CLONING AND FUNCTIONAL CHARACTERIZATION OF ENDOCHITINASE GENE FROM *TRICHODERMA* SPP.

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I. INTRODUCTION

The present day world is facing various problems regarding food security. The traditional agriculture is affected by various problems such as drought, pest and diseases, reduced availability of the land, increase in population. Among them, pests and diseases cause major losses. According to a study conducted by Bowyer (1999), the total loss as a consequence of plant diseases could be as high as 25 per cent of the yield in western countries and almost 50 per cent in developing countries. Of these, one third is due to fungal infection. In addition to causing yield losses, fungal infection reduces the quality of the product due to the presence of toxic metabolites such as aflatoxin and ergotin (Seitz *et al*., 1982; Alderman *et al*., 1996).

Pesticides have been providing effective protection against many diseases but their application results in environmental pollution and emergence of resistant pathogen strains. In addition, chemical control which adds to the cost of production is often beyond the reach of the small farmers in developing nations. Therefore, efforts have been made to breed resistant cultivars and to develop biocontrol agent, for the control of various fungal plant pathogens. *Trichoderma* spp. is a well known biocontrol agent against plant diseases. There are about 41 species in the genus *Trichoderma.* The species and the isolates within the same species differ in their biocontrol potential (Goes *et al*. 2002; Umamaheswari and Sankaralingam, 2005; Upendra, 2006). Therefore, isolation and characterization of *Trichoderma* from different geographical locations is likely to provide an array of diverse isolates within desired biocontrol potential against plant diseases. Though there are several commercial preparations of *Trichoderma* (Trieco, Bio-fungus, Root Pro, and Trichoderma 2000) available for field applications, its performance is limited and highly variable, primarily because of varying environmental conditions. In addition, direct field application of *Trichoderma* is known to inhibit arbuscular mycorrhizal (AM) fungi and vice versa (Wyss *et al.,* 1992; McAllister *et al*., 1994; Siddiqui and Mohamood, 1996).

Advances in molecular biology have laid the foundation for isolation of valuable genes and their transfer to target plants through novel transgenic approach. Chitinases, *ß*-1, 3-glucanases and proteases are the best studied antifungal proteins. Chitinase encoding genes are being used to improve plant defense against fungal pathogens. These enzymes are capable of degrading the linear homopolymer of ß-1, 4-N-acetyl- D-glucosamine, the main cell wall component of most phytopathogenic fungi, showing strong inhibitory activity *in vitro* on germination and hyphal growth (Lorito *et al.,* 1996a). Plants do have chitinases, but are not as effective as microbial chitinases. Therefore, cloning and characterization of genes from biocontrol microbes such as *Trichoderma* is very important. Hence, the present investigation was carried out with the following objectives:

1. Screening of *Trichoderma* isolates for chitinolytic activity
2. Cloning of endochitinase genes from *Trichoderma* spp.
3. Expression of the cloned chitinase gene isolated from *T. virens* in yeast (*Saccharomyces cerevisiae*).

II. REVIEW OF LITERATURE

The ultimate goal of agriculture is to feed the world's growing population. The developing countries like India heavily depend on the agricultural sector, which contributes a quarter of GDP and provide livelihood to two-thirds of the population. But, growth in this sector is influenced by various external factors like monsoon, pest and diseases, economic and social factors.

More than 1, 00,000 kinds of fungi exist, of which about 8,000 fungi can cause diseases in plants, and a relatively small number of them cause disease in humans and livestock (Sweets and Baker, 1994). Almost all the agricultural and horticultural crop species suffer severe yield losses due to fungal diseases. In the Indian context, fungal diseases are rated as the most important factor causing yield losses in major cereal, pulse, fruit and oilseed crops. Various strategies are being employed to control fungal plant pathogens.

2.1 STRATEGIES TO CONTROL PLANT DISEASES

The efforts on effective disease control are being made since the mid 1600, when it was reported that some species and varieties were more resistant to a disease than the others. Knowingly or unknowingly, farmers had been selecting these resistant plants (Fokunang *et al.,* 2004). New technology in all areas has improved agricultural production, but at the cost of environment. So the recent challenge faced by modern agriculture is to achieve satisfactory control of plant diseases in an environment friendly manner (Lorito and Scala**,** 1999). Strategies include biological control, breeding of resistant varieties, improved cultural practices, storage conditions that are less favorable for pathogen attack and survival and integrated pest management (IPM).

Pesticides and organic compounds are widely used to control plant pathogens in many countries. However, the non-degradable components of these compounds have accumulated over the years and entered the food chain, causing toxicity in animals (Cigdem and Merih, 2003; Chet, 1987; Lynch, 1990). Hence, it is compelling to look for alternative disease management practices, which include the use of biocontrol agents, pathogen-resistant crop cultivars and other strategies.

2.2 BIOLOGICAL CONTROL AGENTS (BCAs) FOR PLANT DISEASES

Biological control of plant pathogens is an attractive proposition to decrease heavy dependence of modern agriculture on costly chemical fungicides, which not only cause environmental pollution but also lead to the development of resistant strains (Harjono and Widyastuti, 2001). Biological control of plant disease is defined as the involvement and the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause (Lewis and Papavizas, 1991).

Different biological control agents (BCAs) can be used for the control of diseases. These include bacteria, fungi and actinomycetes. The most important BCAs belong to the genus *Trichoderma*.

2.3 *Trichoderma* AS A BIOLOGICAL CONTROL AGENT

Trichoderma are a class of imperfect fungi, without known sexual stage. They are usually found in uncultivated land sometimes in forest land and to some extent in cultivated land. It is considered as one of the efficient biocontrol agent due to its high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in nutrient utilization, capacity to modify the rhizosphere, strong aggregativeness against the pathogenic fungi and efficiency in promoting plant growth and defense mechanisms (Grondona *et al*., 1997). It is known to control soil borne fungal pathogens belonging to class ascomycete, deutaromycete, bascidiomycete and also certain air borne pathogens (Monte, 2001).

2.4 MECHANISMS OF BIOCONTROL BY BCAs

There are five different mechanisms by which BCAs control other microorganisms; i) direct competition with the target organism ii) antibiosis iii) predation or parasitism of the target organism and iv) induced resistance of the host plant v) inactivation of the enzymes produced by the pathogen. (Wafaa, 2002). Most BCAs apply only one of these four mechanisms; however, some may employ more than one. Some of the mechanisms adopted by *Trichoderma* against plant pathogens are described below.

2.4.1 Competition

Starvation is the most common cause of death for microorganisms occurring due to competition for the limiting nutrients, which results in biological control of fungal phytopathogens (Chet *et al*., 1997). Certain *Trichoderma* produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet and Inbar, 1994). In addition, *Trichoderma harzianum* T35 controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, and become more effective when the nutrient concentration reduces (Tjamos *et al.,* 1992). Additionally, *Trichoderma* has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain energy from the metabolism of different sugars, such as those derived from polymers, wide-spread in fungal environments including cellulose, glucan and chitin among others. *Trichoderma* strongly inhibits *Rhizoctonia solani*, *Pythium ultimum* and *Chalara elegans* when cultured in the same agar medium and the inhibitory action was associated with high rate and extent of CO2 accumulation in comparison with the plant pathogenic fungi (Ozbay and Newman, 2004).

