogens. *T. harzianum* isolates were grown on the chitin which was the sole carbon source (Kivanc 2003). All isolates showed different behavior depending on the physiological tests carried out such as growth in the presence of inhibitory substrates, pH limits of growth and hydrolysis of gelatin (Kivanc, 2003). Chitinase production was significantly stimulated by acidic pH of 5.5 to 6.0(El-Katatny *et al.*, 2000). In the study by Santamarina *et al.* (2002), the antibiotic activity of 70 isolates belonging to the genera *Aspergillus, Penicillium, Fusarium, Alternaria* and *Trichoderma* was tested as preliminary screening. The highest activity was obtained with three *Penicillium oxalicum* isolates, one *Penicillium*

decumbens and a *Trichoderma harzianum* isolate. These five isolates were chosen to study their effect on bacteria, fungi and insects. Extracts from these isolates were obtained and tested for antibiotic activity with positive results, which implies that metabolite production is involved in this antagonistic effect. The highest activity was shown by *T. harzianum* and *P. oxalicum* extracts, but there was high variability among *P. oxalicum* isolates.

Instead of using organisms as a whole, chitinase enzymes were isolated and tested for their antifungal activity (Lorito *et al.*, 1993). Two chitinolytic enzymes from *Trichoderma harzianum* strain P1 were tested for their antifungal activity in bioassays against nine different fungal species. Spore germination (or cell replication) and germ tube elongation were inhibited by all chitin-containing fungi except *T. harzianum strain* p1. It shows that chitinolytic system of *T.harzianum* is not effective on its own spore germination (or cell replication) and germ tube elongation. The ED_{50} values for the endochitinase and chitobiosidases were 35-135µg/ml and 62-180µg/ml, respectively. The two enzymes appeared to be synergistic against pathogens reducing the ED_{50} of a 1:1 mixture of both enzymes to as low as 10µg/ml (Lorito *et al.*, 1993).

2.5.3 Based on Biochemical Estimation

Chitinolytic activity of *Verticillium suchiasporium* and *Verticillium chlamydosporium* was assayed by measuring the release of reducing saccharides from colloidal chitin. Absorbance of the reaction mixture at 582 nm (A_{582}) was measured taking calibration curve of N-acetyl-D-

glucosamine (NAGA) to determine reducing saccharide concentration (Tikhonov *et al.*, 2002).

Endochitinase activity was measured and monitored spectrophotometrically as the release of p-nitrophenol (pNP) from p-nitrophenyl-N-acetyl-D-glucosaminide (Harman *et al.*, 1993). Endochitinase activity can be measured by the reduction in turbidity of a suspension of colloidal chitin by the enzyme solution of *Trichoderma* (Harman *et al.*, 1993). Alternatively, endochitinase activity could be measured by using a microtitre plate assay using p-nitrophenyl-β-D-N, N', N"–acetylchitotriose as the substrate (Harman *et al.*, 1993; Bielka *et al.*, 1984; Heinrikson and Meredith, 1984).

In some cases, 4-methylumbelliferyl β-D-N', N"-diacetylchitotrioside or 4-methylumbelliferyl-N-acetyl-β-D-glucosamide (4-MU-GlcNAc) (Haran *et al.*, 1995) a fluorogenic analogue of chitin was used as substrate for hydrolysis (Haran *et al.*, 1995; Schickler *et al.*, 1998).

2.5.4 DNA Based Techniques

PCR primers were designed for chitinase genes in four γ-proteobacteria in the families Alteromonacae and Enterobacteriacae (group I chitinases) and used to explore the occurrence and diversity of these chitinase genes in cultured and uncultured marine bacteria. PCR primers were designed based on conserved nucleotide sequences of chitinase genes in cultured bacteria (Cottrell *et al.*, 2000). Kim *et al.*, (2002) designed degenerate primers for detection and isolation of glycosyl hydrolase genes.

2.6 PURIFICATION OF CHITINASES

Chitobiosidases and an endochitinase were derived from dialyzed, concentrated culture filtrates of *Trichoderma harzianum* using gel filtration, chromatofocussing and isoelectric focusing (Harman *et al.*, 1993; Takaya *et al.*, 1998). Protein concentration was determined by SDS-PAGE analysis (Kivanc, 2003 and Haran *et al.*, 1995).

The N-acetylglucosaminidase present in the culture-supernatant of *Trichoderma harzianum* was purified to homogeneity by gel filtration and hydrophobic interaction chromatography, as demonstrated by SDS-PAGE analysis. Purification of protein extract from the solid substrate fermentation material with *Trichoderma longiabrachiatum* IMI92027 revealed high chitinolytic activities between pI 5.9 and 4.8, where N-acetyl-beta-d-hexosaminidase and chitinase peaks have been found in the same pI range. Two chitinases of 43.5 and 30 kDa were purified at acidic pI (Kovacs *et al.*, 2004).

2.7 CLONING AND EXPRESSION OF CHITINASE GENES FROM FUNGI

Few fungal chitinase genes have been cloned and characterized as compared to the plant chitinases (Graham and Sticklen, 1994). Kuranda and Robbins (1991) sequenced endochitinase genes, CTS-1 and CTS-2 of a yeast fungus, *Saccharomyces cerevisiae*, following initial cloning by plasmid-based over expression. Analysis of the amino acid sequence suggests that chitinase protein contains four domains: a signal sequence, a catalytic domain, a serine/threonine-rich region, and a chitin-binding domain at the carboxyl terminal. The catalytic domain is

homologous to a pathogenesis-related cucumber chitinase. Two small regions of this domain were conserved not only with the cucumber chitinase but also with several bacterial chitinases, endoglycosidase H, and a mammalian lysosomal chitinase, all of which cleave the β-1, 4 glycosidic bonds between adjacent GlcNAc residues (Kuranda and Robbins, 1991). Most of the fungal chitinases have a conserved catalytic region, which contains invariant aspartic and glutamic residues as in the case of *Trichoderma harzianum* (McCreath *et al.*, 1995).

The first filamentous fungal chitinase sequence was obtained from *Aphanocladium album* by Blaiseau *et al.* (1992). A partial 5' nucleotide sequence of chitinase 1 (*chi 1*) gene was determined using its cDNA library. The transformation of this chitinase fragment to *Fusarium oxysporum* resulted in an expected 39kDa chitinase protein, which cross-reacted with antiserum of *Aphanocladium album* (Blaiseau *et al.*, 1992). Subsequently, the same research group isolated and determined the complete nucleotide sequence of chitinase encoding gene (*chi 1*) from both cDNA and genomic library of *A. album.* Three short introns were detected (Blaiseau and Lafay, 1992). Similar introns were reported for chitinase gene *ech-42* of *Trichoderma harzianum* IMI 206040 strain (Carsolio *et al.*, 1994) and *Rhizopus oligosporus* (*chi 1* and *chi 2*, one for each allelic gene) (Yanai *et al.*, 1992). In contrast, no introns were found in *chit 42* (CECT 2413 strain) (Garcia *et al.*, 1994), and *Th En-42* (from p1 strain) (Hayes *et al.*, 1994) genes of *Trichoderma harzianum.* All the three genes (*ech-42, chit 42* and *Th En-42*) may represent the same chitinase gene as the total number of amino acids for each complete sequence was between 421 and 423. The variation in introns and other amino acids

could be due to different fungal strains used. The average length of complete nucleotide sequence for the published chitinase genes of different fungi was about 1700 base pairs, except *cts2* of *Coccoidiodes immitis* where it is 2580 bps (Pishko *et al.*, 1995) and they possessed CAAT and TATA boxes at the promoter region as common features in eukaryotic genes.

Several researchers made comparison of amino acid sequences of published chitinases from different fungi. When *ech-42* was compared with the previously reported sequence of *Th En-42* by Hayes *et al.* (1994), only 5 amino acids were different at positions 75, 76, 78,121 and 137. A very high homology (73% identity) also was found between the *A. album* (*chi 1*) and *T. harzianum* (*ech-42*) chitinases (Carsolio *et al.*, 1994). Two and three chitinase genes were reported for *Coccoidiodes immitis* (Pishko *et al.*, 1995) and *Candida albicans* (McCreath *et al.*, 1996), respectively. In the case of *Coccoidiodes immitis*, *cts1* has up to five introns in a 1281 bp sequence and *cts2* contains two introns in a 2580 bp open reading frame. Introns were absent in *Candida albicans*. All these fungal chitinase genes share considerable similarities with chitinase genes of different fungi and certain bacteria and plants (Carsolio *et al.*, 1994; Garcia *et al.*, 1994; Kuranda and Robbins 1991; McCreath *et al.*, 1995). The genes encoding chitinases cloned so far from fungal systems are shown in table 1.

Table 1 : Genomic, cDNA, and Protein Sequences of Known Fungal Chitinases

Source	**Type**	**Reference**
Rhizopus oligosporus(chi 1, chi 2)	G, P	Yanai *et al.*, 1992
Aphanocladium album	C, I	Blaiseau *et al.*, 1992
Aphanocladium album(chi 1)	G	Blaiseau and Lafay 1992
Trichoderma harzianum	I,P	Harman *et al.*, 1993
Trichoderma harzianum(Th En-42)	C	Hayes *et al.*, 1994
Trichoderma harzianum(ech-42)	C	Carsolio *et al.*, 1994
Trichoderma harzianum(chit 42)	C	Garcia *et al.*, 1994
Saccharomyces cerevisiae(CTS-1, CTS-2)	G	Kuranda and Robbins 1991
Candida albicans (cht2,cht3)	G	McCreath *et al.*, 1995
Candida albicans (cht 1)	G	McCreath *et al.*, 1996
Coccoidioides immitis (cts 1, cts2)	G,C	Pishko *et al.*, 1995
Coccoidioides immitis	G,C	Zimmermann *et al.*, 1996
Coccoidioides immitis	C	Yang *et al.*, 1996
Trichoderma virens	G	Kim *et al.*, 2002
Rhizopus oligosporus	G, P	Takaya *et al.*, 1998
Paracoccidioides brasiliensis.	C	Santos *et al.*, 2004

G-Genomic sequence; C-cDNA sequence; P-protein sequence; I-incomplete sequence

2.9 TRANSGENIC PLANTS AND ANTIFUNGAL DEFENSE

A chimeric chitinase gene was constructed by replacing the 5' regulatory region of the bean chitinase 5B gene with the promoter region of the cauliflower mosaic virus (CaMV) 35S-RNA and introduced into *Brassica napus* and *Nicotiana tabacum.* Expression in these transgenic plants conferred increased resistance against the disease caused by *R.solani* (Broglie and Broglie, 1993). Induction of bacterial chitinase gene (*Chi A*) from *Serratia marcescens* in transgenic tobacco plants conferred increased resistance to *Alternaria longipes*. This increase in resistance, however, appeared to decrease with the age of the plants (Suslow *et al.*, 1988). The transgenic tobacco plants generated by using promoters from petunia plants showed a higher level of *Chi A* expression (Jones *et al.*, 1988). The *Chi 1* gene from *Rhizopus oligosporus* was transfered to tobacco, which conferred, increased resistance to *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Terakawa *et al.*, 1997). Chitinases of *Trichoderma atroviridae* induced scab resistance and some metabolic changes in two cultivars of apple Galaxy and Ariane. A negative correlation was observed between growth of transgenic lines and endochitinase activity (Faize *et al.*, 2003). Antifungal endochitinase from the mycoparasitic *Trichoderma harzianum* was transferred to tobacco and potato, which conferred resistance to foliar pathogens, *Alternaria alternate, A.solani, Botrytis cinerea,* soil borne pathogen *Rhizoctonia solani.*

Immunocytochemical studies of root tissues of wild type and 35S-CH5B transgenic canola plants infected by *R. solani* were carried out by Benhamou *et al.* (1993). The hyphae of *R. solani* in transgenic plants

appeared physically damaged and suffered increased vacuolization and cell lysis. Fungal colonization was restricted to the cortex in the transgenic plants, while wild type plants were colonized extensively in all root tissues including the xylem elements (Benhamou *et al.*, 1993).

There is evidence that the effectiveness of chitinase against several plant pathogens lies in its synergistic action with glucanases and N-acetylglucosaminidase. In recent years, considerable progress has been made in producing disease-resistant and high-yielding transgenic plants. It may be necessary to integrate different resistance genes together in order to extend the host defense against different fungi.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present study was undertaken to screen the native isolates of various fungi belonging to different genera for chitinase activity and to clone a full-length gene of chitinase from one of the isolates.

3.1 FUNGAL STRAINS

Fungal strains, which were identified in earlier reports to have the chitinase activity, were obtained from different culture collections as listed below (Table 2).

Table 2: Fungal strains used for the study

Strains	**Source**
Trichoderma harzianum (1)	Biocontrol Unit, UAS, Dharwad
Trichoderma harzianum (2)	Biocontrol Unit, UAS, Dharwad
Trichoderma harzianum (3)	Biocontrol Unit, UAS, Dharwad
Trichoderma virens	Biocontrol Unit, UAS, Dharwad
Trichoderma viride	Biocontrol Unit, UAS, Dharwad
Trichoderma koningii	Biocontrol Unit, UAS, Dharwad
Aspergillus oryzae NCIM-1212	National collection of industrial microorganisms, National Chemical Laboratory, PUNE
Metarrhizium verrucasia NCIM-1194	National collection of industrial microorganisms, National Chemical Laboratory, PUNE
Bauveria bassiana NCIM-1216	National collection of industrial microorganisms, National Chemical Laboratory, PUNE
Nomuraea releyi	Dept of Agril. Entomology, UAS, Dharwad

All the fungal cultures were maintained on potato dextrose agar. *Serratia marcescens* strain was obtained from the Department of Agricultural Microbiology UAS, Dharwad, maintained on Luria agar and was used as reference strain (Monreal and Reese, 1969).

3.2 SCREENING THE ISOLATES FOR CHITINOLYTIC ACTIVITY

Chitinolytic activity of the available isolates was estimated as follows:

3.2.1 Screening of Isolates on Mandels and Reese (1965) Agar

Individual spore of fungi and *Serratia* suspensions were inoculated on the media (Appendix - I) containing the colloidal chitin as the sole carbon source. The plate with *Serratia* was incubated at 37±2°C for 24 hrs on Monreal and Reese (1969) medium. It was used as reference strain for testing the validity of the colloidal chitin preparation. The plates with fungal spores were incubated at 28±2°C for 48 hrs on Mandels and Reese (1965). The chitinolytic activity was measured by the zone of solubilization of colloidal chitin, the substrate for chitinase enzymes after one day of incubation.

3.2.2 Screening of the Isolates on Mandels and Reese (1965) Broth

Individual spores of fungi were inoculated to the media containing the colloidal chitin as sole carbon source. The vials with fungal spores were incubated at 28±2°C temperature for 30 days. The chitinolytic activity was measured by the reduction in the colloidal chitin level in the broth. The vial without fungal spores was used as control.

3.2.3 Preparation of Colloidal Chitin

Colloidal chitin was prepared by the method of Roberts and Selintrenikoff (1988) with certain modifications.

5g of chitin powder (Himedia Laboratories Pvt.Ltd.) was added slowly into 60ml of concentrated HCl (Sd. Fine Chem. Ltd.) and left at 4°C overnight with vigorous stirring. The mixture was added to 2 litres of ice-cold 95% ethanol with rapid stirring and kept overnight at room temperature (25°C). The precipitate was collected by centrifugation at 5000xg for 20 minutes at 4°C and was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Colloidal chitin solution (5%) was prepared and stored at 4°C until further use.

3.3 ISOLATION OF GENOMIC DNA

The genomic DNA was isolated from fungi following the protocol of Hegedas and Khazhatourians (1996) with certain modifications.

Three grams of fungal mat grown on potato dextrose media was taken and homogenized using pestle and mortar in 4 ml of 2 percent sodium dodecyl sulfate (SDS) for 5 minutes. To the above solution, 6 ml of lysis buffer (2.5 Mm EDTA, 1% Triton x and 50mM Tris-HCl, pH 8.0) was added. The suspension was extracted with equal volume of phenol:chloroform (1:1) and centrifuged at 10,000 rpm for 10 minutes. The supernatant was taken into a fresh tube and $1/10^{th}$ volume of 3M sodium acetate and 0.54 volume of isopropanol were added at room temperature, mixed by gentle inversion and kept for 30 minutes at 2°C. The DNA was recovered by centrifugation at 10,000 rpm for 10 minutes at 4°C. The DNA pellet was washed with 70 percent ethanol, air-dried

and resuspended in 300µl of $T_{10}E_1$ (10mM tris HCl and 1mM EDTA, pH 8.0).

3.3.1 Purification of Genomic DNA

The genomic DNA isolated was purified according to the protocol described by Maniatis et al, (1982).

To the DNA solution, RNase @ 100 µg per ml was added and this solution was incubated for two hours at 37°C on water bath. The solution was centrifuged at 10,000 rpm for 10 minutes and the suspension was treated with equal volume of buffered phenol (pH 8.0) and centrifuged. The upper aqueous layer was taken in a fresh tube and treated with equal volume of phenol:chloroform (1:1 v/v).This suspension was centrifuged and upper aqueous layer was taken into fresh tube and to this $1/10^{th}$ volume of 3M sodium acetate and 2 volumes of absolute ethanol were added and incubated at 4°C for 2 hrs. The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes. The pellet was washed with 70 percent ethanol, air dried and dissolved in 100 µl of $T_{10}E_1$ buffer and stored at 4°C until further use.

3.3.2 Quantification of Genomic DNA

The genomic DNA isolated was quantified spectrophotometrically (Jenway Genova) as described by Sambrook and Russel (2001). The total DNA isolated was diluted in sterilized nanopure water and the absorbance at 260nm and 280nm was recorded. The concentration of DNA was expressed in µg/µl. The formula used for calculation of concentration of DNA is:

Concentration of DNA (ng/µl) = O.D at 260nm x 50 x dilution factor

3.4 CHARACTERIZATION OF AVAILABLE CULTURES FOR PRESENCE OF CHITINASE GENE

The following fungal culture were characterized

Trichoderma harzianum (2)

Trichoderma koningii

Trichoderma harzianum (1)

Trichoderma harzianum (3)

Trichoderma viride

Trichoderma virens

Aspergillus oryzae

3.4.1 Primers for screening

*Chi*1F- 5'-TCC CAA ATC CCG TTC TCC CA-3'

*Chi*1R-5'-AAC CTG ATG GCC TAT GAC T -3'

The different fungal isolates were screened for the presence *of* chitinase gene by exclusive PCR with the primers designed for 260 bp fragment. The sequences were essentially the ones described by Saiprasad *et al.* (2003). The primers used were conserved for a 260 bp fragment of endochitinase gene across all endochitinase genes isolated so far from *Trichoderma* spp. The procedure followed for amplification is as given below. The purified total DNA extracts (20ng) of the individual isolates were used as template DNA. The two primers used were *chi*1F

and *chi*1R which were custom synthesized by Sigma Chemical Co., USA and were supplied as lyophilized product of desalted oligos.

3.4.1.1 PCR amplification condition for *Chi* 1 in all the available *Trichoderma* species

Stage	Step	Temperature(°C)	Duration(min)	No. of cycles
I	Initial denaturation	94	10	1
II	Denaturation Annealing Extension	94 50 72	1 1 2	24
III	Final extension	72	10	1

3.5 AMPLIFICATION OF FULL LENGTH CHITINASE GENE

3.5.1 Primers Specific for the Full Length Chitinase Gene

The primers specific for full-length endochitinase gene were designed using the reported full length endochitinase gene from database using the GENETOOL software (Table 3).

Table 3: Primers Designed For Full-Length Endochitinase Gene

Primer	F/R	
*Chi*3	Forward	5'CCA ACA TCA CAA GCA ATT CAC C 3'
	Reverse	5'CCG CGA CGA CTT CAA TGT 3'
*Chi*4	Forward	5'AAT AGG AGG CTC CAC AAT C 3'
	Reverse	5'CTC CCT GAT AAT CGT TTC 3'
*Chi*5	Forward	5'GCT CCA CAA TCA CTT ATA AAT ATG CTG *CA 3'*
	Reverse	5'GGG GAA GTA AAA GAA AAA GAT AAG AAG AAG AAA 3'
Chi-vir	Forward	5' TGC CAA CAA TCT GAA AGG GAAG 3'
	Reverse	5' GCT GTG TAT CCC CTG AAA AGA AG 3'

The primers used for amplification of template DNA (20ng) were custom synthesized at Sigma Chemical Co., USA and were supplied as lyophilized product of desalted oligos.

3.5.2 Standardization of Primer Concentration

Primer concentrations viz., 5pM, 10pM, 20pM, 30pM, 40pM, 50pM were used to optimize the amplification. Based on the results, 10pM of *Chi 1, Chi 3, Chi-vir,* gave single intense amplicon and was used for large-scale amplification.

3.5.3 Reaction Mix

Chi 3 primers were used to amplify chitinase gene from all the strains of *T.harzianum. Chi-vir* primers were used to amplify chitinase gene from *T.virens.* The master mix required for all the samples were

prepared afresh to avoid handling errors. The master mix (18µl) was distributed to the tubes and 2µl of template DNA added to make the total reaction volume of 20µl. dNTPs were purchased from Bangalore Genei Pvt. Ltd., Bangalore. Taq polymerase enzyme and Taq assay buffer were used from Department of biotechnology, UAS, Dharwad. $MgCl_2$ was prepared in the laboratory.

3.5.4 PCR Amplification

PCR amplification was done following the PCR condition designed as follows.

3.5.4.1 PCR amplification condition for *Chi* 3 in *T.harzianum* (1)

Stage	Step	Temperature(°C)	Duration(min)	No. of cycles
I	Initial denaturation	94	10	1
II	Denaturation Annealing Extension	94 47.9 72	1 1 2	24
III	Final extension	72	45	1

3.5.4.2 PCR amplification condition for *Chi* 3 in *T.harzianum* (3)

Stage	Step	Temperature(°C)	Duration(min)	No. of cycles
I	Initial denaturation	94	10	1
II	Denaturation Annealing Extension	94 51.9 72	1 1 2	24
III	Final extension	72	45	1

3.5.4.3 PCR amplification condition for *Chi-Vir* in *T.virens*

Stage	Step	Temperature(°C)	Duration(min)	No. of cycles
I	Initial denaturation	94	10	1
II	Denaturation Annealing Extension	94 48.5 72	1 1 2	24
III	Final extension	72	45	1

3.6 SEPARATION OF AMPLIFIED PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

About 10µl of amplified products from each tube along with 2 µl of loading dye was separated on 1 percent agarose gel using 1x TAE buffer along with 1 Kbp ladder (Bangalore Genie Pvt. Ltd, Bangalore) as DNA molecular weight marker and gel image was documented using gel documentation system (UVitec, Cambridge, England)

3.7 CLONING OF ENDOCHITINASE GENE

PCR based cloning was attempted starting from elution of PCR fragment from agarose gel to the confirmation of clones.

3.7.1 Elution of PCR Fragment from Gels

The intense band of 1.3 kb and 1.7 kb PCR fragments obtained with the designed primers were excised from the low melting agarose gel with a sterile sharp scalpel blade by keeping the gel on low intensity (70%) UV transilluminator. The agarose gel piece containing the fragment was collected in a sterile pre-weighed micro-centrifuge tube.

3.7.2 Purification of PCR Fragment

The excised PCR fragments were eluted from agarose gel using Qiagen gel extraction kit. The method of purification was as described in the user's manual.

3.7.3 Quantification of the PCR Product

The eluted, purified PCR product was quantified by ethidium bromide spotting method as described by Sambrook and Russel (2001).

3.7.4 Cloning of PCR Product

The purified PCR products of 1.3 kb, 1.7kb (50ng/µl) were ligated to pTZ57R/T vector (2868bp) as described *in* InsT/A clone™ PCR product cloning kit (#k1214) from MBI, fermentas USA. The map of pTZ57R with multiple cloning sites is shown (Fig. 1). pTZ57R/T was derived from pTZ57R by Eco321 (an isochizomer of EcoRV) and treated with terminal deoxynucleotidyl transferase to create 3'-ddT overhangs at both ends, then PCR fragment with 3'dA overhangs was ligated to the vector. This circular plasmid with inserts of 1.7 kb was directly transformed in *E.coli* DH5α. The inserts of 1.3 kb ligated in plasmid and directly transformed in *E.coli* XL-1 Blue strain.

3.7.5 Ligation

For ligation, optimal molar ratio of ends of 1:3 of Vector:insert was calculated as per the table given in Appendix-II. The components of ligation mixture were mixed in a 0.5 ml micro-centrifuge tube and incubated at 22°C, overnight, in thermal cycler (Dyad *engine, MJ* research, USA). A control ligation reaction was also performed using the PCR fragment provided in the kit by adding components as described in Appendix-II.

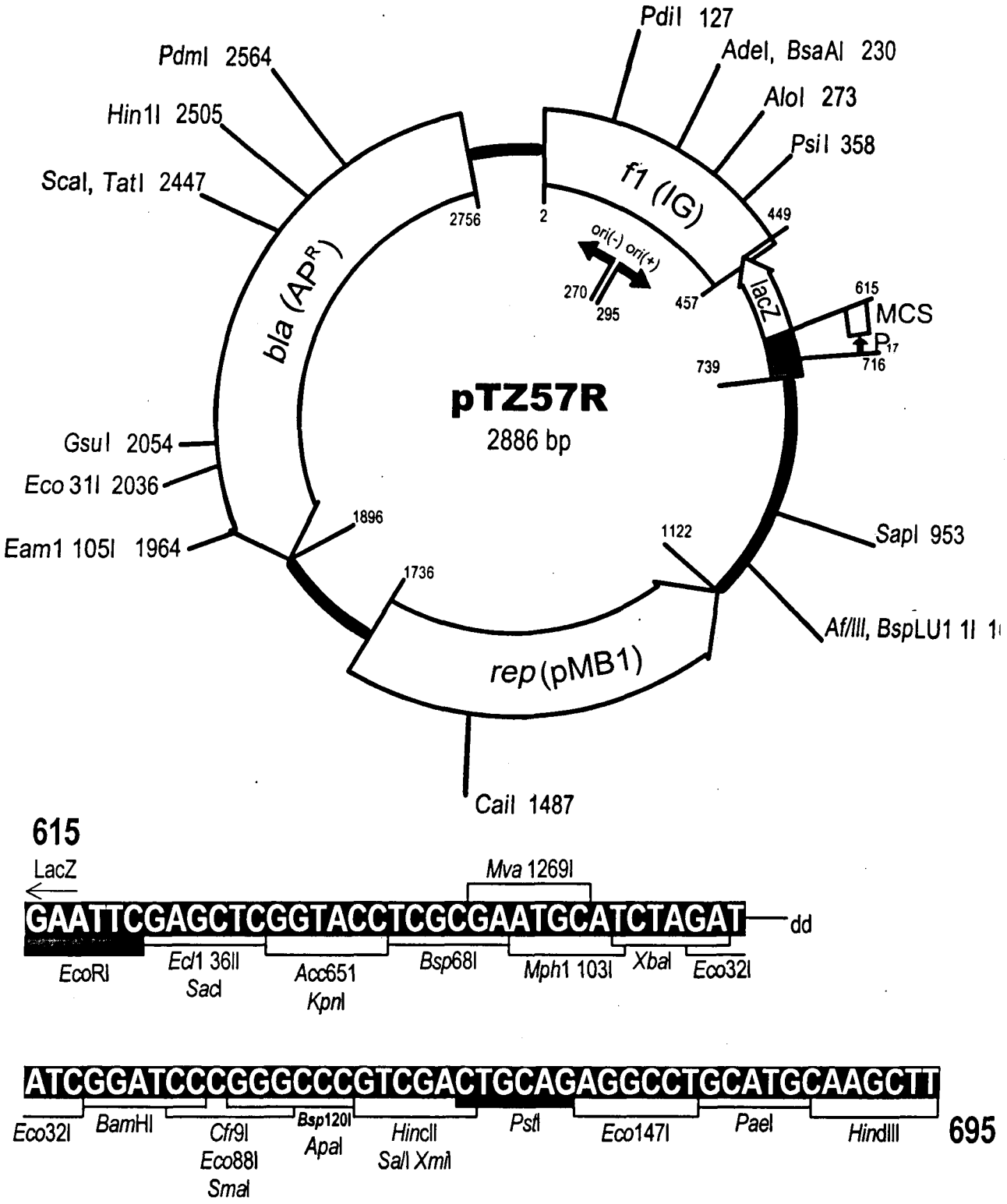


Fig. 1 : Restriction map and multiple cloning site of vector pTZ57R

3.7.6 Transformation

Transformation was done as described in the InsT/A clone™ PCR product cloning kit (#k1214) from MBI, Fermentas USA.

3.7.6.1 Step-I: Preparation of bacterial overnight culture

About 2 ml of transform Aid™ C-medium was inoculated with XL-1 Blue and DH5α bacteria (frozen stock or a colony) and incubated overnight at 37°C over a shaker (Thrmoforma orbital shaker). 1/10th volume of this overnight culture was added to pre-warmed 3 *ml of* C-medium and incubated the tube over a shaker at 37°C for 2 hours.

3.7.6.2 Step-II: Preparation of competent cells

Transform Aid™ T-solution was prepared by mixing equal volume of T-solution (A) and T-solution (B). 1.5 ml of fresh DH5α and XL-1 Blue culture was dispensed in 1.5 ml micro-centrifuge tubes and spun at maximum speed for 1 minute at 4°C. Pelleted cells were resuspended in 30µl of Transform Aid T-solution. Tubes were incubated on ice for 5 minutes. Cells were spun again at 4°C for 1 minute. Cells were then resuspended in 120µl of Transform Aid™ T-solution and incubated on ice for 5 minutes.

3.7.6.3 Step-III: Preparation of DNA for transformation

DNA was prepared for transformation by dispensing 2.0µl of supercoiled (10-100pg) or 2.5µl of ligation mixture (10-20ng of vector DNA) in a fresh micro-centrifuge tube and incubated on ice for 2 minutes.

3.7.6.4 Step-IV: Transformation

For transformation 50µl of the resuspended competent cells were dispensed in each of the tubes containing DNA and incubated on ice for 5 minutes. Control transformation was carried out using 2.0µl of super coiled plasmid DNA of pTZ57R supplied in the kit. Cells were plated on pre-warmed Luria (Appendix-II) agar plates with *ampicillin*, X-gal and IPTG (Appendix-II) and incubated overnight at 37°C.

3.7.7 Clone Selection

After overnight incubation, the recombinant clones were identified by the white colonies since the vector is genetically marked with Lac Z and insertionally inactivated by the cloned fragment. Few of the white colonies were randomly picked up for further studies.

3.7.8 Confirmation of Clones

Confirmation for the presence of desired DNA fragment cloning vector was done by different methods as described below.

3.7.8.1 Plasmid isolation from the recombinants

Plasmid isolation from the recombinants was carried out by alkaline lysis method as proposed by Sambrook and Russel (2001).

White colonies were inoculated to 2.0 ml Luria broth with ampicillin (100µg/ml) and incubated over night at 37°C at 175 rpm, on a shaken 1.5 ml of over night culture was centrifuged at 15,000 rpm for 30 seconds at 4°C and the bacterial pallet was resuspended in 100µl of ice cold alkaline lysis solution I (Appendix-II) by vigorous vortexing. 200µl of freshly prepared alkaline lysis solution II (Appendix-II) was added to the

bacterial suspension and mixed by inverting the tubes five times 150 µl of ice cold alkaline lysis solution III (Appendix-II) was added, mixed by inverting the tubes several times and incubated on ice for 3 to 5 minutes. The bacterial lysate was centrifuged at 15000 rpm for 5 min at 4°C and the suspension was treated with equal volume of phenol: chloroform (Appendix-II). The aqueous layer was transferred to fresh tube and plasmid DNA was recorded by adding 2 volumes of absolute ethanol, mixed the solution by inverting and allowed to stand at room temperature for 2 min. The DNA was precipitated by centrifugation for 15,000 rpm at 4°C. The DNA pallet was washed with 70 per cent ethanol, air-dried and dissolved in 50µl of $T_{10}E_1$ (pH 8.0) containing 20µl per ml DNase free RNase.

Plasmid DNA was visualized on 0.8 per cent agarose gel as described earlier against the 1 Kb ladder.

3.7.8.2 PCR confirmation of recombinants

The recombinant clones were confirmed with the specific primers taking plasmid DNA as template along with control vector and genomic DNA of various fungal strains. The amplification of desired fragments from respective recombinants was considered for confirmation of clones.

3.8 SEQUENCE ANALYSIS OF THE CLONE

3.8.1 Sequencing of the Clone

The clones were sequenced at BioServe Biotechnologies India Pvt. Ltd. The sequencing was done with M13 Primers.