Soil treatment with *T. harzianum* spores suppressed the infection by *Fusarium oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis*. (Sivan and Chet, 1989). In case of black pepper, *T. harzianum* and *T. virens* were found to be efficient in controlling *Phytophthora capsici* which causes foot rot (Rajan *et al*., 2002). Direct application of *T. virens* into the soil infested with *F. oxysporum f. sp. gladioli* reduced the pathogen colonies significantly (5.2 log CFU/gram of soil) compared to control (6.1 log CFU/g of soil) (Mishra *et al*., 2004).

2.4.2 Biofertilization and Stimulation of Plant Defense Mechanisms

Trichoderma strains are known to associate with plant roots and root ecosystems. They are also plant symbiont and opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi producing compounds that stimulate growth and plant defense mechanisms (Harman *et al.,* 2004). This mechanism includes plant root colonization and rhizosphere modification.

2.4.2.1 Plant Root Colonization

Trichoderma strains must colonize plant roots prior to stimulation of plant growth and protection against infections. Colonization implies the ability to adhere and recognize plant roots, penetrating and withstanding toxic metabolites produced by the plants in response to invasion by a foreign organism, whether pathogen or not. Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Arora *et al*., 1992). In cucumber, inoculation of *T. harzianum* spores increased the production of peroxidase activity and chitinase activity (Yedidia *et al,* 2001). In cotton, *T. virens* treatment increased the terpenoids desoxyhemigossipol (dHG), hemigossypol (HG) and gossypol (G), which have strong inhibitory action against cotton pathogens such as *R. solani* (Howell *et al.,* 2000). The experiments carried out in greenhouses confirmed considerable yield increase when plant seeds were previously treated with spores from *Trichoderma* (Arora *et al*., 1992). Increase in the yield was due to production of phytohormones such as indole acetic acid (IAA), ethylene, zeatin and gibberellin (GA3) (Arora *et al.,* 1992; Osiewacz, 2002)

2.4.2.2 Rhizosphere Modification

The soil environment influences spore germination, chlamydospore formation and the production of secondary metabolites, such as siderophores (Eisendle *et al.,* 2004) antibiotics (Chet and Innbar, 1994) and enzymes (Arst and Penalva*,* 2003). The pH of the microbial environment is one of the major factors affecting the activity of both *Trichoderma* and pathogenicity factors secreted by different microorganisms. Some antibiotics are degraded at high pH; air drying and low pH may induce enzyme degradation by acidic proteases (Delgado *et al*., 2000, 2002); and the growth of many fungi is inhibited by weak acids, such as sorbic acid, due to rapid decline in cytosolic and vacuolar pH (Arst and Penalva*,* 2003). Since *Trichoderma* is adapted to acidic soil, it has a capacity to inhibit the pathogen by controlling the external pH; in such a way that the altered pH does not affects its own secreted enzyme (McIntyre *et al*., 2004).

2.4.3 Antibiosis

Antibiosis occurs during interactions involving low molecular weight diffusible compounds called antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. For the first time, Weindling (1934) showed the lethal principle produced by *T. lignorum* and secreted to the medium. It was toxic to *R. solani,* and *Sclerotinia americana,* which was named as gliotoxin (Weindling, 1941). Gliviridin is another antibiotic produced by *Gliocladium virens,* which has inhibitory effect on *Pythium ultimum* and *Phytophthora* spp. (Howell and Stipanovic, 1983). Apart from these two antibiotics, it produces volatile and nonvolatile toxic metabolites that impede colonization by antagonized microorganisms. Among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described (Vey *et al.,* 2001). Apart from the gliotoxin and gliviridin, the peptaibols are another class of antibiotics secreted by *Trichoderma*. These are linear peptides that have strong antimicrobial activity against gram-positive bacteria and fungi, act synergistically with cell wall degrading enzymes (CWDEs) to inhibit the growth of fungal pathogens and elicit plant resistance response to pathogens. Wiest *et al.* (2002) showed that the exogenous application of the peptaibols triggered the defense response to tobacco mosaic virus (TMV) in tobacco.

2.4.4 Mycoparasitism

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack, subsequent penetration and killing of the host. *Trichoderma* spp. may exert direct biocontrol by parasitizing a range of fungi; detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of CWDEs, mostly chitinases, glucanases and proteases (Harman *et al*., 2004). Mycoparasitism involves morphological changes, such as coiling and formation of appressorium- like structures, which serve to penetrate the host (McIntyre *et al.,* 2004; Dennis and Webster, 1971; Sivan and Chet, 1989). *Trichoderma* attaches to the pathogen via its cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria and subsequent secretion of CWDEs and peptaibols (Howell, 2003). The CWDEs of *Trichoderma* such as different chitinolytic enzymes and glucanases have been suggested as the key enzyme in mycoparasitism (Elad *et al*., 1982; Cherif and Benhamou, 1990). Endochitinase (42-kDa), chitobiosidase (40-kDa) and N-acetyl- b-D-glucosaminidase (73-kDa) from *T. atroviride* strain P1 and *T. virens* strain 41 were reported to have a substantial inhibitory effect on the germination of spores and hyphal elongation of several fungal pathogens, viz., *Botrytis cinerea, Fusarium* spp., *Alternaria* spp., *Ustilago avenae*, *Uncinula necator* and virtually on all fungi containing chitin in their cell-wall (Di Pietro *et al.,* 1993; Lorito *et al.,* 1993, 1994, 1996a; Schirmbock *et al.,* 1994). However, some experiments showed that mutant strain of *T .virens* that are unable to produce antibiotic, gliotoxin and mycoparasitism, had the same effect as that of wild type against *R. solani* and *P. ultimum*, when cotton seedling were treated (Howell, 2002; 2000; 1995). This clearly showed that neither mycoparasitism nor antibiosis was the sole biocontrol mechanism.

2.4.5 Inactivation of the Pathogen's Enzymes

Inactivation of the pathogen's enzymes is another biocontrol mechanism by *Trichoderma* spp. Enzymes of *B. cinerea*, *viz*., pectinases, cutinase, glucanase and chitinase were suppressed through the action of secreted protease (isolate T39) on plant surfaces (Elad *et al*., 1999). The *in vitro* inhibitory ability of *T. harzianum* on the phytopathogen *Alternaria alternata* (*Alternaria alternata* (Fr.) Keissl.) was investigated in the presence of growth regulators. *A. alternata,* a pathogenic fungus secretes endo-polygalacturonase (*endo-PG*) and pectate lyase (PL), which are responsible for the hydrolysis of pectic components of the plant cell wall. The presence of *T. harzianum* decreased *endo-PG* secretion by *A. alternata* to 50 per cent, and this inhibitory effect was independent of the presence of growth regulators (Roco and Perez, 2001).