3.8.2 Sequence Analysis

The sequence obtained with the M13 primers were subjected for homology search at nucleotide level (BLASTn) and at protein level using the translated query sequence Vs protein database (BLASTx) programmes available at NCBI website http//: www.ncbi.nlm.nih.gov. Conserved domains were studied using Conserved Domain Database search programme (rpsBLAST) available at NCBI website http//: www.ncbi.nlm.nih.gov. The sequences were further analyzed in BTI Software GENETOOL for the analysis of restriction sites, exons and ORFs.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The present study was conducted to screen various fungal isolates for chitinolytic activity and to clone the endochitinase gene from fungal strains. The results of various experiments designed and conducted are presented below.

4.1 SCREENING OF FUNGAL STRAINS FOR CHITINOLYTIC ACTIVITY

On Monreal and Reese (1969) medium with colloidal chitin, *S. marcescens* produced clear zone of hydrolysis. In the case of fungi, zone of hydrolysis was not visible on agar medium due to confluent growth of mycelia.

On M and R (1965) broth with colloidal chitin, inoculated fungal spores grew and the level of clearance of the medium was visually scored 30 days after inoculation.

Complete clearance of colloidal chitin was observed in both the vials inoculated with *Trichoderma koningii* whereas, no clearance of colloidal chitin was observed in the case of *Nomureae releyi* (Table 4 and Plate 1).

4.2 DETECTION OF ENDOCHITINASE GENE IN FUNGAL STRAINS

The genomic DNA was extracted from fungi following the protocol of Hegedas and Khazhatourians (1996) with certain modifications. The

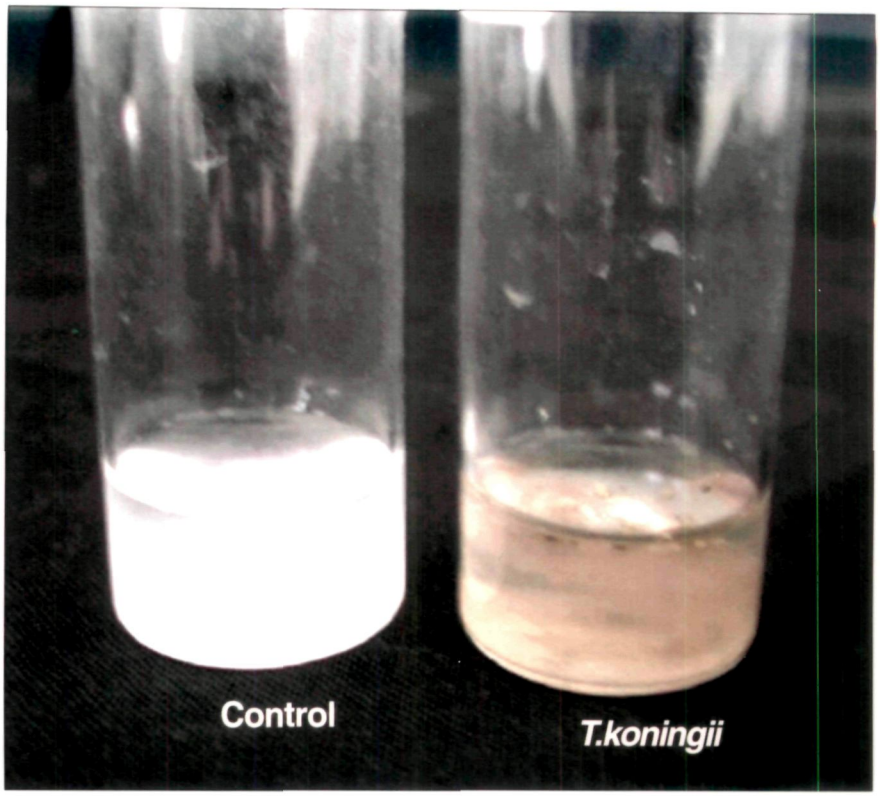


Plate 1. Clearance of colloidal chitin by *Trichoderma koningii* in M & R (1965) broth

Table 4: Clearance of Colloidal Chitin in M and R (1965) Broth

Fungal isolates	**Replication I**	**Replication II**
Trichoderma harzianum (1)	++	++
Trichoderma harzianum (2)	+	+
Trichoderma harzianum (3)	+	+
Trichoderma koningii	++++	++++
Trichoderma virens	++	++
Trichoderma viride	+	+
Metarrhizium verrucasia (NCIM 1194)	+	+
Bauveria bassiana (NCIM 1216)	+	+
Aspergillus oryzae (NCIM 1212)	+++	+++
Nomuraea releyi	-	-
Control	-	-

LEGEND:

- : no clearance of colloidal chitin

+: slight clearance of colloidal chitin

++: slight to medium clearance of colloidal chitin

+++: medium clearance of colloidal chitin

++++: complete clearance of colloidal chitin

quality of the genomic DNA isolated was checked by subjecting the DNA for electrophoresis in 0.7 per cent agarose gel (Plate 2).

Spectrophotometer concentration of DNA isolated from various fungal strains ranged from 3.50 to 7.3 µg/µl and diluted to required concentration with buffer.

Chi 1 primers designed for amplification detection of endochitinase gene encoding 42kDa protein (Saiprasad *et al.,* 2003) were used for screening.

Different concentration of *chi* 1 primers (5, 10, 20, 30, 40 and 50 pM/µl) were used for amplification against 20ng of template DNA of *Trichoderma harzianum* (2) strain. It was found that 10pM/µl of primer was optimum for getting a single unique band of 260 bp. All the genomic DNA samples of fungal strains showed a unique band of 260bp except *Trichoderma viride* and *Aspergillus oryzae* (Plate 3).

4.3 PCR AMPLIFICATION OF ENDOCHITINASE GENE

Different concentrations of respective primers specific for full-length gene were used to find out the optimum concentration. The primer concentrations (pM/µl) used were 5, 10, 20, 30, 40 and 50. Though amplification of desired length was observed in all the cases, at 10pM/µl concentration, a good unique band was observed without leaving primer dimers in all the three templates with respective primers in a reaction volume of 20 µl.

Two pairs of the endochitinase specific primers; *chi 3* designed for amplification of full length gene in *Trichoderma harzianum* and *chi-vir* designed for amplification of full length gene in *Trichoderma virens* were

LEGEND

Plate 2

M. λ DNA *EcoRI / HindIII* Double Digest
1. *Trichoderma harzianum* (1)
2. *Trichoderma harzianum* (2)
3. *Trichoderma harzianum* (3)
4. *Trichoderma koningii*
5. *Trichoderma virens*
6. *Trichoderma viride*
7. *Aspergillus oryzae*

Plate 3

M. 100 bp Ladder
1. *Trichoderma harzianum* (1)
2. *Trichoderma harzianum* (2)
3. *Trichoderma harzianum* (3)
4. *Trichoderma koningii*
5. *Trichoderma virens*
6. *Trichoderma viride*
7. *Aspergillus oryzae*

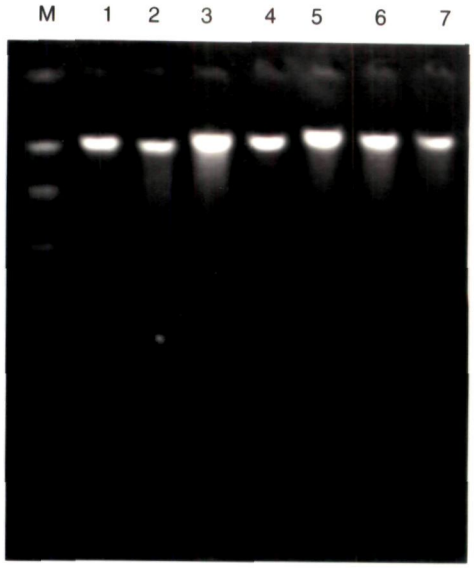


Plate 2. Genomic DNA of fungal isolates

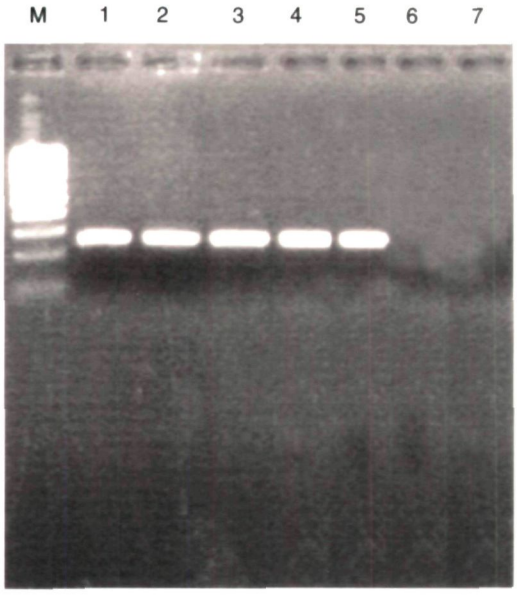


Plate 3. Amplification of 260 bp fragment of endochitinase gene in fungal isolates

used. These primers were designed to amplify the endochitinase gene encoding 42kDa protein. The template DNA of *Trichoderma virens* gave an amplification of 1.7 Kb and templates of *Trichoderma hariznum* (1) and (3) gave an amplification of 1.3 Kb fragment with respective primers (Plates 4, 5 and 6).

4.4 CLONING OF ENDOCHITINASE GENE

purified PCR fragments from large-scale amplification of endochitinase full-length (1.7 Kb) gene from *Trichoderma virens* and 1.3 Kb gene from *Trichoderma harzianum* strains was done using chil15-VIR and chi 3 primers respectively, were cloned by the standard T/A Cloning Procedure.

The amplicons were ligated to pTZ57R/T cloning vector. The recombinant product of vector pTZ57R/T+ 1.7KB amplicon from *Trichoderma virens* and vector +1.3KB amplicon from *Trichoderma harzianum* were used to transform to competent *E.coli* strain DH5α, separately.

4.4.1 Confirmation of clones and sequencing

The transformed cells were picked up, streaked on Luria agar with ampicillin$_{100}$ containing X-GAL and isopropyl-β-D-thiogalactosidase for clonal selection. Recombinant cells were selected based on Blue/White colony assay (Plate 7). The clones from *Trichoderma virens* were named pSAV. These 11 clones from white colonies confirmed for the presence of recombinant plasmid (Plate 8) by PCR amplification with *Chi*1-*VIR* primers. All these clones had the expected 1.7 Kb insert. Similarly, the recombinant clones containing *T. harzianum* chitinase gene (Plate 9) from

LEGEND

Plate 4
M. 1 kb Ladder
1. 44.0°C Annealing temperature
2. 45.8°C Annealing temperature
3. 46.9°C Annealing temperature
4. 48.5°C Annealing temperature
5. 53.3°C Annealing temperature
6. 54.1°C Annealing temperature
7. 55.0°C Annealing temperature
8. 56.5°C Annealing temperature

Plate 5
M. 1 kb Ladder
1. 44.0°C Annealing temperature
2. 45.8°C Annealing temperature
3. 46.9°C Annealing temperature
4. 47.9°C Annealing temperature
5. 51.9°C Annealing temperature
6. 53.3°C Annealing temperature
7. 54.1°C Annealing temperature
8. 55.0°C Annealing temperature

Plate 6
M. 1 kb Ladder
1. 44.0°C Annealing temperature
2. 45.8°C Annealing temperature
3. 46.9°C Annealing temperature
4. 48.2°C Annealing temperature
5. 51.9°C Annealing temperature
6. 53.3°C Annealing temperature
7. 54.1°C Annealing temperature
8. 55.0°C Annealing temperature

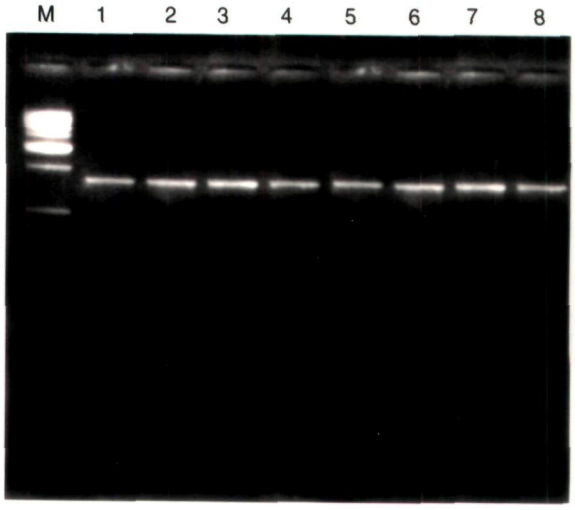


Plate 4. PCR amplification of 1.7 kb fragment from *T. virens*

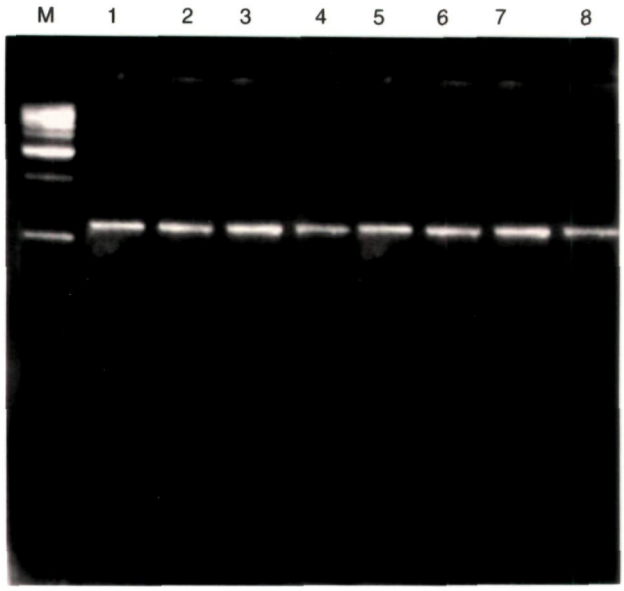


Plate 5. PCR amplification of 1.3 kb fragment from *T. harzianum* (1)

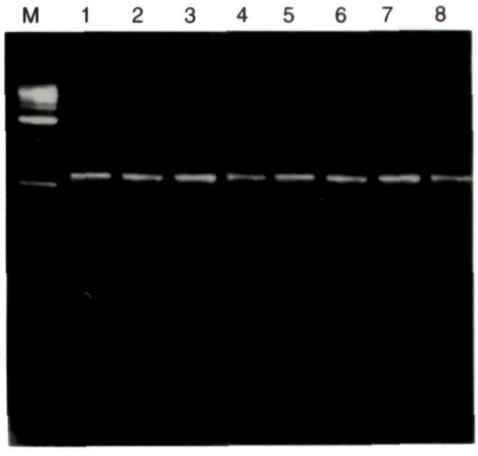


Plate 6. PCR amplification of 1.3 kb fragment from *T. harzianum* (3)

LEGEND

Plate 7
Blue and White colonies of recombinant

Plate 8
M. 1 kb Ladder
1. pSAV104
2. pSAV204
3. pSAV304
4. pSAV404
5. pSAV504
6. pSAV604
7. pSAV704
8. pSAV804
9. pSAV904
10. pSAV1004
11. pSAV1104
12. pTZ57R

Plate 9
M. 1 kb Ladder
1. *pSAH$_1$204*
2. pSAH$_1$504
3. pSAH$_1$1804
4. pSAH$_1$2104
5. pSAH$_1$2304
6. pSAH$_3$204
7. pSAH$_3$604
8. pSAH$_3$2704
9. pSAH$_3$2904
10. pSAH$_3$3304
11. pTZ57R