2.5 *Trichoderma* AND CHITINASE

Trichoderma, which are efficient producers of chitinase, can be exploited to get plant resistance to various pathogens. *Trichoderma* chitinase (EC 3.2.1.1.14) belongs to hydrolase group can hydrolyze chitin, a polymer of β-1, 4 linked N-acetyl glucoseamine (Kas, 1997; Kitamura and Kamei, 2003). The Chitinases are classified into two families *viz.,* family 18 and 19, based on amino acid sequence similarities (Henrissat and Bairoch, 1993). Family 18 includes chitinases found in bacteria, fungi, viruses, and animals, and class III and V of plant chitinases. Family 19 includes class I, II and IV chitinases of plant origin only, with the exception of chitinase C from *Streptomyces griseus* HUT 6037 (Ohno *et al*., 1996) and chitinases F and G from *Streptomyces coelicolor* (Saito *et al*., 1999). The *Trichoderma* chitinase belongs to family 18 and are mainly classified into three categories (Harman *et al*., 1993; Sahai and Manocha, 1993). Endochitinases (EC 3.2.1.14) cleave internal bonds within chitin, releasing chitotetraose, chitotriose, and chitobiose. Exochitinase (chitobiosidase) catalyzes the release of chitobiose without the formation of oligo- or monosaccharides. β-N-

Acetylhexosaminidases (N-acetyl-β–D-glucosaminidases, EC 3.2.1.52) cleave chitobiose, chitotriose, and chitotetraose to N -acetylglucosamine monomers in a manner similar to exochitinase (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996).

2.6 REGULATION OF CHITINASE GENE IN *Trichoderma*

Depending on the strain, the chitinolytic system of *T. harzianum* may contain five to seven individual enzymes (Haran *et al*., 1995). In the well-characterized strain *T. harzianum* TM, this system comprises two β- (1, 4) -N - acetylglucosaminidases (102 and 73 kDa), four endochitinases (52, 42, 33, and 31 kDa), and one exochitinase (40 kDa) (Haran *et al*., 1996; Lorito *et al*., 1993). Various components of the chitinolytic system of *T. harzianum,* likely include enzymes that are mutually complementary in terms of their action (Lorito *et al*., 1993).

Most studies on the regulation of chitinase formation in *Trichoderma* spp. have identified chitinases only by enzyme assays and have not addressed the possibility of differential regulation for the various isozymes. According to early reports, genes encoding endochitinase 42 (*ech42*), endochitinase 33 (*chit33*), and N-acetyl-β-D-glucosaminidase (*nag1*) are induced by fungal cell walls or colloidal chitin (Carsolio *et al*., 1994; Limon *et al*., 1995; Garcia *et al*., 1994; Peterbauer *et al*., 1996), as well as carbon starvation (Limon *et al*., 1995; Margolles *et al*., 1996). The expression of *ech42*, associated with light-induced spore germination was inhibited by carbon catabolites (Lorito *et al*., 1996b; Carsolio *et al*., 1994); where as the transcription of *nag1* (exochitinase) was induced by of N-acetyl-β-D-glucosamine (GlcNAc) (Carsolio *et al*., 1994; Limon *et al*., 1995; Garcia 1994; Peterbauer *et al*., 1996).

2.7 ISOLATION OF *Trichoderma* spp. FROM SOIL

Trichoderma have different mechanisms to control the phytopathogenic fungus. The different species and isolate belonging to same species differ in their ability to control plant diseases. Therefore, isolation and screening of *Trichoderma* spp. from different soil environment is important.

Trichoderma are the filamentous fungi, usually present in uncultivated land or waste lands. The most efficient method for the isolation of *Trichoderma* was described by the Kader *et al.* (1999) in which soil sample was mixed with sterile distilled water and serial dilutions were prepared. From the dilution, 0.5 ml was plated on potato dextrose agar (PDA) and incubated at 30^{O}C for three days. The fungi isolated were subcultured until a pure isolate was obtained.

Cigdem and Merih (2003) used thirty one soil samples from different agricultural land and forest soil, from Esksehir inoculated on PDA, malt extract agar, rose bangal agar and oat flour agar and incubated at 28°C for 5 days. Purified colonies of *Trichoderma* after subculture were identified according to Watts *et al.* (1988) and Rifai (1969). These soil samples contained *T. harzianum* and other strains of the *Trichoderma.* Shalini *et al.* (2006) isolated seventeen strains of *Trichoderma* from various parts of India. For isolation, serial dilution was used and one ml of an aliquot was plated on the PDA. The plates were incubated at 28^{O}C and pure cultures were subcultured. These cultures included *T. viride, T. harzianum* and *T. aureoviride.* In our laboratory, Upendra (2006) isolated 23 *Trichoderma* isolates from 51 soil samples collected from the different parts of India, using serial dilution method followed by plating on PDA. The isolate included; *Trichoderma* spp. *viz*., *T. viride, T. virens, T. polysporum, T. harzianum, T. pseudokoningii* and *T. koningii*

2.8 SCREENING OF *Trichoderma* spp.

2.8.1 Inhibition of Pathogens

Goes *et al*. (2002) tested fourteen isolates of the *Trichoderma* (six of *T. viride*, six of *T. harzianum*, one of *T. polysporum*, one of *T. pseudokoningii*) against the pathogenic fungus *Rhizoctonia solani.* They used 5 mm discs, placed on PDA plate in opposite direction at 7 cm from each other, and incubated at 26°C. *Trichoderma* isolates demonstrated high antagonistic action over *R. solani*; the isolates of *T. harzianum* were the most aggressive. The isolates of *Trichoderma* overlapped the colony of *R. solani* within 120 hours, except for the three isolates (2596, 3086 and 2745). The three isolates (3302, 3601 and Tm,) start overlapped the colony of *R. solani* earlier than the other isolates. The isolate *T. pseudokoningii* T14, was not able to overlap the colony of *R. solani*, but inhibited its growth.

Umamaheswari and Sankaralingam (2005) isolated six *Trichoderma* spp. from rhizosphere and phyllosphere of vegetable crops, and did *in vitro* screening for their antagonistic potential against *A. alternata*, the causal agent of leaf blight of watermelon. Though all the *Trichoderma* spp. were antagonistic to *A. alternata, Trichoderma* spp. CIAH-175 recorded maximum reduction in mycelial growth of pathogen upto 86.3 per cent.

Kucuk and Kivanc (2004) identified efficient *Trichoderma* isolates by using dual plate assay against *Gaeumannomyces graminis* var. tritici, *Fusarium culmorum* and *F. moniliforme*. About 7 mm diameter of mycelial disk of *T. harzianum* and pathogen were placed on opposite side of the PDA plate. After incubation at 28^{O}C for five days, growth of cultures was determined. Among the isolates, *T. harzianum* T15 were more efficient than T8 and T11 in retarding growth and sporulation of the *G. graminis*. Inhibition of these pathogens was mainly due to production antibiotics *viz*., trichodermin, trichodermol, harzianum A and harzianolide, and also due to chitinase and glucanase which are thought to be closely related to mycoparasitism. Recently, sixteen isolates were tested for the antagonistic activity by Upendra (2006). Among these isolates, four *T. virens* isolates were highly effective against *S. rolfsii* and none of the isolates were able to inhibit the growth of *F. solani* completely. Walter *et al.* (2006), screened nine genera of the fungi which included *Alternaria, Cladosporium, Epicoccum, Gliocladium, Trichoderma, Ulocladium, Aureobasidium, Cryptococcus* and *Saccharomyces*, and six unidentified microorganisms from Switzerland, against pathogenic *B. cinerea* isolate B1, B2, B4 and B7, using the method described by the Köhl *et al.* (1997). Mycelial inoculum (10 mm plugs) was cut using a cork borer from the leading edge of a colony. One plug each of *B. cinerea* B1 and a test isolate were placed 50 mm apart in an 85 mm diameter petri dish containing PDA, and the plates were incubated at 20°C with a 12 hours photoperiod and assessed visually after 15 and 30 days. Among them, only four test isolates, two *Epicoccum* spp., one *Trichoderma* spp. and one unidentified micro-organism completely overgrew the pathogen. One isolate of *Trichoderma* stopped the growth of the pathogen prior to contact, this isolate was considered as highly antagonistic.

Trichoderma spp. are very efficient in controlling *S. rolfsii* when the mycelial disc of phytopathogen and *Trichoderma* were placed on the PDA plate. Mean growth of *Trichoderma* was 3.25 cm for the isolate J10 and 0.35 cm for isolate Jn22. The most effective isolates J10, T36, and T33. N38 were formed the clear zone around the *S. rolfsii* after 48 hours indicating potential mycoparasitism ability (Radwan *et al*., 2006).

2.8.2 Substrate Hydrolysis in Media

Most fungi and bacteria produce chitinase when grown on a medium containing chitin such as colloidal chitin, dried fungus mycelium and glycol chitin. The 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 or colloidal chitin.

Kovacs *et al*. (2004) screened thirty *Trichoderma* strains representing 15 species within the genus for extracellular production of chitinolytic enzymes through solid substrate fermentation. Among all the isolates *T. longibrachiatum* IMI 92027 (ATCC 36838) gave the highest yield (5.0 IU/g of dry matter of substrate) after three days of fermentation on wheat bran-crude chitin (9:1 mixture) medium. The optimal moisture content (66.7per cent), chitin content (20per cent), initial pH of the medium (2.0-5.0) and time course (5 days) of solid substrate fermentation were determined for strain IMI 92027. Cellulase, xylanase, alpha-amylase, and beta-xylosidase activities were also detected. Aishwarya (2004) screened six *Trichoderma* spp, *Metarrhizium verrucasia, Bauveria bassiana, Aspergillus oryzae, and Nomurea releyi* using Mendals and Reese (1965) medium supplemented with colloidal chitin as a sole carbon source. *T. koningii* showed the highest hydrolytic activity and Nomurea *releyi* showed the least activity.

2.8.3 Biochemical Estimation

Biochemical estimation of chitinase activity includes substrate degradation and estimation of reducing sugar. Endochitinase activity can 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), and also in some cases p-nitriphenyl -β- N-acetyl - β -D- glucosamine (pNPGlcNAC), p-nitrophenyl-β-D-N-N'-diacetylchitobiose (pNPGlcNAc)2 (Inbar and Chet, 1991 and Viterbo *et al*., 2002) and 4-methylumbelliferyl β-D-N', N"-diacetylchitotrioside, 4-methylumbelliferyl-N-acetyl-β-D-glucosamide (4-MU-GlcNAc), a fluorogenic analogue of chitin was used as substrate for hydrolysis (Cottrell *et al*., 2002; Haran *et al*., 1995). Some researchers also used 4 -Methyl umbelliferone – D-N-N'-N"- try acetyl chitotriose, N-acetyl glucose amine (Kim *et al*., 2002, Lorito *et al*., 1998) as a substrate.

Sugar conversion assay or colorimetric assay is a simple and sensitive assay for the estimation of the chitinase. Katatny *et al*. (2001) assayed the chitinase activity of the *T. harzianum* as described by the Molano *et al*. (1977) with minor modification. The assay mixture contained 1 ml of 0.5 per cent chitin in 50mM acetate buffer pH 5.2 and 1 ml of enzyme solution. The mixture was incubated for 7 hours at 37°C with shaking and reaction was stopped by addition of 1 ml of the dinitrosalicylate (DNS) reagent. The amount of reducing sugars released was calculated from standard curve recorded for N-acetylglucosamine, and chitinase activity was expressed in pkat (pmol s^{-1}).

Omumasaba *et al.* (2001) assayed the chitinase activity as described by Imoto and Yagishita (1971), where the reaction mixture contained 1.4 ml of 0.2 per cent glycol chitin in 0.1 M acetate buffer, pH 5.0 and 0.1 ml of diluted enzyme. This mixture was incubated at 45°C for 20 minutes. The reaction was stopped by the addition of 2 ml of 0.05 per cent potassium ferricyanide. The amount of reducing sugar liberated was determined. One unit of chitinase activity was defined as the amount of enzyme that produced 1 mmol of reducing sugar per minute under standard assay conditions.

Kapat *et al.* (1996) used the colorimetric assay to determine the chitinase activity of the *T. harzianum.* The reaction mixture contained 0.5 cm^3 of 5.0 kg/m^3 swollen chitin and 0.5 cm^3 of enzyme solution. This mixture was incubated for 1 hour at 40^OC without agitation. To obtain a product profile, the reaction mixture was incubated at 40^OC for different periods of time, ranging from 0 to 120 minutes. After the incubation, products released from the chitin were estimated as N-acetyl-D-glucosamine equivalent. The reducing sugar was measured by the dinitrosalisylic method as described by the Miller (1959). One unit of enzyme activity was defined as the amount of enzyme that releases 1 gmol of N-acetyl-D-glucosamine (NAcGlc) from the substrate in 1 minute per ml of culture filtrate at a defined temperature.

Ike *et al.* (2006) used the substrate chitisan 7B and chitosan 10B for the determination of chitinase activity. The reaction mixture contained 1 ml of enzyme in 50mM sodium acetate buffer (pH 5.5) and the substrate, incubated at 30^OC for appropriate time. This reaction was terminated by immersing the reaction mixture in boiling water for 10 minutes and reducing sugar released was estimated.

Harjono and Widyastuti (2001) assayed the *T. reesei* endochitinase activity on the basis of reduction of turbidity of suspension of colloidal chitin as described by the Tronsomo and Harman (1993); where as colloidal chitin was prepared as described by the Vessey and Pegg (1973) from crab shell chitin. One enzyme unit was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5 per cent at 510 nm (Harman *et al*., 1993; Tronsmo and Harman, 1993).

2.8.4 Glycol Chitin Plate Assay for Screening Chitinolytic Activity

The potential use of chitinase as a biochemical marker to find out efficient biocontrol agent requires screening of large number of native isolates. The current detection methods include colorimetric assay (Molano *et al*., 1977), direct detection of activity after polyacrylamide gel electrophoresis (Pan *et al*., 1991; Trudel and Asselin, 1989) HPLC (Koga *et al.,* 1998), reflectant spectroscopy (Roberts *et al*., 1994). Recently, Luis and Ray (2004) reported that glycol chitin plate assay for chitinase activity in plants is very efficient and less time consuming. This method is based on the affinity of calcofluor white M2R for chitin (Maeda and Ishida, 1967) using glycol chitin as a substrate for endochitinase (Pan *et al*., 1991). Glycol chitin embedded in an agarose gel provides a homogeneous substrate for the reaction to take place (Zou *et al.,* 2002). Glycol chitin serves as substrate for chitinase (Koga and J., 1983) binds to fluorescent brightener 28 by affinity (Maeda and Ishida, 1967). After proper incubation and enzymatic activity, the brightener is bound only to undigested glycol chitin (Trudel and Asselin, 1989). The result is easily visualized as well defined dark area on a fluorescent background when viewed under UV trans illuminator.