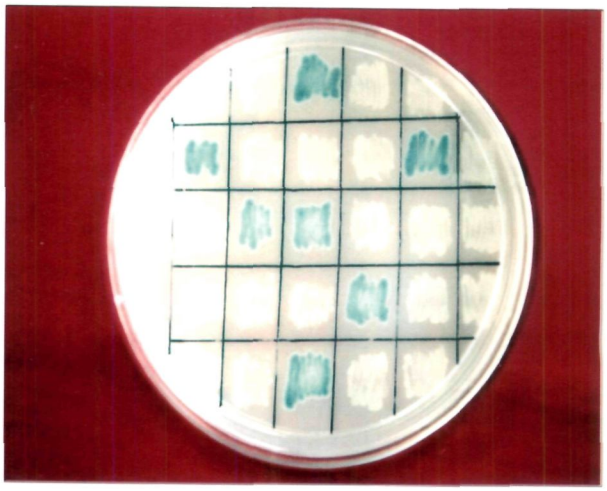


Plate 7: Blue-white screening of recombinants on LA + X-gal + IPTG + Amp_{100}

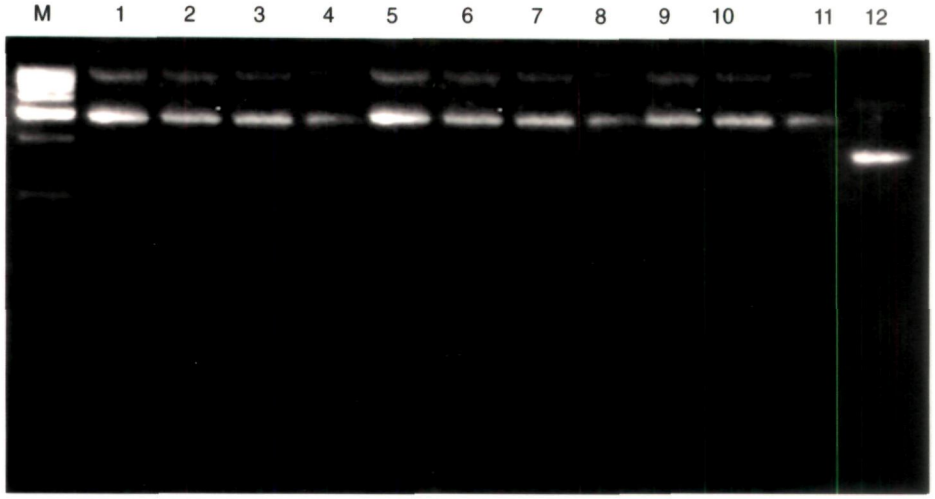


Plate 8. Mini preparation of plasmids from pSAV clones

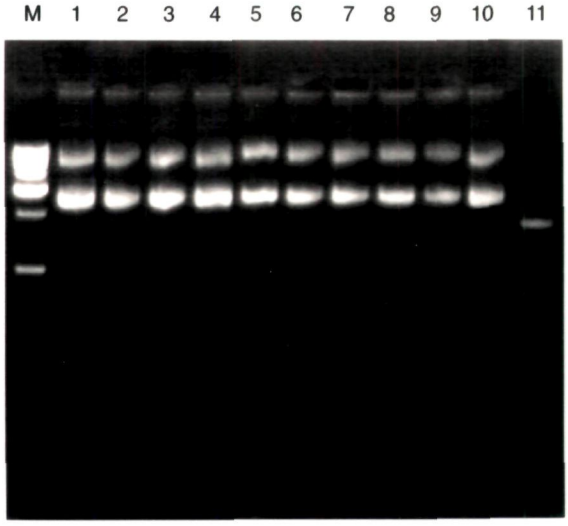


Plate 9. Mini preparation of plasmids from $pSAH_1$ and $pSAH_3$ clones

isolates 1 ($pSAH_1$) and ($pSAH_3$) had the expected 1.3 Kb insert (Plates 10, 11 and 12).

4.5 SEQUENCE ANALYSIS OF pSAV

Out of 11 pSAV clones confirmed to have 1.7 kb insert, pSAV804 containing a 1.7 kb insert was sequenced at BioServe Biotechnologies India Pvt. Ltd. with the M13 primers, the complete sequence of insert was obtained (Appendix-III).

4.5.1 BLASTn Search

The sequence of cloned fragment was subjected to BLASTn search, and the homology results are presented in Table 5. This sequence showed 98 per cent homology to complete coding sequence of *Trichoderma virens chit-G1* gene for endochitinase-G1 and 97 per cent homology to complete coding sequence of *Trichoderma virens* chitinase gene itself, the sequence using which the primers were designed (AAL78813.1). The BLASTn search has shown differences of 33 bp with (AAL78813.1). Gaps were observed at three positions i.e. 549th (1121), 1320th (460), and 1616th (54) positions. An insertion of C was present at 1596th (74) position. The list of changed bases is given in Table 6.

4.5.2 BLASTx Search

The sequence was also subjected to BLASTx search for homology search in protein database at the Translated Query vs. Protein Database search programme available at NCBI website http//: www.ncbi.nlm.nih.gov. The gaps were introduced in the *translated* sequence by BLASTx to maximize the homology. The homology results

LEGEND

Plate 10

M. 1 kb Ladder
1. pSAV104
2. pSAV204
3. pSAV304
4. pSAV404
5. pSAV504
6. pSAV604
7. pSAV704
8. pSAV804
9. pSAV904
10. pSAV1004
11. pSAV1104
12. Positive Control
13. Negative Control

Plate 11

M. 1 kb Ladder
1. $pSAH_1204$
2. $pSAH_1504$
3. $pSAH_11804$
4. $pSAH_12104$
5. $pSAH_12304$
6. Positive Control
7. Negative Control

Plate 12

M. 1 kb Ladder
1. $pSAH_3204$
2. $pSAH_3604$
3. $pSAH_32704$
4. $pSAH_32904$
5. $pSAH_33304$
6. Positive Control
7. Negative Control

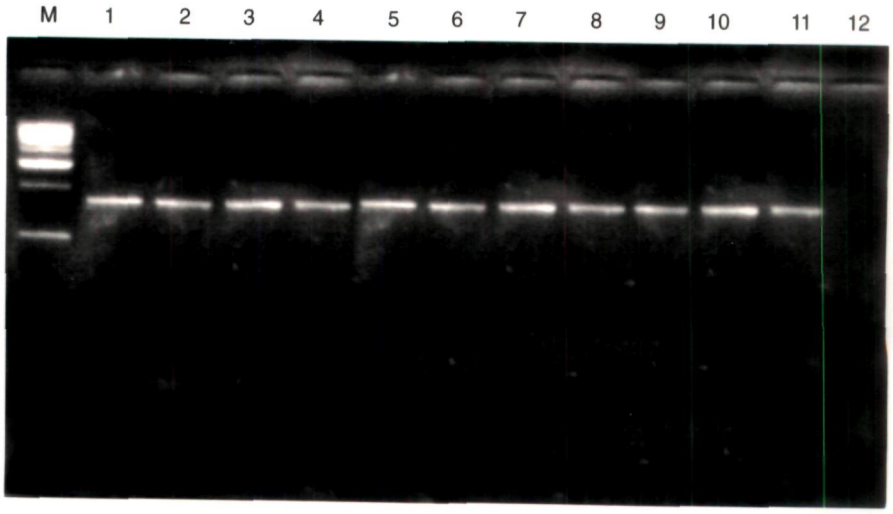


Plate 10. PCR confirmation of 1.7 kb insert in pSAV recombinants

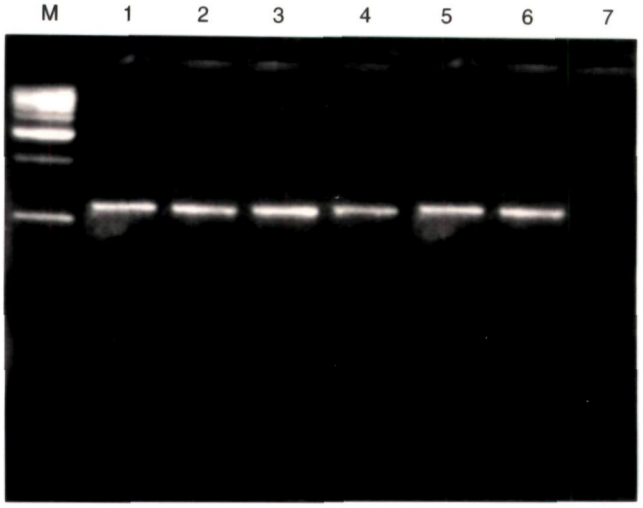


Plate 11. PCR confirmation of 1.3 kb insert in $pSAH_1$ recombinants

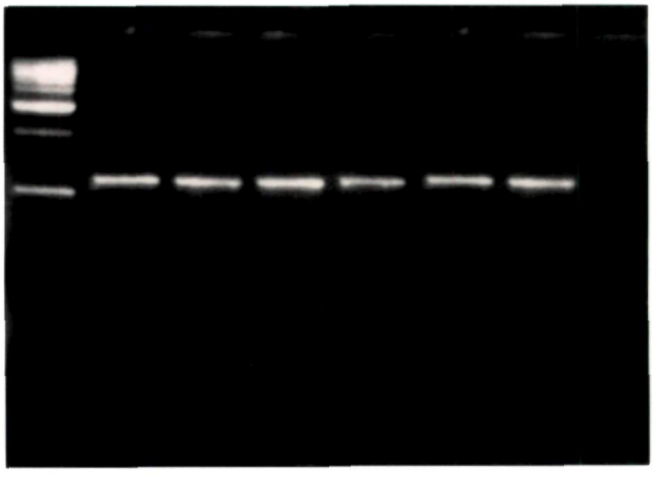


Plate 12. PCR confirmation of 1.3 kb insert in $pSAH_3$ recombinants

Table 5: Nucleotide-Nucleotide Blast (BLASTn) Results of pSAV804

Accession No.	Homologous Sequence	Homology (%)	Score bits	e-value
AB041749.1	*Trichoderma virens* chit-G1 gene for endochitinase-G1 complete cds.	98	3146	0.0
AF050098	*Trichoderma virens* chitinase gene, complete cds.	97	2999	0.0
AF397020.1	*Hypocrea virens* class V chitinase	99	1905	0.0
AB041754.1	*Trichoderma hamatum* chit-HAM gene for endochitinase-HAM, complete cds.	92	1417	0.0
AB041752.1	*Trichoderma harzianum* chit-HAR2 gene for endochitinase-HAR2, complete cds.	91	1326	0.0

Table 6: Base Pair Changes Observed in the Sequence of pSAV804 in BLASTn Search

Changed from	Changed to	Positions
T	C	146 (201), 164 (1506), 739 (931), 1039 (631)
C	T	538(1132), 655 (1015),1117 (553), 1456 (214)
T	A	470 (1200), 1370 (300), 1348 (322)
C	G	942 (728), 1663 (7)
T	G	536 (1134),1167 (503), 1480 (190)
A	G	566 (1104),572 (1098),762 (908),910 (760)
G	T	1149 (521), 1305 (365)
A	C	185 (1485), 1437 (233)
G	A	187 (1483), 479 (1191), 1143 (527),1335 (335), 1336 (334)
G	C	539 (1131)
G	GAP	549 (1121)
T	GAP	1616 (54)
C	GAP	1320 (350)
GAP	C	1596 (74)

NOTE: The numbers in brackets show the positions of the nucleotides in the correct orientation (5' to 3') sequence.

are presented in Table 7. The translated sequence showed 98.3 per cent homology with endochitinase-G1 of *Hypocrea virens* (BAB40587.1).

4.5.3 rpsBLAST Search

The translated sequence was subjected to Conserved Domain Search at the Conserved Domain Database Search (rpsBLAST) programme available at NCBI website http//: www.ncbi.nlm.nih.gov. The conserved domains were found to be between 8 to 280 amino acids. The homology results of rpsBLAST of pSAV804 ORF is given in Table 8. The gaps are introduced to maximize the homology by rpsBLAST. There was 77.9 per cent homology with the conserved sequences. The results of conserved domain search are given in Fig. 4.

4.5.4 Sequence Analysis at GENETOOL

The sequences were subjected to further analysis in BTI software GENETOOL, for finding Open Reading Frames (ORFs), Restriction sites and exons.

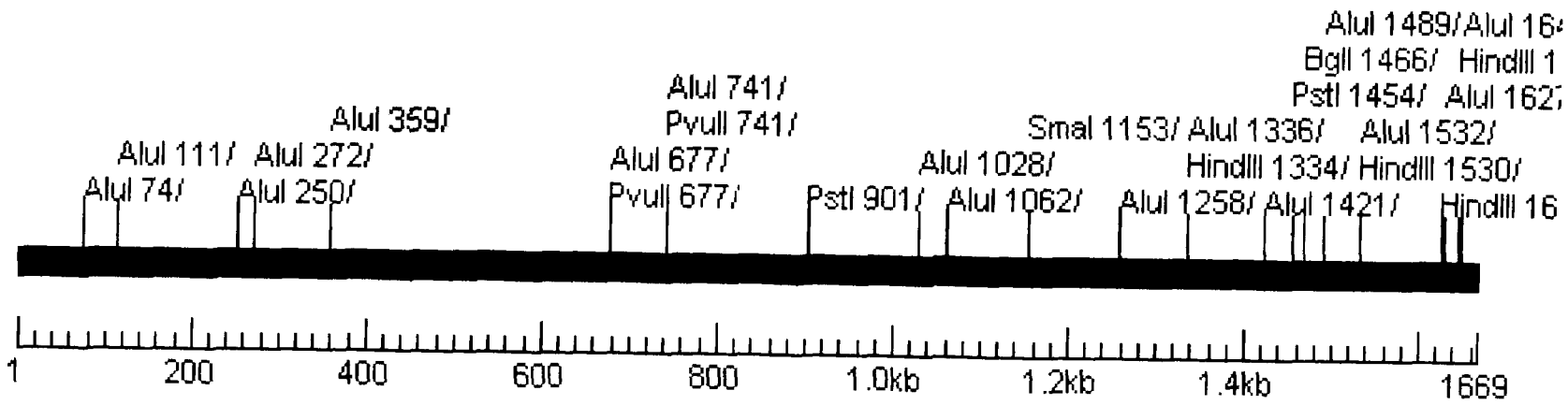
4.5.4.1 Restriction sites of pSAV804

The sequence has shown the presence of two internal unique restriction sites for *BglI* at 1466 and *SmaI* at 1153. The restriction sites are given in 3' → 5'. There is no internal restriction site for *BamHI and XbaI*. The restriction map of the sequence and clone are given in Fig. 2 and 3.

4.5.4.2 Open reading frame (ORF) of pSAV804

The sequence comprised of two ORFs in frame-2 corresponding to endochitinase gene. One between the region of 223-1047 which deduces

Fig. 2 : Restriction Map of pSAV804 insert



Restriction Sites of Unlimited Cuts with Common Enzymes

Sites of restriction for unique cuts:

SmaI at 1153

BglI at 1466

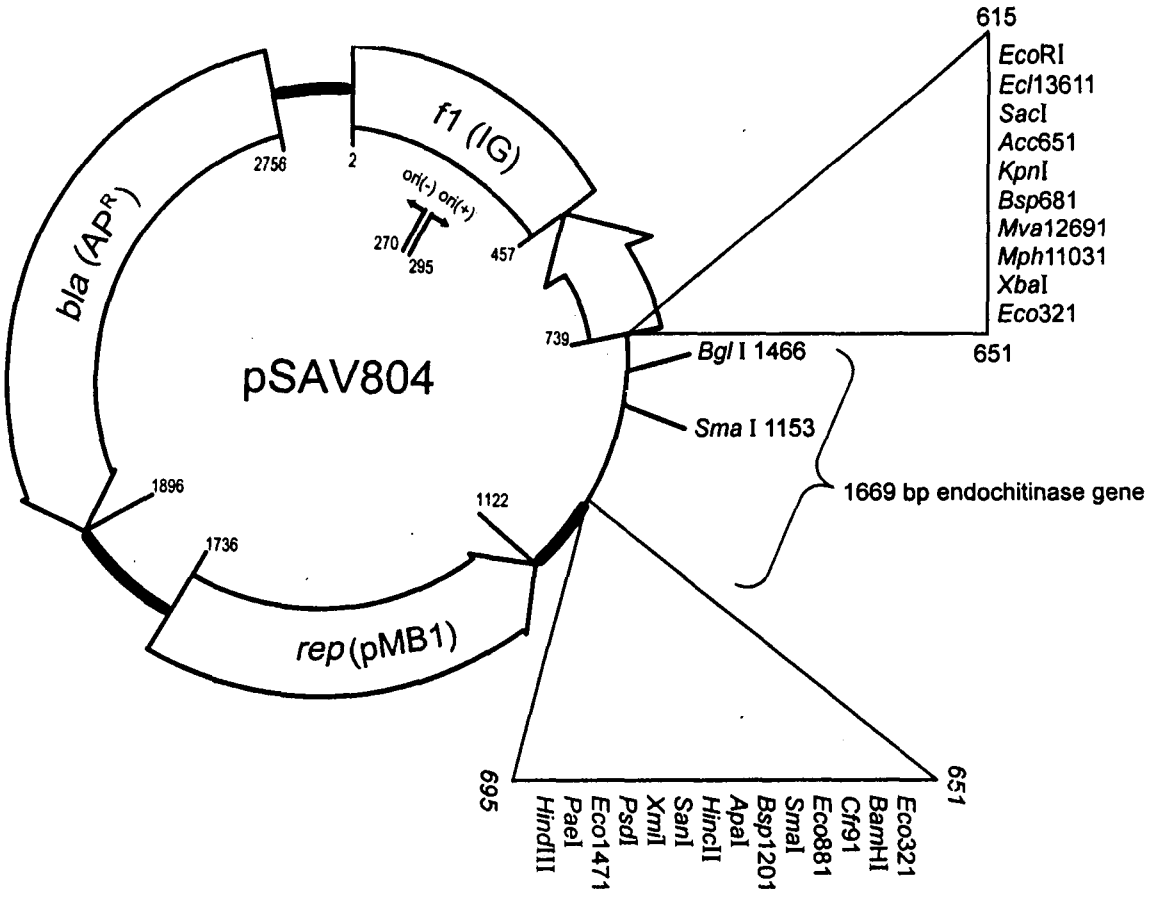
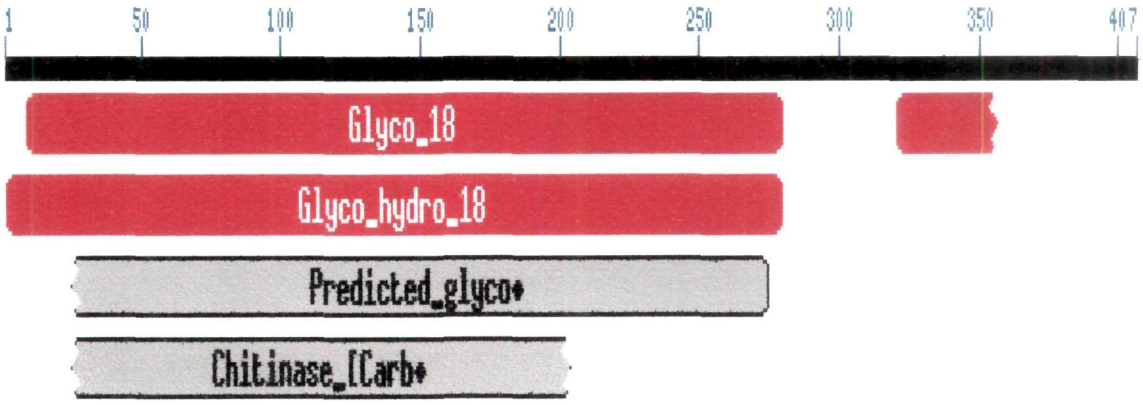


Fig. 3 : Restriction map of construct pSAV804 containing endochitinase gene.