The glycol chitin plate assay was also used to analyze the chitinase activity from *Pantoea dispera* (Goel *et al*., 2004). The polyacrylamide gel electrophoresis was carried out using 15 per cent gel as described by Sambrook *et al.* (1989) and gel was run at 4^O C . After SDS electrophoresis, the gel was incubated at 37 ^OC for four hours in sodium acetate buffer (0.2 M, pH 5.0) containing 1 per cent (V/V) TritonX-100 to remove SDS and the gel was washed with distilled water. This gel was then transferred to each chitin plate containing a different dye. Thin layer of acetate buffer (0.2 M, pH 5.0) was added to hasten the diffusion of chitinase from polyacrylamide gel to agar plate. These plates were incubated at 37^O C under dark condition. The dark bands appear against the fluorescein background on chitin agar plate with calcofluor white M2R and fluorescein isothiocynate after 7 hours. These bands were observed under UV- transilluminator (Goel *et al*., 2005).

2.9 CLONING OF THE CHITINASE GENES FROM *Trichoderma* spp.

Chitinases mainly belongs to two Families *viz.,* Family 18 and Family 19, and *Trichoderma* chitinase belongs to family 18. Family 19 contains mainly plant chitinase and Streptomyces chitinase. In *Trichoderma* also there are several types of chitinases such as *ech42, ech46, chit36, chit37, ech30* etc. (Verena *et al*., 2005; Klemsdal *et al.,* 2006).

For cloning chitinase genes from *Trichoderma* mainly two approaches viz., genomic DNA approach and cDNA approach were used. In genomic DNA approach, mostly PCR amplification with specific primers was followed. Genes encoding endochitinase were cloned from different *Trichoderma* spp such as *T. harzianum ech 42* (Baek *et al*.,1999, Woo *et al*., 1999, , Carsolio *et al*., 1999, Lorito *et al*.,1998 ; Viterbo *et al*., 2002; Garcia *et al*., 1994; Limon *et al*., 1995) *T. asperellum* (*chit36*) (Viterbo *et al*., 2002) and *T. atroviride* (*chit36*) (Viterbo *et al*., 2002; Verena *et al*., 2005 Klemsdal *et al*., , 2006; Reithner *et al*., 2005), *T. hamatum* (Steyaert *et al*., 2004; Giczey *et al.,* 1998; Fekete *et al*., 1996), *T. reesei* (Ike *et al*., 2006) and *T. virens* (Kim *et al*., 2002; Baek *et al*., 1999). In other cases cDNA approach was used for cloning the gene coding for endochitinase from *T. harzianum* (Carsolio *et al,* 1994, Garcia *et al*., 1994), *T. reesei* (Ike *et al.,* 2006), *T. virens* (Kim *et al*., 2002).

The amino acid analysis of endochitinase shows that it belongs to glycosyl hydrolases family 18 which contains two conserved motifs, chitinase family active site ([LIVMFY] - [DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-X-E) and chitin binding domain (XXXSXGG) (Terwisscha *et al*., 1996; Boot *et al*., 1995; Renkema *et al*., 1998). This gene also contains signal peptide which is not highly conserved and varies in length, and the possible site for the cleavage is Ala-Ser site. The presence of N-terminal signal peptide indicated that they were targeted for the secondary pathway (Emanuelsson *et al*., 2000; Nielsen *et al*., 1997).

2.10 EXPRESSION OF CHITINASE GENE IN YEAST

It is essential to confirm the expression of cloned gene, before going for plant transformation. Yeast, *Saccaromyces cervisiae* is a model organism for the expression of eukaryotic genes. The yeast expression vectors pYES2/CT (Invitrogen) is the most commonly used vector for the expression of genes cloned into yeast. Draborg *et al.* (1995) constructed the *T. harzianum* cDNA library in pYES2. Further in 1996 they transferred plasmid DNA from a cDNA library pool of 15,000 colonies into *S. cerevisiae* W3124 (Hazel *et al*., 1992) by electroporation (Becker and Guarente, 1991), and the transformants were plated on SC agar plates (Sherman, 1991) containing 2 per cent glucose. After incubation at 30 ^{O} C for 3 to 4 days, the colonies were replicated onto SC plates containing 2 per cent galactose and 0.2 per cent colloidal hydrated chitin. The yeast strains containing the endochitinase were selected based on the hydrolysis zone on colloidal chitin plate. The endochitinase enzyme was purified using the culture supernatant. The purification was done by anion exchange chromatography and reverse phase high performance liquid chromatography (RP-HPLC). NH_2 terminal amino acid sequencing revealed that the endochitinase was homologous to sequence obtained from the native endochitinase (Hermam *et al.,* 1993) from *T. harzianum*. It clearly indicated that the endochitinase is secreted into growth medium by yeast and protein processing is identical to the fungal processing.

Song *et al*. (2005) cloned *ech42* from the *T. aureoviride.* The chitinase gene was cut from pMD18-T vector with *Eco*R I and *Bam*H I and then ligated into *Eco*R I and *Bam*H I sites downstream of the *GAL*1 promoter of pYES2. The resulting plasmid (pYES2/ech42) was characterized by restriction analysis. The pYES2/ech42 plasmid was transformed into *S. cerevisiae* H158 by the lithium acetate method, as described by Krautwurst *et al.* (1998). These yeast transformants were grown on the minimal medium containing raffinose as the sole carbon source for growth of yeast and induced by supplying the galactose as carbon source. Further, chitinase activity of the recombinant yeast was assayed as described by Miller (1959) with some modification. Chitinase activity was estimated colorimetrically using colloidal chitin as substrate. The reaction mixture, consisting of 0.5 ml colloidal chitin (0.5 per cent, w/v) and 0.5 ml enzyme solution, was incubated at 50°C in a water bath for 30 minutes. The reaction was stopped by adding 0.5 ml dinitrosalicylic acid and the reaction mixture was immediately boiled for 5 minutes. One unit of chitinase activity was defined as the amount of enzyme which produced 1 µmol min^{-1} reducing N-acetyl D-glucosamine. After cooling, the reducing sugars released due to chitinase activity were measured at 540 nm. The maximum activity of the chitinase in yeast was found at 48 hours (0.5 U/ml).