Fig. 4 : rpsBLAST results of pSAV804



smart00636, Glyco_18, Glyco_18 domain

```
            CD-Length = 335 residues,  85.7% aligned
            Score =  260 bits (665), Expect = 2e-70

Query:  8    NAYGCVKQLFKVKKANRGLKVLLSIGGWTW-STNFPSAASTDANRKNFARTAITFMKDWG  66
Sbjct:  49   ADIGNFLQLKALKKKNPNLKVLLSIGGWTEGSDNFSSMLSDPASRKRFIDSIVSFLRKYG  108

Query:  67   FDGIDVDWEYPADSTQA-SNMILLLKEVRSQLDAYAAQYAPGYHFLLTIAAPAGKDNYSK  125
Sbjct:  109  FDGIDIDWEYPGARGDDEENYTLLLKELREALDKEEAE---GKGYLLTAAVPAGPDKIDK  165

Query:  126  LR-LADLGQVLDYINLMAYDYAGSFSPLTGHDANLFATHRTPMPLPNPSNPNATPFNTDS  184
Sbjct:  166  GYDLPAISKYLDFINLMTYDFHGAWDNPTGHNAPLYGGPGDP-----------EYFNVDY  214

Query:  185  AVKDYIKGGVPANKIVLGMPIYGRSFQNT----------------------AGIWDYKA  221
Sbjct:  215  AVKYYLCKGVPPSKLVLGIPFYGRGWTLVDGSNNGPGAPFTGPATGGPGTWEGGVVSYRE  274

Query:  222  LPRA--GATIKYDDVAKGYYSYNSNTKELISFDTPDMINTKVAYLKSLGLGGSMFWEASA  279
Sbjct:  275  ICKQLGGATVVYDDTAKGPYAYNPGTGQWVSYDDPRSIKAKADYVKDKGLGGVMIWELDG  334

Query:  280  D  280
Sbjct:  335  D  335
```

Table 7: Translated Query Vs. Protein Database Blast (BLASTx) Search Results of pSAV804

Accession No.	Homologous Sequence	Homology (%)	Score bits	e-value
BAB40587.1	Endochitinase-G1 (*Hypocrea virens*)	98.3	335	0.0
AAL78812.1	class V chitinase (*Hypocrea virens*)	97	335	0.0
AAC05829.1	chitinase (*Hypocrea virens*)	94	332	0.0
BAB40592.1	endochitinase-HAM (*Trichoderma hamatum*)	92	331	0.0
BAB40590.1	endochitinase-HAR2 (*Trichoderma harzianum*)	91	332	0.0

Table 8: rpsBLAST results of pSAV804 ORF

Sl.No.	Accession No.	Protein	Homology (%)	e-value
1.	CDD/24813	smart 00636, Glyco-18, Glyco-18 domain	77.9	2e-61
2.	CDD/25596	pfam 00704, Glyco-hydro-18, Glycosyl hydrolases family 18	78	1e-48
3.	CDD/13173	CoG 3858, Predicted glycosyl hydrolae (General function predicted)	45.6	9e-07

to 274 codons and another between 1351-1497 which deduces to 48 codons (Fig. 5).

4.5.4.3 Exons of pSAV804 ORF

The ORF comprises of single exon between the region 190-803 in 3' → 5' in frame-2. The information of exons is given in Fig. 6.

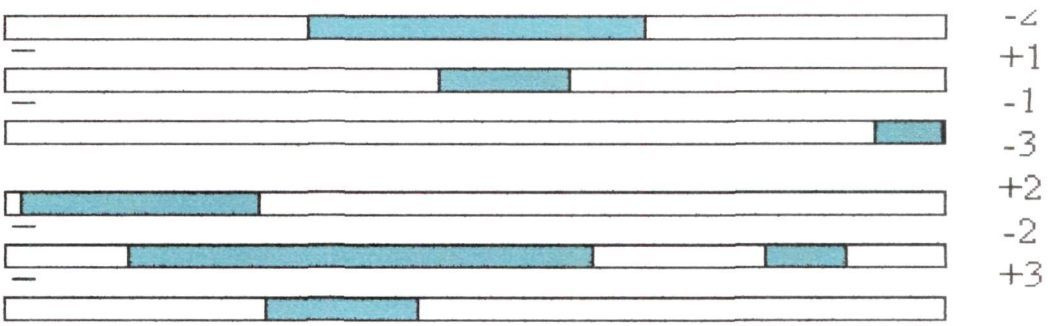
4.6 SEQUENCE ANALYSIS OF $pSAH_1$

Out of 5 $pSAH_1$ clones confirmed to have 1.3 Kb insert, $pSAH_12104$ was selected for sequencing. The clone was sequenced at BioServe Biotechnologies India Pvt. Ltd. A sequence of 1264bp was available with M13PR (Appendix-III). The available sequence information from cloned fragment was subjected to BLASTn search. The available sequence information did not show homology with any of the Endochitinase genes. The sequence has shown homology with chromosomal sequences of other plant pathogenic fungi. The sequence has shown homology with the hypothetical protein from different fungi other than the *Trichoderma* spp. when subjected to BLASTx search. The results are shown in Table 9. The ORF of the sequence showed no conserved domain in the rpsBLAST search. The database did not have the information. The sequence was further subjected to study the restriction sites. The restriction map of the sequence is given in Fig. 7.

4.7 SEQUENCE ANALYSIS OF $pSAH_3$

Out of 5 $pSAH_3$ clones of *T. harzianum* confirmed to have 1.3 Kb insert, $pSAH_32704$ was selected for sequencing. The clone was sequenced at BioServe Biotechnologies India Pvt. Ltd. A sequence of 1266bp was available with M13 primers (Appendix-III). The available sequence

Fig. 5 : ORF and the deduced amino acid sequence of pSAV804.



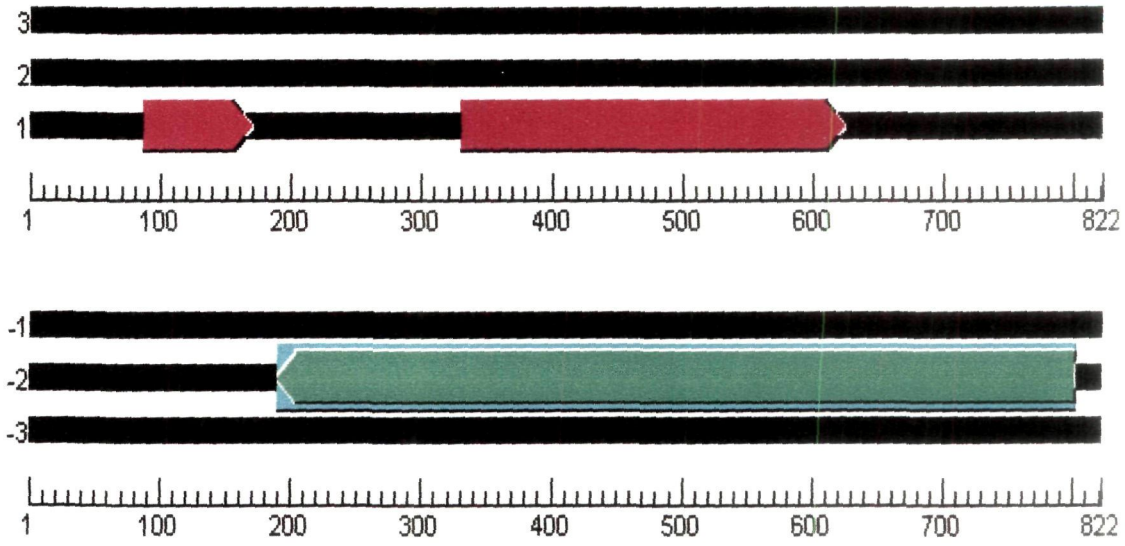
Note: Two ORFs in frame -2: 223-1047 (825 bp)
: 1351-1497 (147 bp)
The deduced amino acid sequence of 825 bp *ORF*

```
1047 atgcttacgatcacaatagcttggaatgatgtcggcaccaatgcc
     M  L  T  I  T  I  A  W  N  D  V  G  T  N  A
1002 tatggctgcgtcaagcagttgttcaaggtgaagaaggccaaccga
     Y  G  C  V  K  Q  L  F  K  V  K  K  A  N  R
 957 ggcctcaaggttttgctctccattggtggctggacctggtccacc
     G  L  K  V  L  L  S  I  G  G  W  T  W  S  T
 912 aacttcccttctgcagcaagcaccgatgccaaccgaaagaacttt
     N  F  P  S  A  A  S  T  D  A  N  R  K  N  F
 867 gcacgaactgccattacattcatgaaggattggggtttcgatggt
     A  R  T  A  I  T  F  M  K  D  W  G  F  D  G
 822 attgatgtcgattgggagtaccccgcagacagcacccaggcctcc
     I  D  V  D  W  E  Y  P  A  D  S  T  Q  A  S
 777 aacatgatccttctgctcaaggaagtccgatctcagctggatgcc
     N  M  I  L  L  L  K  E  V  R  S  Q  L  D  A
 732 tatgccgcacaatacgcccctggctaccacttcctcctcaccatt
     Y  A  A  Q  Y  A  P  G  Y  H  F  L  L  T  I
 687 gccgccccagctggcaaggacaactactctaaattgcgcctggct
     A  A  P  A  G  K  D  N  Y  S  K  L  R  L  A
 642 gatcttggccaagtcctcgactacattaacctcatggcctacgac
     D  L  G  Q  V  L  D  Y  I  N  L  M  A  Y  D
 597 tacgctggttccttcagccccttaccggacacgacgccaatctg
     Y  A  G  S  F  S  P  L  T  G  H  D  A  N  L
 552 ttcgcaacccatcgaaccccaatgccactcccttcaacacggatt
     F  A  T  H  R  T  P  M  P  L  P  S  T  R  I
 507 ctgctgtcaaggattatatcaagggaggtgttcccgctaacaaga
     L  L  S  R  I  I  S  R  E  V  F  P  L  T  R
 462 ttgttcttggcatgcccatttatggacgatcattccagaacaccg
     L  F  L  A  C  P  F  M  D  D  H  S  R  T  P
 417 ctggtattggccagacttacaacggagttggaggtggcggtggtg
     L  V  L  A  R  L  T  T  E  L  E  V  A  V  V
 372 gctccactggaagctgggaggccggtatctgggattacaaggccc
     A  P  L  E  A  G  R  P  V  S  G  I  T  R  P
 327 tccccagggccggtgctaccatcaagtacgatgatgtggcaaagg
     S  P  G  P  V  L  P  S  S  T  M  M  W  Q  R
 282 gctactacagctacaactccaacaccaaggagcttatctctttcg
     A  T  T  A  T  T  P  T  P  R  S  L  S  L  S
 237 ataccctgacatga 223
     I  P  L  T  *
```

The deduced amino acid sequence of 147 bp ORF

```
1497 atgttgagcttcctcggcaaatccgtggccttgctggctgcactg
       M  L  S  F  L  G  K  S  V  A  L  L  A  A  L
1452 caggctaccctcagcgctgcaagccctctagctacagaggagcac
       Q  A  T  L  S  A  A  S  P  L  A  T  E  E  H
1407 tccgttgagaagagagccaatggatacgcaaactctgtctacttc
       S  V  E  K  R  A  N  G  Y  A  N  S  V  Y  F
1362 accaactggtaa 1351
       T  N  W  *
```

Fig. 6 : Exon Map of pSAV804 ORF



Note : Exon in the region between 190 – 803 in frame-2

information from cloned fragment was subjected to BLASTn search. The sequence did not show homology with any of the endochitinase genes. The sequence has shown homology with the chromosomal regions of the plant pathogenic fungi instead it has shown homology with the hypothetical protein from different fungi other than the *Trichoderma* spp. when subjected to BLASTx search, the results are shown in Table 10. The ORF of the sequence when subjected for rpsBLAST, no conserved domains were found with any of the protein. The database lacked the information. The restriction map of the sequence is given in Fig. 8.

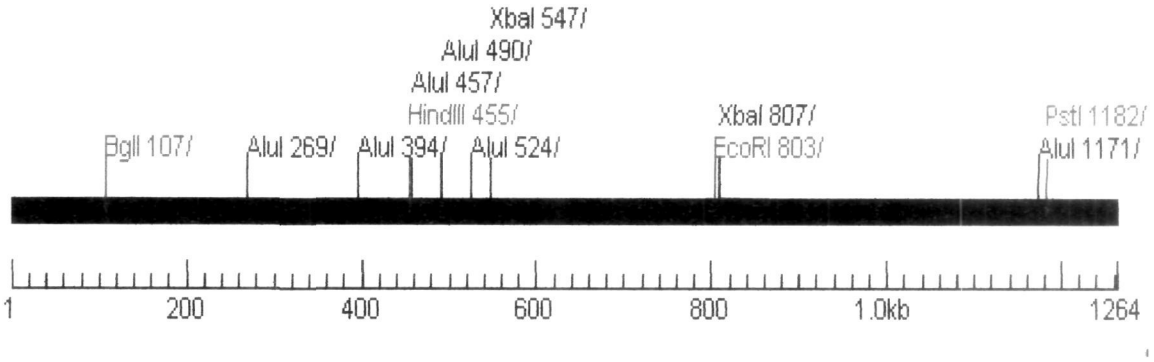
Table 9: BLASTx results of pSAH$_1$2104

Sl.No.	Accession No.	Protein	Homology (%)	e-value
1.	EAA75053.1 XP-386287.1	Hypothetical protein FG06111.1 (*Gibberella zeal* PH-1)	50	1e-08
2.	EAA52837.1 XP-369499.1	Hypothetical protein MG05965.4 (*Magnaporthe grisea* 70-15)	46	2e-05
3.	XP-328499.1 (*Neurosporae crassa*) CAC28592.1 EAA28848.1	Conserved hypothetical protein MG05965.4 (*Magnaporthe grisea* 70-15)	45	2e-28

Table 10: BLASTx results of pSAH$_3$2704

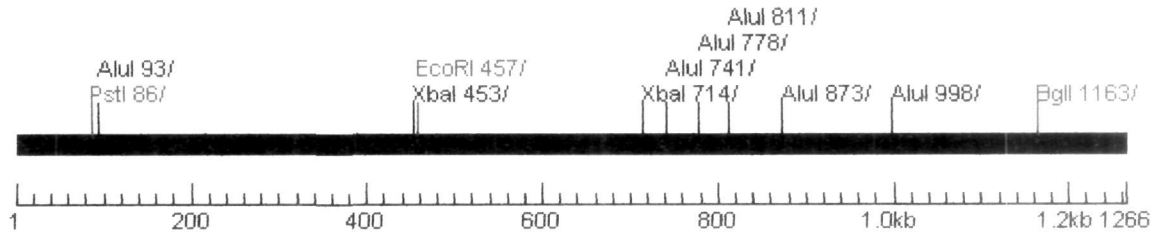
Sl.No.	Accession No.	Protein	Homology (%)	e-value
1.	XP-328499.1 (*Neurosporae crassa*) CAC28592.1 EAA28848.1	Conserved hypothetical protein MG05965.4 (*Magnaporthe grisea* 70-15)	50	2e-28
2.	EAA75053.1 XP-386287.1	Hypothetical protein FG06111.1 (*Gibberella grisea* 70-15)	46	2e-05
3.	EAA52837.1 XP-369499.1	Hypothetical protein MG05965.4 (*Magnaporthe grisea* 70-15)	45	2e-28

Fig. 7 : Restriction Map of the pSAH$_1$2104



Sites of unique cuts: BglI at 107, HindIII at 455, EcoRI at 803, Pst at 1182.