2.11 EXPRESSION OF ENDOCHITINASE GENE IN PLANT

Several efforts have been made to test the efficiency of *Trichoderma* endochitinase in plant. Lorito *et al.* (1998) expressed an endochitinase-encoding gene from *T. harzianum* in tobacco and potato. High expression levels of the fungal gene were obtained in different plant tissues, which had no visible effect on plant growth and development. Substantial differences in endochitinase activity were detected among transformants. Selected transgenic lines were highly tolerant or completely resistant to the foliar pathogens; *A. alternata*, *A. solani*, *B. cinerea*, and the soilborne pathogen *R. solani*. The high level and the broad spectrum of resistance obtained with a single chitinase gene from *Trichoderma* overcame the limited efficacy of transgenic expression of chitinase genes isolated from plants and bacteria in plants. Liu *et al*. (2004) transformed rice plant with a gene encoding endochitinase (*ech42*) from the biocontrol fungus *T. atroviride.* The transformed plants showed increased resistance to sheath blight caused by *R. solani* and rice blast caused by *Magnaporthe grisea*. In case of apple, endochitinase gene from *T. harzianum* was transferred through *Agrobacterium* mediated transformation. The presence of the gene in apple was confirmed by southern analysis. Eight plants were used for the bioassay with *Venturia inaequalis* which causes the apple scab. The disease severity was compared with the control and there was a reduction in the number of lesions (0-99.7 per cent) and per cent leaf area infected (0-90 per cent). However, the endochitinase had negative effect on the growth of the plant (Jyoti *et al.,* 2000). Chandrakanth *et al*. (2003) transferred the 42 kDa endochitinase from *T. virens* to cotton. Transgenic plants showed resistance to the pathogens i.e., *A. alternata* and *R. solani*. Recently, Shah *et al*. (2005) transformed tobacco plants with *ech42* gene cloned from an Indian isolate of *T. virens* and confirmed the integration of endochitinase into tobacco genome by PCR amplification using specific primers.

III. MATERIALS AND METHODS

The present study was conducted to isolate and screen *Trichoderma* isolates, clone full length endochitinase gene and to study the expression of a cloned gene in *Saccharomyces cerevisiae*

3.1. ISOLATION OF *Trichoderma* spp. FROM THE SOIL AND THEIR MAINTENANCE

Trichoderma species were isolated from soil samples collected from different parts of Western Ghat which are available in the department. Soil suspensions were prepared by adding 1 g homogeneously mixed soil to 10 ml sterile distilled water and mixed for 15 minutes. Immediately, each suspension was serially diluted to 10^{-5}. From the 10^{-5} dilution, 0.1 ml was spread on potato dextrose agar (PDA) (Appendix-IA) plate supplemented with propionic acid, which was then incubated at 28°C for 7- 10 days. Based on the appearance, *Trichoderma* colonies were transferred to PDA plates till pure cultures were obtained. The isolates were sent to the Department of Mycology and Plant Pathology, Agharkar Research Institute, Pune, for final identification at species level. All the confirmed *Trichoderma* cultures were further maintained on PDA at 4^0C and subcultured once in three months.

3.2 SCREENING OF *TRICHODERMA* FOR CHITIONOLYTIC ACTIVITY

The chitinase specific plate assay was done as described by Luis and Ray (2004) with some modification. Screening was done for the isolates available in the Institute (Upendra, 2006), which included isolates representing the seven different *Trichoderma* spp.

3.2.1 Preparation of Colloidal Chitin

Colloidal chitin was prepared by the method of Roberts and Selintrenikoff (1988) with certain modifications. 5 g of chitin powder (HiMedia Laboratories Pvt. Ltd., Mumbai) was added slowly into 60 ml of concentrated HCl (Sd. Fine Chemicals Ltd., Mumbai) and left for vigorous shaking overnight at 4°C. The mixture was added to two liters of ice-cold 95 per cent ethanol with rapid stirring and kept overnight at room temperature (25°C). The precipitate was collected by centrifugation at 5,000g for 20 minutes at 4°C and then washed with sterile distilled water until the pH of the colloidal chitin turned neutral (pH 7.0). Later, colloidal chitin solution (5 per cent) was prepared and stored at 4°C for further use.

3.2.2 Preparation of Culture Filtrate for Plate Assay

Trichoderma spores were inoculated on 25 ml of 0.5 X PDA (Appendix-IA) and incubated at 28°C for 3-4 days or until the full growth of mycelium. After incubation, mycelium was washed several times with distilled water. Clean mycelium was then transferred to Mandels and Reese (1965) (Appendix-IC) broth containing 0.3 per cent colloidal chitin as sole carbon source and incubated at 28°C under shaking condition at 100 rpm. One ml of culture supernatant was taken at the intervals of 24, 48, 72 and 96 hours, in clean micro centrifuge tube. This was centrifuged at 13,000 rpm for one minute at 4°C and supernatant was transferred to micro centrifuge tube and stored at -20°C for further use

3.2.3 Preparation of the Substrate and Assay for Chitinase Activity

One per cent (w/v) agarose solution was prepared in sodium phosphate (0.01 M pH 5.5) and heated to boiling point. One ml of 1 per cent glycol chitin solution (Appendix-IX) was added to 100 ml of agarose solution. The resulting suspension was stirred to ensure homogeneous distribution of the substrate, and 30 ml aliquot was poured into polypropylene petri dishes (90 cm diameter). The agarose was allowed to cool and solidify for 20-25 minutes. Small wells (3 mm diameter) were created using cork borer in agarose gels at 1.5 cm from each other to form a grid. About 30 µl of crude enzyme solution (culture filtrate prepared in the previous section) was loaded into each well. The plate was incubated at 7 hours at 37^{o} C. After incubation 50ml of 0.5M solution of Tris-Cl (pH 8.9) with 0.01 per cent calcofluor white M2R (Sigma)(Appendix IX) was added to the petri dish to stop the reaction and stain the plate. The gel was rinsed twice with water followed by overnight color development in the dark.

3.3 ISOLATION OF GENOMIC DNA FROM FUNGUS

The *T. viride* (IABT1012, IABT1013,) and *T. harzianum* IABT1015 were inoculated in potato dextrose broth for 2-5 days at 30^{o}C. About 100 mg of fungus mycelium was taken in 2.0 ml micro centrifuge tube and 500µl of lysis buffer (Appendix-X) was added. Mycelium was finely macerated using micro-pestle and vortexed for 5 minutes. The suspension was extracted with equal volume of phenol: chloroform: IAA (25:24:1) and centrifuged at 10,000 rpm for 10 minutes. The supernatant was taken into a fresh tube and equal volume of isopropanol was added at room temperature, mixed by gentle inversion and kept for 10 minutes at room temperature. The DNA was recovered by centrifugation at 10,000 rpm for 10 minutes at 4°C. The DNA pellet was washed with 70 per cent ethanol, air dried and resuspended in 50µl of $T_{10}E_1$ (10 mM Tris-Cl and 1 mM EDTA, pH 8.00). Concentration of DNA was estimated using ethidium bromide spotting method as described by Sambrook and Russel (2001).

3.4 AMPLIFICATION OF FULL LENGTH ENDOCHITINASE GENE

3.4.1 Specific Primers for the Full Length Endochitinase Gene

Specific primers for full length endochitinase genes were designed based on the reported full length endochitinase gene from *T. viride* (Acc. No. BAB40593) and *T. harzianum* (Acc. No. S72483.1) from NCBI database using the FastPCR software (Kalendar 2006).

TVR-1 primer was synthesized for the amplification of the endochitinase gene from pSUM1C clone (which was already cloned in our laboratory, Upendra, 2006) to remove the extra length upstream to the coding region. The pSUM1C contains endochitinase which has 173 bp upstream to the coding region. The start codon is underlined in the primer

Trichoderma harzianum

THR-3

FORWARD-5'-CACCATGTTGAGCTTCCTCG-3'

REVERSE-5'-ACTTCATGACCCTGCTCCTCCA-3'

Trichoderma viride

TVD-I

FORWARD-5'-CGTTGGTTCCCATTCAGCGCTTC-3'

REVERSE-5'- TCCAAGAGCATTTCCCCGCAACA- 3'

Trichoderma virens **(For pSUM1C)**

TVR-1

FORWARD-5'-ACC**ATG**TTGAGCTTCCTCGGCA -3'

REVERSE-5'-TCCCCTGAAAAGAAGCCACCT-3'

The primer sequences designed are listed below.

The primers used for amplification of template DNA (20ng) were custom synthesized at Integrated DNA technologies, Inc. USA and supplied as lyophilized product of desalted oligos. **4.2 PCR Amplification Condition for Endochitinase (*Chit 46*) Gene from *T. viride, T. harzianum* and *T. virens* (pSUM1C)**

Separation of the amplified product by agarose gel electrophoresis is described below.

Stage	Step	Temperature(°C)	Duration (minutes)	No. of cycles
I	Initial denaturation	94	5	1
II	Denaturation	94	1	
	Annealing			
	TVD	55	1	40
	THR and TVR	59		
	Extension	72	2	
III	Final Extension	72	20	1
IV	Incubate	4		Hold

3.5 AGAROSE GEL ELECTROPHORESIS

About 20 µl of the amplified product from each tube along with 3 µl of loading dye were loaded onto 1.0 per cent agarose gel along with one kb ladder (New England Biolabs Inc.) as DNA molecular weight marker. Electrophoresis was done at 50V for initial 30 minutes and then 70V for 1 hour. The buffer used was 1x TAE at pH 8.0. The DNA bands in the gel were visualized on a UV-transilluminator and documented using a gel documentation system (Uvitec Cambridge, England).

3.6 CLONING OF ENDOCHITINASE GENE

3.6.1 Elution and Purification of PCR Fragment

The intense band of 1.6 kb PCR fragment obtained from two strains of *T. viride* IABT1012 and IABT1013 and 1.5 kb fragment from *T. harzianum* IABT1015 using gene specific primers were excised from the low melting agarose gel (0.7 per cent) (Appendix-II) with a sharp sterile scalpel blade by keeping the gel on low intensity (70 per cent) UV-transilluminator. The agarose gel piece containing the fragment was collected in a sterile pre-weighed micro centrifuge tube. The 1.5 kb amplification obtained from the pSUM1C (extra length removed) was also eluted as described above.

The excised PCR fragments were eluted out using Eppendorf gel extraction kit according to manufactures instruction. The purified PCR product was quantified by ethidium bromide spotting method as described by Sambrook and Russel (2001).

3.6.2 Cloning Of PCR Product

The purified PCR product of 1.6 kb (50ng/µl) from three strains of *T. viride* IABT-1012 and 1013 , 1.5 kb of *T. harzianum* IABT1015 and 1.5kb of pSUM1C were ligated to pTZ57R/T vector (2886 bp) as described in InsT/A clone™ PCR product cloning kit (#k1214) from MBI, Fermentas USA. 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 into a 0.5 ml micro centrifuge tube and incubated overnight at 16°C in thermal cycler (Thermo EC, USA). A control ligation reaction was performed using control PCR fragment provided in the kit by adding components described in Appendix-IIIC. The ligation products were used to transform *E. coli* DH5α.

3.7 TRANSFORMATION OF *E. coli* DH5α WITH RECOMBINANT CLONE

3.7.1 Preparation of Competent Cells

The competent cells of *E. coli* DH5α were prepared following the protocol mentioned by Sambrook and Russel (2001) with minor modification as described below.

An isolated colony from *E. coli* DH5α plate was inoculated into 5 ml Luria broth and incubated at 37°C overnight at 200 rpm. The next day, the culture was diluted to 1:100 using Luria broth *i.e.*, 0.5 ml of culture was added to 50 ml of Luria broth. It was incubated for 2 to 3 hours till it attained the OD of 0.3 - 0.4 at 600 nm. The culture was chilled in ice for 30 minutes and 25 ml of culture was dispensed into two 50 ml centrifuge tubes. The cells were pelleted at 6,000 rpm for 5 minutes, the supernatant was discarded and pellet was resuspended in 12.5 ml of ice- cold 0.1 M calcium chloride. The centrifuge tubes were again kept in ice for 45 minutes and later centrifuged at 4000 rpm for 10 minutes. The pellet was dispensed in 1 ml of 0.1 M calcium chloride. About 200 µl of cells were distributed to each pre-chilled 1.5 ml micro centrifuge tubes and immediately used.

3.7.2 Transformation of *E. coli* DH5α

About 100µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 µl of ligated mixture was added into the tube and mixed gently. The mixture was chilled in ice for 45 minutes. Later, heat shock was given by shifting the chilled mixture to pre-heated 42°C water bath for exactly 2 minutes. Immediately it was transferred to ice bucket to chill for 5 minutes. To this 800 µl of Luria broth was added and incubated at 37°C at 200 rpm for 45 minutes, to allow bacteria to recover and express the antibiotic marker encoded by the

plasmid. The culture was centrifuged at 13,000 rpm for 1 minute and about 700 µl of supernatant was discarded and the pellet was dissolved in the remaining supernatant and spread on Luria agar plates with Amp_{100}, X-gal and IPTG (Appendix-IV), and incubated overnight at 37°C.

The recombinant clones were identified by blue/white colony assay. After incubation, only white colonies, having recombinant vectors were picked up and streaked on plates having Luria agar (Appendix IB) with Amp_{100}, X-gal and IPTG, and incubated at 37°C overnight for confirmation and further use.

3.7.3 Confirmation of Clones

Confirmation for the presence of desired DNA fragment in the cloning vector was done by PCR amplification using specific primers and restriction analysis.

A) Isolation of Recombinant Plasmid

The alkaline lysis protocol of Birnboin and Doly (1979) with certain modifications was used for isolation of recombinant plasmids.

White colonies were inoculated in 10 ml Luria broth with ampicillin (100µg/ml) and incubated over night at 37°C under shaking conditions at 175 rpm. Overnight grown cultures were centrifuged at 5,000 rpm for 2 minutes at 4°C in 2.0 ml micro-centrifuge tubes. The supernatant was removed and the pellet was washed with 2.5 ml of STET (Appendix-V). It was centrifuged at 5,000 rpm for 2 minutes. Again, supernatant was removed and the pellet was resuspended in 200 µl of ice-cold alkaline lysis solution I (Appendix-V) by vigorous vortexing. Later, 400 µl of freshly prepared alkaline lysis solution II (Appendix-V) was added to each tube and the contents were mixed by inverting the tubes for 4 to 5 times and kept in ice for 5 minutes. To this suspension, 300 µl of alkaline lysis solution III (Appendix-V) was added and again mixed thoroughly by gently inverting the tubes for 4-5 times. The tubes were kept in ice for 5 minutes and centrifuged at 13,000 rpm for 8 min at 4°C. The supernatant was transferred to fresh tubes and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to avoid proteins and was mixed well. Further, it was centrifuged at 13,000 rpm for 10 minutes at 4°C. The aqueous layer was transferred to a fresh centrifuge tube and two volumes of isopropanol were added. The contents were mixed by inverting the tubes 4 to 5 times and allowed to stand for 2 minutes at room temperature. The solution was later centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was washed with 70 per cent ethanol and spun for 1 minute at 13,000 rpm to recover the plasmid. The supernatant was discarded, pellet was dried completely and dispensed into 25 µl of $T_{10}E_1$ (pH 8.0) containing 3 µl of RNase (10 mg/ml). The solution was kept at 50°C for 15 minutes and then stored at -20°C. Plasmid DNA was visualized on 0.7 per cent agarose gel as described earlier.