Fig. 8 : Restriction Map of the pSAH$_3$2704



Sites of unique cuts: PstI at 86, EcoRI at 457, BglI at 1163.

DISCUSSION

V. DISCUSSION

The productivity of agricultural systems in the developing world is declining. For the past few decades, world agriculture has relied on the input of expensive agrochemicals, many of which are becoming less effective, due to development of resistance among pathogens for various fungicides, evolution of new strains and development of conditions that are favourable for newer pathogens. New solutions and products are needed to achieve the necessary increase in food production and employment.

The use of biocontrol agents (BCA) to combat disease have become popular as they are highly economical, environment friendly. Self-propagating and self-perpetuating and no development of resistance.

The most frequently studied biocontrol agent (BCA) of fungal diseases is *Trichoderma* spp. BCA has been shown to act on the disease in different ways; mycoparasitism, competition, antagonism, etc. (Elad, 1996). Mycoparasitism involves the host cell wall degradation with the help of extracellular enzymes such as chitinases and glucanases, which can dissolve the fungal cell wall, allowing parasitic hyphae to penetrate the host cells (Elad, 1995). Proteases are also involved in biocontrol (Geremia *et al.*, 1993). The purified enzymes from *Trichoderma* are substantially more antifungal than chitinolytic and glucanolytic enzymes purified thus far, from any other source when assayed under the same conditions (Lorito *et al.*, 1993).

Several *Trichoderma* strains have been reported to be effective in controlling plant diseases (Krauss and Soberanis, 2001). Strain

selection and mutation have produced some fungal isolates that are as effective as fungicides in certain cultural conditions (Harman and Hayes, 1996) i.e. upto 100 times more active than the corresponding plant enzymes and effective on a much wider range of pathogens and non toxic to plants even at high concentrations (Lorito *et al.*, 1998).

Despite the interest in biological control agents, the use of these agents is limited due to low field remnance. New technologies and resources are needed for the development of sustainable crop protection systems by different control strategies against various pathogens. The introduction of genes encoding the enzymes responsible for disease resistance to plants is an important alternative available to be included in Integrated Disease Management. However, with conventional breeding, it is a time consuming task and involves many drawbacks. Modern gene transfer approaches include various techniques of genetic engineering. The genes that have been transferred to plants conferring disease resistance are chitinases, glucanases, proteases, etc. The search for other disease-resistance genes in many plant species is underway. Plants that produce more chitinase than usual have been developed and they show increased resistance to fungal diseases (Lorito *et al.*, 1998).

Isolation and cloning of the chitinase gene is the first step in development of transgenics. In the present investigation, an attempt was made to clone full-length endochitinase gene from one of the native *Trichoderma* isolate.

Screening of fungal isolates for chitinolytic activity in Mandels and Reese (1965) agar medium with colloidal chitin as the sole carbon source showed no zone of hydrolysis where as plate inoculated with

Serratia marcescens showed clear zone of hydrolysis in Monreal and Reese (1969). On the agar medium, over growth of mycelia did not permit visualization of the zone of hydrolysis. It was decided to screen the isolates in broth media only. Screening of fungal isolates for chitinolytic activity in Mandels and Reese (1965) broth with colloidal chitin as the sole carbon source showed wide variation among the fungal isolates. Under the experimental conditions, *Trichoderma koningii* showed highest chitinolytic activity, followed by three of the strains of *Trichoderma harzianum* and *Trichoderma virens*. The most inefficient fungal isolate was *Nomuraea releyi*. This may be because *Nomuraea releyi* is biocontrol agent, which controls insects, and genes encoding chitinases might not have been induced in the presence of chitinase from other source. Monreal and Reese (1969) found *Trichoderma viride* to display an efficient chytinolytic system when they grown on different chitin substrates. Other *Trichoderma* species were not included in study. Similar to present study, Grabowski (1999) reported that *Trichoderma koningii* was most effective in biocontrol of *Monilia fructigena* on Apple fruits. On the contrary, *Trichoderma koningii* was found to be less effective on pathogens when compared to other species of *Trichoderma* on Karnal Bunt of Wheat (Amer *et al.*, 1998). This difference may be because of difference in strains used for the study and the target organism.

As a further step in identifying the strain for cloning endochitinase gene, the fungal isolates were analysed for the endochitinase gene profile via PCR amplification with the primers specific for endochitinase gene sequence reported by Saiprasad *et al.* (2003). The isolates exhibited a wide variation in the amplified

fragment length. An amplicon of expected length (260 bp) was observed in all the three strains of *Trichoderma harzianum, Trichoderma koningii and Trichoderma virens,* but not in other organisms tested. It appears that the sequence information of endochitinase gene in these strains is different at nucleotide level.

Further attempts were made to clone full-length endochitinase genes from the isolates, which showed amplification of 260 bp diagnostic fragment. The *Trichoderma koningii* was selected for isolating the full-length endochitinase gene. But, no unique band was observed with *Trichoderma koningii* DNA as template with the designed primers. So, it was decided to explore the other two species, *Trichoderma harzianum* and *Trichoderma virens.* The genes were amplified using primers designed specific to it from *Trichoderma virens* (1.7 kb) and two strains of *Trichoderma harzianum* (1.3 Kb). The amplicon of 1.7 Kb and 1.3 Kb from respective species were obtained and cloned into pTZ57R, Eco32I site containing T overhangs through the T/A cloning strategy. Of the several clones obtained, three clones, pSAV804 having 1.7 Kb, $pSAH_12104$ and $pSAH_32704$ having 1.3 Kb inserts were analysed further. The presence of insert itself in them was confirmed by size of the plasmid and PCR amplification of the recombinant plasmid DNA by specific primers.

Homology search of sequence obtained from pSAV804 using M13 primers revealed the presence of 1669 bp insert showing 94-98per cent homology at nucleotide level with other reported endochitinase gene (ACC NOS.BAB40587, AAL78813.1, AAC05829.1). The nucleotide sequence differed for 33 bp at various positions. The average length of chitinase genes cited in literature is around 1700 bp

(Manocha and Govindswamy, 2002) and such differences in base pairs were observed in many of the genes cloned so far (Hayes *et al.*, 1994; Carsolio *et al.*, 1994). The deduced amino acid sequences showed 98 per cent homology at protein level with reported endochitinase proteins (ACC. NOs. BAB40587.1, AAL78813.1, AAC05829.1 etc.). BLASTx introduced the gaps in amino acid sequence. Comparison between protein of the published chitinase genes was done by many researchers (Hayes *et al.*, 1994; Carsolio *et al.*, 1994). When *ech-42* was compared with the previously reported sequence of Th *En-42* by Hayes *et al.* (1994), only 5 amino acids were different at positions 75, 76, 78, 121 and 137.

The information obtained in rpsBLAST programme has shown 85.7 per cent conservation in the protein. The conserved domain was the catalytic domain which showed 83 per cent homology with glycosyl hydrolase family 18 to which the chitinases belong. Most of the fungal chitinases have a conserved catalytic region which contains invariant aspartic and glutamic residues as in the case of *T. harzianum* (McCreath *et al.*, 1995).

The position of primer sequence in the insert showed that, the insert is in reverse orientation in the vector. The sequence was further analysed in BTI software GENETOOL. The internal restriction sites were *BglI* and *SmaI*. The absence of internal *BamHI* and *XbaI* would enable the release of the insert from the vector and clone it into an expression vector for further studies. It can also be cloned in the plant transformation vector by restricting with same enzymes. The ORF of the sequence comprises of 822 base pairs and 274 codons. But the ORF of the Acc. No. AF050098 using which, the primers were

designed, comprises of 1016 bp which deduces 339 codons (Baek *et al.*, 1999). Another ORF of 147 base pairs was observed between the region 1351-1497 which deduces 48 codons which also corresponds to chitinase. This might be because of a change of nucleotide which resulted in occurance of a stop codon in the ORF.

The ORF of the pSAV804 comprises of single exon with no introns. In earlier reported genes introns were present in *Trichoderma harzianum* endochitinase gene (Carsolio *et al.*, 1994).

With the available sequence information it can be concluded that the gene is novel. The effect of differences in the sequence observed with this analysis will be seen only when the expression studies are conducted in future.

Homology search of two clones from *Trichoderma harzianum* strains 1 and 3, $pSAH_1 2104$ and $pSAH_3 2704$ respectively, did not show any homology with reported endochitinase nucleotide sequences. Homology search at protein level has shown 50 per cent homology to a hypothetical, unnamed, proteins from fungi other than *Trichoderma* spp. The primers might have picked up some other sequences that are not as yet defined. There is a need to study the nature of the protein by expression studies in eukaryotic expression system.

SUMMARY

VI. SUMMARY

The genus of *Trichoderma* encompasses several species, which are a valuable biopesticides and a source of and endochitinase genes that encode enzymes responsible for disease resistance. In present study, fungal isolates were analyzed and attempts were made to clone endochitinase gene.

- Among the fungal isolates for chitinolytic activity a strain of *Trichoderma koningii* was found to be the most potent based on hydrolytic activity.
- The PCR, set to amplify a diagnostic 260 bp fragment, showed that isolates exhibited differences. *Trichoderma harzianum* strains 1, 2, 3, *Trichoderma koningii* and *Trichoderma virens* showed amplification of the expected 260bp fragment with the same primer pair.
- The primers for full-length gene were designed using the available sequences in the database.
- *Trichoderma harzianum* strains 1 and 3, *Trichoderma koningii* and *Trichoderma virens* were used for amplification of full length gene. As *Trichoderma koningii* did not show amplification of desired length, the amplicons obtained from *Trichoderma harzianum* strains 1 and 3 and *Trichoderma virens* were cloned into pTZ57R containing T overhangs at Eco32I site and clone in each was analysed further.
- The recombinant clone pSAV804 of *T. virens* insert contained 1.7 kb endochitinase gene in reverse orientation.

- The recombinant clone pSAH2704 and pSAH2104 of *T. harzianum* contained insert of 1.3 kb, respectively, in reverse and correct orientation.

- Analysis of the sequence at BTI software GENETOOL and BLASTn, BLASTx and rpsBLAST programmes available at NCBI website http//: www.ncbi.nlm.nih.gov showed that the nucleotide sequence and deduced amino acid sequences of pSAV804 had 98 per cent homology with other reported endochitinase genes and proteins. $pSAH_12104$ and $pSAH_32704$ showed no homology with other reported endochitinase genes. Instead they have shown homology with the hypothetical proteins of some other fungi.

- The cloned fragment of pSAV804 now need to be transferred to eukaryotic expression vector and plant transformation vector to study their potential to develop transgenics that are resistant to fungal diseases.

REFERENCES

VII. REFERENCES

AMER, G.A.M., SINGH, D.V., AGGARWAL, R. AND DUREJA, P., 1998, Microbial antagonism to *Neovossia indica*, causing Karnal bunt of wheat. *Ph.D. thesis*, PG School, IARI, New Delhi.

ANONYNMOUS, 1999, *Annual Report for 1998-99*, National Research centre on Sorghum, Hyderabad, pp. 110-115.

BAEK, J. M., HOWELL, C. R. AND KENERLEY, C. M., 1999, The role of an extracellular chitinase from *Trichoderma virens* GV29-8 in the bio-control of *Rhizoctonia solani*. *Current Genetics*, **35:** 41-50.

BARTNICKI-GARCIA, S., 1973, Fundamental aspects of hyphal morphologenesis. *In Microbial Differentiation*, pp. 245-267.

BENHAMOU, N., BROGLIE K., CHET, I. AND BROGLIE, R., 1993, Cytology of infection of 35S bean chitinase in transgenic canola plants by *Rhizoctonia solani:* cytochemical aspects of chitin breakdown *in vivo*. *The Plant Journal*, **4:** 295-305.

BIELKA, H., DIXON, H.B.F., KARLSON, P., LIEBECQ, C., SHARON, A., VAN LENTEN, E.J., VELICK, S. F. AND VLIEGENTHART, J. F. G., 1984, *Enzyme nomenclature*. Academic Press, New York.

BLAISEAU, P. L., KUNZ, C., GRISON, R., BERTHEAU, Y. AND BRYGOO, Y., 1992, Cloning and expression of a chitinase gene from the hyperparasitic fungus *Aphanocladium album. Current Genetics*, **21:** 61-66.

BLAISEAU, P. L. AND LAFAY, J. F., 1992, Primary structure of a chitinase encoding gene (*chi 1*) from the filamentous fungus *Aphanocladium album. Gene*, **120:** 243-248.

BOOT, R. G., RENKEMA, G. H., STRIJLAND, A., VAN ZONNEVELD, A.J. AND AERTS, J.M.F.G., 1995, Cloning of a cDNA encoding chititriosidase, a human chitinase produced by macrophages. *Journal of Biological Chemistry*, **270:** 26252-26256.

BROGILE, R. AND BROGILE, K., 1993, Chitinase gene expression in transgenic plants: a molecular approach to understanding plant defence responses. *Phil. Trans. R. Soc. Lond., B*, **342 :** 265-270.

CARSOLIO, C., GUITIERREZ, A., JIMENEZ, B., VAN MONTAGU, M. AND HERERA-ESTRELLA, A., 1994, Characterization of ech-42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proceedings of National Academy Science,* USA, **91:** 10903-10907.

COLLINGE, D. B., KRAGH, K. M., MIKKELESEN, J. D., NIELSEN, K. K., RASMUSSEN, U. AND VAD K., 1993, Plant chitinases. *Plant Journal*, **3:** 31-40.

COTTRELL, M. T., WOOD, D. N., YU, J. AND KIRCHMAN, D. L., 2000, Selected chitinase genes in cultured and uncultured marine bacteria in the α- and √- subclasses of the proteobacteria. *Applied and Environmental Microbiology*, **66:** 1195-1201.

DI PIETRO, A., LORITO, M., HAYES, C. K., BROADWAY, R. M. AND HARMAN, G. E., 1993, Endochitinase from *Gliocladium virens*: isolation, characterization and synergistic antifungal activity in combination with gliotoxin. *Phytopathology*, **83:** 308-313.

ELAD, Y., 1995, Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection. *Biological Reviews*. **72:** 381-422.

ELAD, Y., 1996, Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *European Journal of Plant Pathology*, **102:** 719-732.

ELAD, Y., 2000, Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection*, **19:** 709-714.

ELAD, Y., ZVIECH, Y. AND CHET, I., 1986, Biological control of *Macrophomina phaseolina* (Tassi) Goid. by *Trichoderma harzianum*. *Crop Protection*, **5:** 288-292.

EL-KATATNY, M. H., SOMITSCH, W., ROBRA, K .H., EL-KATATNY, M. S. AND GUBITZ, G. M., 2000, Production of chitinase and 1,3-glucanase, by *Trichoderma harzianum* for control of the phytopathogenic fungus *Sclerotium rolfsii*. *Food Technology Biotechnology*, **38:** 173-180.

FAIZE, M., MALNORY, M., DUPUIS, F., CHEVALIER, M., PARISI, L. AND CHEVREAU, E., 2003, Chitinases of *Trichoderma atraviride* induce scab resistance and some metabolic changes in two cultivars of apple. *Phytopathology*, 93: 1496-1504.

FLACH, J., PILET, P. E. AND JOLLES, P., 1992, What's *new in chi*tinase research? *Experientia*, **48:** 701-716.

GAFFER, A., 1968, Interaction of soil fungi with *M. phascolina* (Maubl.) Ashby the cause of root rot of cotton. *Mycopathologia Mycologia Applicata*, **34:** 196-201.

GARCIA, I., LORA, J. M., DE LA CRUZ, J., BENITEZ, T., LLOBELL, A. AND PINTOR-TORO, J. A., 1994, Cloning and characterization of a chitinase (*chit42*) cDNA from the mycoparasitic fungus *Trichoderma harzianum. Current Genetics.* **27:** 83-89.

GEREMIA, R. A., GOLDMAN, G. H., JACOBS, D., ARDILES, W. B., VILLA, S., MONTAGU, M. V., HERRERA – ESTRELLA, A., 1993, Molecular characterization of the proteinase – encoding gene *prb*1, related to mycoparasitism by *Trichoderma harzianum. Molecular Microbiology*, **8:** 603-613.

GOODAY, G. W., 1990, Physiology of microbial degradation of chitin and chitosan. *Biodegradation*, **1:** 177-190.

GRABOWSKI, M.M., 1999, Research on Possibilities of Application of Selected Bacteria and Fungi in Biological Control of *Monilia fructigena* (Aderh. et Ruhl.) on Apple Fruits. Bulletin of the *Polish Academy of Sciences: Biological Sciences*, pp. 2-4.

GRAHAM, L. S. AND STICKLEN, M. B., 1994, Plant chitinases. *Canadian Journal of Botany*, **72:** 1057-1083.

GROVER, A. AND GOWTHAMAN, R., 2003, strategies for development of fungus-resistant transgenic plants. *Current Science*, **84:** 330-340.

HARAN, S., SCHICKLER, H., OPPENHEIM, A. AND CHET, I., 1995, New components of the chitinolytic system of *Trichoderma harzianum*. *Nucleic Acid Research*, **99**: 447-450.

HARMAN, G. E., HAYES, C.K., LORITO, M., BROADWAY, R. M., DI PIETRO, A., PETERBAUR, C. AND TRONSMO, A., 1993, Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. *Phytopathology*, **83:** 313-318.

HARMAN, G.E. AND HAYES, C.K., 1996, Report to the Office of Technology Assessment (U.S. Congress, Washington D.C.), pp. 34-96.

HAYES, C. K., KLEMSDAL, S., LORITO, M., DI PIETRO, A., PETERBAUER, C., BAKAS, P. J., TRONSMO, A. AND HARMAN, G. E., 1994, Isolation and sequence of an endochitinase-encoding gene from a cDNA library of *Trichoderma harzianum*. *Gene*, **138:** 143-148.

HEGEDAS, D. D. AND KHAZHATOURIANS, G. G., 1996, Defection of entomopathogenic fungus *Bauveria bassiana* within infected migratory grasshoppers (*Melanoplus sanguipiper*) using polymerase chain reaction and DNA probe. *Journal of Invertebrate Pathology*, **67:** 21-27.

HEINRIKSON, R. L. AND MEREDITH, S. C., 1984, Amino acid analysis by reverse-phase high performance liquid chromatography: Precol derivation with phenylisothyosinate. *Anals of Biochemistry*, **136:** 65-74.

HENRISSAT, B. AND BAIROCH, A. (1993). New families in the classification of glycosyl hydroclases based on aminoacid sequence similarities. *Biochemical Journal*, **293**: 781-788.

HOWELL, C. R., 2004, Cotton seedling disease control with biological / chemical seed treatments. Proceedings of beltwide cotton conferences. January 5-9, 2004, San Antonio, Texas. *National Cotton Council*, 2004, cdrom.p.421.

HSU, S. C. AND LOCKWOOD, J. L., 1975, Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied microbiology*, **29**: 422-426.

HUMPHREYS, A. M. AND GOODAY, G. W., 1984, Phospholipid requirement of microsomal chitinase from *Mucor mucedo*. *Current Microbiology*, **11:** 187-190.

HUYNH, Q. K., HIRONAKA, C. M., LEVIN, E. B., SMITH, C. E., BORGMEYES, J. R. AND SHAH, D. M., 1992, Antifungal proteins from plants. *Journal of Biological Chemistry*, **267:** 6635-6640.

JACH, G., GORNHARDT, B., MUNDY, J., LOGEMANN, J., PINSDORF, E., LEACH, R., SCHELL, J. AND MAAS, C., 1995, Enhanced quantitative resistance against fugal disease by combinatorial expression of different barely anti-fungal proteins in transgenic tobacco, *Plant Journal.* **8:** 97-109.

JONES, J.D.G., DEAN, C., GILBERT, D., BOND-NUTTER, D., LEE, R., BEDBROOK, J., AND DUNSMUIR, P., 1988, Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. *Molecular Genetics*, **212:** 536-542.

KAUFFMAN, S., LEGRAND, M., GEOFFROY, P. AND FRITIG, B., 1987, Biological function of "pathogenesis-related proteins": four PR proteins of tobacco have 1, 3-β-glucanase activity. *EMBO Journal*, **6:** 3209-3212.

KIM, D. J., BAEK, J. M., URIBE, P., KENERLEY, C. M. AND COOK, D. R., 2002, Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens. Current Genetics*, **40:** 374-84.

KIVANC, M., 2003, Isolation of Trichoderma spp. and determination of their antifungal, biochemical and physiological features. *Turkish Journal of Biology*, **27:** 247-253.

KOVACS, K., SZALEACS, G., PUSZTAHELYI, T. AND PANDEY, A., 2004, Production of chitinolytic enzymes with Trichoderma longiabrachiatum IMI 92027 in solid substrate fermentation. *Applied Biochemistry and Biotechnology,* **118 :** 189-204.

KRAUSS, U. AND SOBERANIS, W., 2001, Biocontrol of Cocoa pod diseases with mycoparasite mixtures. *Biological Control* **18:** 235-242.

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.

LEGRAND, M., KAUFFMAN, S., GEOFFROY, P. AND FRITIG, B., 1987, Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. *Proceedings of National Academy Sciences,* USA, **84:** 6750-6754.

LORITO, M., 1998, Chitinolytic enzymes and their genes In: Harman G.E., Kubicek C. P. (eds). *Trichoderma* and *Gliocladium,* Vol 2 Enzymes, biological control and commercial application, pp.73-99. Taylor and Fransis, London UK.

LORITO, M., FARKAS, V., REBUFFAT, S., BODO, B. AND KUBICEK, C. P., 1996. Cell wall synthesis is a major target of mycoparasitic antogonism by *Trichoderms harzianum. Journal of Bacteriology,* **178:** 6382-6385.

LORITO, M., HARMAN, G. E., HAYES, C. K., BROADWAY, R. M., TRONSMO, A., WOO, S. L. AND DI PIETRO, A., 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal Activity of Purified Endochitinase and Chitobiosidose. *Phytopathology,* 83: 302-307.

LORITO, M., PETERBAUER, C., HAYES, C. K.AND HARMAN, G. E., 1994, Synergistic interaction between fungus cell wall degrading enzymes and different antifungal compound enhances inhibition of spore germination. *Microbiology,* **140:** 623-629.

MANDELS, M. AND REESE, E. T., 1965, Inhibition of cellulases. *Annual Review of Phytopathology,* **30:** 43-47.

MANIATIS, T., FRITSCH, E. F. AND SAMBROOK, J., 1982, Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

MANOCHA, M. S. AND BALASUBRAMANIAN, R., 1988, *In vitro* regulation of chitinase and chitin synthase activity of two mucoraceous hosts of a mycoparasite. *Canadian Journal of Microbiology,* **34:** 1116-1121.

MANOCHA, M. S. AND GOVINDASWAMY, V., 2002, Chitinolytic enzymes of fungi and their involvement in biocontrol of plant pathogens in plant microbe interaction.

MAUCH, F., MAUCH-MANI, B. AND BOLLER, T., 1988. Antifungal hydrolases in P tissue II. Inhibition of fungal growth by combinations of chitinase and beta-1,3-gluconase. *Plant Physiology,* **88:** 936-942.

McCREATH, K. J., SPECHT, C.A. AND ROBBINS, P. W., 1995, Molecular cloning and characterization of chitinase genes from *Candida albicans. Proceedings of National Academy Sciences,* USA, **92:** 501-504.

McCREATH, K. J., SPECHT, C.A., LIU, Y. AND ROBBINS, P. W., 1996, Molecular cloning of a third chitinase gene (*Chit 1*) from *Candida albicans. Yeast,* **12:** 501-504.

MILEWSKI, S., O'DONNEL, R. W. AND GOODAY, G.W., 1992, Chemical modification studies of the active centre of Candida albicans chitinase and its inhibition by allosamidine. *Journal of General Microbiology,* **138:** 2545-2550.

MONREAL, J. AND REESE, E. T., 1969, Chitinases of *Serratia. Canadian Journal of Microbiology,* **15:** 689-696.

NEUHAUSS, J. M., AHL-GOY, P., HINZ, U., FLORES, S. AND MEINS, F., 1991. High level expression of tobacco chitinase gene in *Nicotitna sylvestris*: Susceptibility of transgenic plants to *Cercospora nicotianae* infection. *Plant Molecular Biology,* **16:** 141-151.

ORTELLI, S., WINZELER, H., WINZELER, M., FRIED, M. AND OSBERGER, J., 1996, Leaf rust resistance gene Lr 9 and winter wheat yield reduction: yield and yield components. *Crop Science,* **36:** 1590-1595.

PISHKO, E. J., KIRKLAND, T. N. AND COLE, G.T., 1995, Isolation and characterization of two chitinase-encoding genes (*cts1, cts2*) from the fungus *Coccidiodes immitis. Gene,* 167: 173-177.

PUNJA, Z. K. AND RAHARJO, S. H. T., 1996. Response of transgenic cocumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. *Plant Diseases*, **80:** 99-105.

RAST, D. M., HORSCH, M., FURTER, R. AND GOODAY, G.W., 1991, A complex chitinolytic system in exponentially growing mycelium of Mucor rouxii: properties and function. *Journal of General Microbiology*, **137:** 2797-2810.

REDDY, B.N. AND HINDUMATHI, 1997, VAM fungi as an agent of biological control of charcoal rot of Sorghum. Paper presented at the *Golden Jubilee International Conference on Integrated Plant Disease Management for Sustainable Agriculture.* New Delhi, Nov. 10-15, 1997, p. 186.

RIFAI, M.A., 1969, A regression of the genus *Trichoderma*, Common Wealth Mycological Institute, Kew Survey, London, U.K., *Mycological Paper*, **116:** 1-56.

ROBERTS, W. K. AND SELITRENNIKOFF, C. P., 1988, Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology*, **134:** 169-176.

ROBY, D., BROGLIE, K., CRESSMAN, R., BIDDLE, P., CHET, I. AND BROGLIE, R., 1990, Activation of bean chitinase promoter in transgenic tobacco plants by pathogenic fungi. *Plant Cell*, **2:** 999-1007.

SAHAI, A. S. AND MANOCHA, M. S., 1993, Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. *FEMS Microbiological Reviews*, **11:** 317-338.

SAIPRASAD, G. V. S., MYTHILI, J. B., LALITHA, A., SUNITHA, C. AND RASHMI, H. J., 2003, Isolation of endochitinase cDNA clone from fungus *Trichoderma harzianum.* Unpublished. Accession No. gi : 29539651/BQ172872. T22SAI.

SAMBROOK, J. AND RUSSELL, D. W., 2001, Molecular cloning : A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

SANTAMARINA, M. P., ROSELLO, J., LLACER, R. AND SANCHS, V., 2002, Atagonistic activity of Penicillium oxalicum Correi and Thom, Penicillium decumbens Thom and Trichoderma harzianum Rifai isolates against fungi, bacteria and insects *in vitro. Reviews of Iberoam Micology,* **19:** 99-103.

SANTOS, M.O., PEREIRA, M., FELIPE, M. S., JESUINO, R. S., ULHOA, C. J., SOARES, R. D. E. B. AND SOARES, C. M., 2004, Molecular cloning and characterization of a cDNA encoding the N-acetyl-beta-D-glucosaminidase homologue of *Paracoccidioides brasiliensis. Medical Mycology,* **42:** 247-53.

SCHICLER, H., HARAN, S., OPPENHEIM, A., CHET, I., 1988a, Induction of the *Trichoderma harzianum* chitinolytic system is triggered by the chitin monomer N-acetyl glucosamine. *Mycological Research,* **102:** 1224-1226.

SCHUMBAUM, M. A., MAUCH, F., VOGELI, U. AND BOLLER, T., 1986, Plant chitinases are protein inhibitors of fungal growth. *Nature,* **324:** 365-367.

SHARMA, J.K. AND SANKARAN, K.V., 1988, biocontrol of rust and leaf spot diseases. *Biocontrol of Plant Diseases*, **2** : 1-24.

SHARP, G. L., MARTIN, J. M., LANNING, S. P., BLAKE, N. K., BREY, C. W., SIVAMANI, E. Q. R. AND TALBERT, L.E., 2002, Field evaluation of transgenic and classical sources of wheat streak mosaic virus resistance. *Crop Science*, 42: 105-110.

SUSLOW, T.V., MATSUBARA, D., JONES, J., LEE, R., AND DUNSMUIR, P., 1988, Effect of expression of bacterial chitinase on tabacco susceptibility to leaf brown Spot. *Phytopathology*, **78** : 1556.

TAKAYA, N., YAMAZAKI, D., HORTUCHI, H., OHTA, A. AND TAKAGI, M., 1998, Intracellular chitinase gene from *Rhizopus oligosporus* is molecular cloning and characterization. *Microbiology*, **144** : 2647-2654.

TERAKAWA, T., TAKAYA, N., HORTUCHI, H., KOIKE, M. AND TAKAGI, M., 1997, A fungal chitinase gene from *Rhizopus oligosporus* confers antifungal activity to tobacco. *Plant Cell Reports*, **16:** 439-443.

TIKHONOV, V.E., LOPEZ-LLORCA, L. V., SALINAS, J. AND JANSSON, H., 2002, Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium. Fungal Genetics and Biology*, **35:** 67-78.

VERBURG, J. G., AND HUYNH, O. K., 1991, Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana. Plant physiol.*, **95** : 450-455.

YANAI, K., TAKAYA, N., KOJIMA, N., HORIUCHI, H., OHTU, A. AND TAKAGI, M., 1992, Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. *Journal of Bacteriology,* 174: 7398-7406.

YANG, C., ZHU, Y., MAGEE, D. M. AND COX, R. A., 1996, Molecular cloning and characterization of the Coccoidiodes immitis complement fixation/chitinase antigen. *Infection and Immunology,* **64:** 1992-1997.

ZIMMERMANN, C. R., JOHNSON, S. M., MARTENS, G. W., WHITE, A. G. AND PAPPAGIANIS, D., 1996, Cloning and expression of the complement fixation antigen-chitinase of Coccoidiodes immitis. *Infection and Immunology,* **64:** 4967-4975.

APPENDICES

APPENDIX - I

1) Potato Dextrose Agar (PDA)

Peeled Potato	200 g/l
Dextrose	20 g/l
Yeast Extract	0.1 g/l
Agar (for solid medium)	20 g/l
Distilled Water	1000 ml

2) Luria Agar

Tryptone	10 g/l
Yeast Extract	5 g/l
Sodium chloride	5 g/l
Agar (for solid medium)	18 g/l
PH	7.2

3) Monreal and Reese (1969)

Components	**g/l**
Colloidal chitin	5 g
Yeast extract	0.5 g
$(NH_4)_2SO_4$	1.0 g
$HgSO_4$ $7H_2O$	0.3 g
KH_2PO_4	1.36 g

4) MANDELS AND REESE (1965) MEDIUM

COMPONENTS	ml/l	(mg/l)
$(NH)_2 SO_4$(10%)	14.0	(0.14)
KH_2PO_4 (1M)	15.0	(0.02)
UREA (10%)	3.0	(0.03)
$CaCl_2$ (10%)	3.0	(0.03)
$MgSO_4$. $7H_2O$ (10%)	3.0	(0.03)
TRACEMETAL STOCK	1.0	
TWEN 80(OPTIONAL)	2.0	(0.02)
DW	1000	

TRACE METAL STOCK

COMPONENTS	mg/l
$FeSO_4$ (2.5 g)	1.0
$MnSO_4.H_2O$	0.5
$ZnSO_4$ (0.83mg)	0.80
$CoCl_2$ (1.0mg)	0.50
HCl CONCENTRATED (5ml)	
DW	495ml

pH of the media adjusted to 5.5.

APPENDIX - II

1) Phenol : chloroform (1:1)

Tris saturated phenol :
Chloroform : Isoamyl alcohol (24:1 V/V)

2) Master mix for PCR reaction (20 µl/tube)

Sterile double distilled water	10.5 µl
10x Taq assay buffer	2.0 µl
$MgCl_2$ (25 mM)	1.0 µl
Forward primer (10pM)	1.0 µl
Reverse primer (10pM)	1.0 µl
dNTPs (1 mM)	2.0 µl
Taq DNA polymerase (3U/µl)	0.6 µl
Total	**20 µl**

3) Loading dye (6x)

0.25 Bromophenol blue

40% (W/V) sucrose in water

Started at 4°C

4) 50x TAE (Tris-Acetate EDTA) (Miniatis *et al.*, 1982)

Tris base	242 g/l
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Distilled water	1000 ml

5) Insert : Vector ratio calculation

Length of DNA fragment (bp)	**Pmoles of ends for 1 μg of DNA**	**Quality of PCR fragments for ligation reaction in μg (0.54 pM ends)**
100	30	0.018
300	10	0.054
500	6.0	0.090
1000	3.0	0.180
2000	1.5	0.360
3000	1.0	0.540

6) Sample ligation mixture (30 μl)

Plasmid vector pTRZ57R/T DNA (0.165 μg, 0.18 pM ends)	1 μl
Purified PCR fragment (≈ 0.54 pM ends)	6 μl
10x ligation buffer	3 μl
PEG 4000 solution	3 μl
Deionized water	16 μl
T_4 DNA ligase (5u)	1 μl
Total	**30 μl**

7) Control ligation mixture (30 µl)

Plasmid vector pTRZ57R/T DNA (0.165 µg, 0.18 pM ends)	3 µl
Control PCR fragment (≈ 0.54 pM ends)	4 µl
10x ligation buffer	3 µl
PEG 4000 solution	3 µl
Deionized water	16 µl
T_4 DNA ligase (5u)	1 µl
Total	**30 µl**

8) Chemicals for Transformation

1. Ampicillin (100 µg/ml) – Amp_{100}

100 µg of ampicillin antibiotic dissolved in 1 ml of sterile water, filter sterilized and stored at 0°C. 25 µl per plate was used.

2. X-gal (20 mg/ml)

20 mg of X-gal (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside) dissolved in 1 ml N, N-dimethyl formamide. Stored at 0°C. 40 µl per plate was used.

3. IPTG (200 mg/ml)

200 mg of IPTG(Iso propyl β-D-thiogalactosidase) dissolved in 1 ml of sterile water, filter sterilized and stored at 0°C. 5 µl per plate was used.

9) Alkaline lysis solution I

Glucose	50 mM
Tris-HCl (pH 8.0)	25 mM
0.5 M EDTA (pH 8.0)	10 mM
Autoclaved and stored at 4°C	

10) Alkaline lysis solution II

NaOH	0.2 N
SDS	1% (W/V)
Prepared fresh and used at room temperature	

11) Alkaline lysis solution III

5M potassium acetate NaoH	60 ml
Glacial acetic acid	11.5 ml
Double distilled water	28.5 ml
Autoclaved and stored at 4°C	

APPENDIX - III

1) Complete Nucleotide sequence of pSAV804

BASE COUNT 388 a 390 c 466 g 425 t

ORIGIN

1	gctgtgtatc	ccctgaaaag	aagccacctt	agttgagacc	cttcctgatg	ttgtcatact
61	tggagttggg	gtagctcagc	aggttctgag	ttgagtccag	acttccaaga	gctctgtgac
121	ttgttccaat	cagcgagtca	gttcccttct	tatcggctga	ggcctcccag	aacatgctac
181	ctcccaagcc	aagagacttg	aggtaggcca	ccttggtgtt	gatcatgtca	ggggtatcga
241	aagagataag	ctccttggtg	ttggagttgt	agctgtagta	gccctttgcc	acatcatcgt
301	acttgatggt	agcaccggcc	ctggggaggg	ccttgtaatc	ccagataccg	gcctcccagc
361	ttccagtgga	gccaccaccg	ccacctccaa	ctccgttgta	agtctggcca	ataccagcgg
421	tgttctggaa	tgatcgtcca	taaatgggca	tgccaagaac	aatcttgtta	gcgggaacac
481	ctcccttgat	ataatccttg	acagcagaat	ccgtgttgaa	gggagtggca	ttggggttcg
541	atgggttgcg	aacagattgg	cgtcgtgtcc	ggtaaggggg	ctgaaggaac	cagcgtagtc
601	gtaggccatg	aggttaatgt	agtcgaggac	ttggccaaga	tcagccaggc	gcaatttaga
661	gtagttgtcc	ttgccagctg	gggcggcaat	ggtgaggagg	aagtggtagc	caggggcgta
721	ttgtgcggca	taggcatcca	gctgagatcg	gacttccttg	agcagaagga	tcatgttgga
781	ggcctgggtg	ctgtctgcgg	ggtactccca	atcgacatca	ataccatcga	aaccccaatc
841	cttcatgaat	gtaatggcag	ttcgtgcaaa	gttctttcgg	ttggcatcgg	tgcttgctgc
901	agaagggaag	ttggtggacc	aggtccagcc	accaatggag	agcaaaacct	tgaggcctcg
961	gttggccttc	ttcaccttga	acaactgctt	gacgcagcca	taggcattgg	tgccgacatc
1021	attccaagct	attgtgatcg	taagcatgtg	tacatagcgg	agctagagat	gaggaacccc
1081	tacaatcatc	ggcatagtgc	ttctcgaaat	cggcgtaggt	atcgccagag	acactgaaag
1141	acagttagta	cccgggaagt	aactttgaga	gaatatatcc	tctccgagtt	tctgacttac
1201	acagtgccgt	ctgcctggag	gttcatgaat	gagtagatga	catgagtgac	atctgaagct
1261	accaaatcgg	caggctggaa	gttgcgctcg	taaattcccc	ttcaaggacg	tgttgtcagc
1321	agtgaatcta	tggaagcttg	ttggtttcac	ttaccagttg	gtgaagtaga	cagagtttgc
1381	gtatccattg	gctctcttct	caacggagtg	ctcctctgta	gctagagggc	ttgcagcgct

1441 gagggtagcc tgcagtgcag ccagcaaggc cacggatttg ccgaggaagc tcaacatggt

1501 gaattgcttg tgatgtgaat gaatctttca agcttgccac ttctgtgctg aattttcttc

1561 tgggatgccg aatgagaacc caagatgttt atatatctgg ctgtggagcc tccagatgct

1621 ctccaagctt gaggctcact tatgaagctt ccctttcaga ttgttggca

2) Sequence Statistics of pSAV804

Sequence Data

Sequence: pSAV804 DNA Sequence.

Total Number of Bases	1669
Total Number of Degenerates	0
Percent GC Density	51.3%
Percent AT Density	48.7%
Sense Strand Mol Weight as DNA	517.02 kilodaltons
Antisense Strand Mol Weight as DNA	514.32 kilodaltons
Weight of DNA Duplex	1.03 megadaltons
Sense Strand Mol Weight as RNA	537.77 kilodaltons
Antisense Strand Mol Weight as RNA	535.58 kilodaltons
Weight of RNA Duplex	1.07 megadaltons
UV Absorbance as ssDNA	OD(260) 1.0 ~ 40 ug/mL = 77.37 nM
UV Absorbance as dsDNA	OD(260) 1.0 ~ 50 ug/mL = 48.48 nM
Melting Temp. as dsDNA ([K+] = 50 mM)	80.38 C

3) Complete Nucleotide Sequence of pSAH$_1$2104

LOCUS
SOURCE Trichoderma harzianum
ORGANISM Trichoderma harzianum
BASE COUNT 340 a 300 c 311 g 312 t 1 others
ORIGIN

1 ccgcgacgac ttcaatgtgc cggacctggg ggttaaagac agcattgagc gggttgttga
61 cattgcgtcc ccgtaccctt gggcttcgtt acttgtggct gccgccgcgg ctgtcacaac
121 tagccttgct cttgcgcctt tggatctcat ccgcacgagg tttgtgacat gactactcag
181 gtttaatgta ctctgtgact gacatgacgc cagattgatt ttaaccaacc ccttcaaggg
241 acagcgaagg acgattgcaa gccttcgagc tcttccatct tactactgcc ctcccgccat
301 cgtcgctcct accatcctcc actcactcat caaccccatc ttcaccctat ccactccctt
361 ggctttgaag accaagttta tggtcacaag cgagcttgcg cccatgacat tctccgttgc
421 caagtttgtt gcctcttcag ttggaatact aattaagctt ccactggaga ctgctctgcg
481 cagaggtcag ctctccgtgc tctctgagcc tgagtatttg gaagctctca acggsgacga
541 gccggctcta gagaccattg tgcccattgg aaaatactac ggcgttttcg gtacaatgta
601 ccacatcgtc acggaagagg gcaatcggga gatcccaccc aaacccgtag taagcaagcg
661 aggcaaactc aagaccaaga acctccagcc gacatacaag aagggtcaag gtatggaagg
721 tctctggaga ggatggcgaa tcggctggtg gggactggtt ggcctttggg gagccaacat
781 ggttggacac gggggcgatg gtgaattcta gacgaaatga aaatgaaaca ctatataggt
841 cgaaacggac tgtgtatacc attgagaatt ggaagtcggg aagaaaaggg aaaggacgaa
901 gggaaagata tcgatacaca tcttctaagg acgacagaac aaaaggaaac tctccgcttc
961 aaacgaattt tatgctggga cgacgacatc atgaccatac cttcattcat acgcatgcac
1021 gaatagcctt gaaaactatc cgttctttta ctagaaagca gagcatttgg tagtatatgt
1081 tgcctatttt aggattttgc tctgtattgc tgatatagcg tagaatgagt gcaaatacaa
1141 atacaattac acttacaagc aaatagcaga gctgagactg cagtagtata ggtgaagatg
1201 agagaaaaga tcactccagg ctcgtataat ttagttgaat tgggtgaatt gcttgtgatg
1261 ttgg

4) Complete Nucleotide Sequence of pSAH$_3$2704

LOCUS
SOURCE Trichoderma harzianum
ORGANISM Trichoderma harzianum
BASE COUNT 318 a 310 c 299 g 339 t
ORIGIN

1 ccaacatcac aagcaattca cccaattcaa ctaaattata cgagcctgga gtgatctttt
61 ctctcatctt cacctatact actgcagtct cagctctgct atttgcttgt aagtgtaatt
121 gtatttgtat ttgcactcat tctacgctat atcagcaata cagagcaaaa tcctaaaata
181 gacaacatat actaccaaat gctctgcttt ctagtaaaag aacggatagt tttcaaggct
241 attcgtgcat gcgtatgaat gaaggtatgg tcatgatgtc gtcgtcccag cataaaattc
301 gtttgaagcg gagagtttcc ttttgttctg tcgtccttag aagatgtgta tcgatatctt
361 tcccttcgtc ctttcccttt tcttcccgac ttccaattct caatggtata cacagtccgt
421 ttcgacctat atagtgtttc attttcattt cgtctagaat tcaccatcgc ccccgtgtcc
481 aaccatgttg gctccccaaa ggccaaccag tccccaccag ccgattcgcc atcctctcca
541 gagaccttcc ataccttgac ccttcttgta tgtcggctgg aagttcttgg tcttgagttt
601 gcctcgcttg cttactacgg gtttgggtgg gatctcccga ttgccctctt ccgtgacgat
661 gtggtacatt gtaacgaaaa cgccgtagta tttttcaaat gggcacaatg gtctctagag
721 ccggctcgtc gccgttgaga gcttccaaat actcaggctc agagagcacg acggagagct
781 gacctctgcg cagaacagtc tccagtggaa gctaattagt attccaactg aagaggcaac
841 aaacttggca acggagaatg tcatgggcgc aagctcgctt gtgaccataa actggtcttc
901 aaagccaagg gagtggatag ggtgaagatg gggtttgatg agtgagtgga ggatggtagg
961 agcgacgatg gcgggagggc agtagtaaga tggaagagct cgaaggcttg caatcgtcct
1021 tcgctgtccc ttgaaggggt tggttaaaat caatctggcg tcatgtcagt cacagagtac
1081 attaaacctg agtagtcatg tcacaaacct cgtgcggatg agatccaaag gcgcaagagc
1141 aaggctagtt gtgacagccg cggcggcagc cacaagtaac gaagcccaag ggtacgggga
1201 cgcaagtcaa caacccgctc aatgctgtct ttaaccccca ggtccggcac attgaagtcg
1261 tcgcgg

5) Sequence Data of the $pSAH_12104$

Sequence Data

Sequence: 2104-cured

Total Number of Bases	1264
Total Number of Degenerates	1 (0.1%)
Percent GC Density	48.3%
Percent AT Density	51.6%
Sense Strand Mol Weight as DNA	390.87 kilodaltons
Antisense Strand Mol Weight as DNA	390.18 kilodaltons
Weight of DNA Duplex	781.05 kilodaltons
Sense Strand Mol Weight as RNA	406.71 kilodaltons
Antisense Strand Mol Weight as RNA	405.63 kilodaltons
Weight of RNA Duplex	812.34 kilodaltons
UV Absorbance as ssDNA	OD(260) 1.0 ~ 40 ug/mL = 102.34 nM
UV Absorbance as dsDNA	OD(260) 1.0 ~ 50 ug/mL = 64.02 nM
Melting Temp. as dsDNA ([K+] = 50 mM)	79.08 C

6) Sequence Data of the $pSAH_32704$

Sequence Data

Sequence: 2704-cured

Total Number of Bases	1266
Total Number of Degenerates	0
Percent GC Density	48.1%
Percent AT Density	51.9%
Sense Strand Mol Weight as DNA	390.83 kilodaltons
Antisense Strand Mol Weight as DNA	391.45 kilodaltons
Weight of DNA Duplex	782.28 kilodaltons
Sense Strand Mol Weight as RNA	406.33 kilodaltons
Antisense Strand Mol Weight as RNA	407.25 kilodaltons
Weight of RNA Duplex	813.57 kilodaltons
UV Absorbance as ssDNA	OD(260) 1.0 ~ 40 ug/mL = 102.35 nM
UV Absorbance as dsDNA	OD(260) 1.0 ~ 50 ug/mL = 63.92 nM
Melting Temp. as dsDNA ([K+] = 50 mM)	78.98 C

CLONING OF ENDOCHITINASE GENE FROM NATIVE ISOLATES OF *Trichoderma* spp.

Aishwarya R. Anigol **2004** **Dr. Sumangala Bhat**
Major Advisor

ABSTRACT

The present study was conducted to screen native fungal isolates for chitinolytic activity and clone the endochitinase gene. Among the fungal isolates (*Trichoderma harzianum* strains *1,2,3, Trichoderma koningii, Trichoderma virens, Trichoderma viride, Metarrhizium verrucasia, Bauveria bassiana, Aspergillus oryzae* and *Nomurea releyi), Trichoderma koningii* showed highest hydrolytic activity when screened in Mandels and Reese (1969) salts supplemented with colloidal chitin as the sole carbon source followed by *Aspergillus oryzae. Nomuraea releyi* showed least activity. Others were on par with each other. Molecular screening with endochitinase gene specific primers, showed the presence of 260 bp fragment *Trichoderma harzianum* isolates *1,2,3, Trichoderma koningii* and *Trichoderma virens* but absence in *Trichoderma viride and Aspergillus oryzae.*

Further attempts were made to clone endochitinase gene from *Trichoderma* species, *harzianum, koningii* and *virens.* Desired length of amplicon was not obtained in *Trichoderma koningii. Trichoderma harzianum* and *Trichoderma virens* showed desired amplicons with the designed primers. A 1.7 Kb amplicon from *Trichoderma virens* and 1.3 Kb from *Trichoderma harzianum* were cloned into pTZ57R/T. The clones were confirmed through plasmid minipreparation, PCR amplification, sequencing and homology search. The clones were sequenced and analysed for homology at nucleotide, protein and domains of protein. *Trichoderma harzianum* clones showed no homology with the endochitinase genes. Among the 11 clones obtained, pSAV804 was sequenced There was 98% homology at both nucleotide and protein levels with the endochitinase genes from *Trichoderma spp.* The protein was conserved at 85.7%. The primer sequence position in the insert and homology search confirmed the reverse orientation of the insert in the vector. The sequence consisted of two ORFs corresponding to the endochitinase gene one with 274 codons and the other 48 codons. It may be because of occurance of stop codon during PCR. With the available sequence information it can be concluded that the gene is novel.