B) Confirmation of Recombinant Clones

The confirmation of the presence of cloned fragment was done by PCR amplification of clones with respective primers. The total DNA and cloning vector were used as positive and negative controls in the PCR. The confirmation was also done through comparative restriction analysis of selected clones and the control vector. For *T. viride* clones *Hin*d III and *Eco*R I and for *T. harzianum* clones *Kpn* I and *Hin*d III endonuclease were used for restriction. The construct containing endochitinase cloned from *T. viride* IABT1012 and IABT1013 were named as pGSK12 and pGSK13 respectively. The construct carrying endochitinase cloned from *T. harzianum* IABT1015 and pSUM1C were named as pGSK15 and pGSK01, respectively.

3.8. SEQUENCING AND *In silico* ANALYSIS OF THE CLONES

The insert in pGSK12, pGSK13 and pGSK15 were sequenced using M13 primers by employing primer walking technique, at Bangalore Genei Private Ltd., Bangalore.

Removal of vector sequences and restriction analysis were done using GENE TOOL and VecScreen service of NCBI. Homology search was done using BLAST search available at http://www.ncbi.nlm.nih.gov. *In silco* translation was done using NCBI BLAST by selecting the CDS feature and pair wise alignment in BLAST option. Multiple alignments for homology search were performed using the Clustral W algorithm in Bioedit and Phylogenetic analysis was done using MEGA3.1 (Kumar *et al*., 2004) software. For out group, protein sequence of putative alliin lyase from *Aegilops tauschii* (AAM69848.1) was used.

The general features of the protein (molecular weight, pI, amino acid composition) were assessed using the Prot Param tool and the presence of a putative signal sequence was predicted using Signal P (Verson 3.0). All other bioinformatics like searching domain and constructing 3D structure of the protein were performed using tools that are accessible via different links on the proteomics service of the Swiss Institute of Bioinformatics.

3.9 CLONING OF *T. virens* ENDOCHITINASE GENE IN YEAST EXPRESSION VECTOR (pYES2/CT)

The endochitinase gene cloned from *T. virens* was used for expression in yeast. Both the constructs (with and without extra length in the upstream region) were used in this study. The pYES2/CT (Invitrogen) and *Saccharomyces cerevisiae* INVSc1 were used for this purpose.

3.9.1. Preparation and Maintenance of pYES2/CT

About 20 µg of lyophilized plasmid pYES2/CT (Fig. 1) was supplied by Invitrogen. 20 µl of distilled water was added to prepare a stock of 1µg/µl and stored at -20^{o}C for further use. Then it was transferred to *E. coli* DH5α as per the procedure mentioned in section 3.7.2.

3.9.2 Preparation and Maintainence of *Saccharomyces cerevisiae* (INVSc1)

Saccharomyces cerevisiae INVSc1 was supplied by Invitrogen as a stab, and maintained on YPD medium (Appendix VI). The genotype and phenotype of *S. cerevisiae* INVSc1 host are given below:

Genotype: *his3Δ1/his3Δ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*

Phenotype: His^{-}, Leu^{-} , Trp^{-} and Ura^{-}

INVSc1 is a diploid strain auxotrophic for histidine, leucine, tryptophan and uracil. The strain cannot grow on SC minimal medium deficient in histidine, leucine, tryptophan and uracil

3.9.3 Vector and Clone Isolation

The plasmid from pSUM1C, pGSK01 clones and pYES2/CT were isolated as described in the section (3.7.3A)

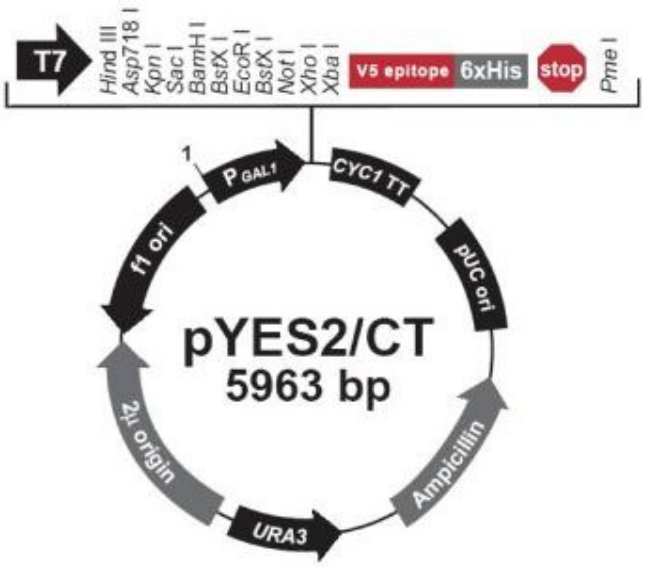

Fig_1_Restriction map of pYES2CT

3.9.4 Restriction, Elution and Ligation of Chitinase Gene

Simultaneous digestion of pYES2/CT, pGSK01 and pSUM1C were done with two restriction enzymes *Bam*H I and *Xba* I. The 1.6 kb fragment of the pSUM1C, 1.5kb fragment of pGSK01 and linearized pYES2/CT were eluted from one percent agarose gel using Eppendorf gel extraction kit. The purified vector DNA, insert from pSUM1C and pGSK01 were quantified by ethidium bromide spotting method. The ligation reaction was carried out with an optimal molar ratio of 1:3 (vector: insert). The components of the ligation mixture were mixed into a 0.5 ml micro centrifuge tube and incubated at 16°C for 16 hours.

3.9.5 Transformation and Confirmation of Endochitinase Gene

The competent cells of *E. coli* DH5α were prepared as described in section 3.7.1 the ligated mixture was used for transformation of *E. coli* DH5α competent cells as mentioned in section 3.7.2. For confirmation of clones, the plasmid was isolated from the clones by following the protocol mentioned earlier in section 3.7.3A. The presence of insert in clones was confirmed by PCR using respective primers. It was further analyzed by complete restriction of clones using *Bam*H I and *Xba* I enzymes. The Constructs were named as pGSY1 for pGSK01 and pGSY2 for pSUM1C.

3.10 YEAST (*Saccharomyces cerevisiae)* TRANSFORMATION

Saccharomyces cerevisiae INVSc1 was inoculated in 10 ml of YPD broth for overnight at 30^{o}C under shaking condition at 200 rpm. After overnight growth it was diluted to get 0.4 OD at 600nm in 50ml of YPD broth and incubated for additional three to four hours. Then it was centrifuged at 15,000g and resuspended in 40ml of 1X TE (appendix VII). Again it was centrifuged at 1,500g for 10 minutes and dissolved in 2ml of 1X LiAc/0.5X TE (Appendix VII). This was incubated at room temperature. For each transformation, 1µg of plasmid DNA and 100 µg of denatured sheared salmon sperm DNA with 100 µl of yeast suspension were

mixed. To this mixture 700µl of 1X LiAc/40% PEG-3350/1X TE (Appendix-VII) was added and thoroughly mixed. It was incubated for 30 minutes at 30°C and 88 µl of DMSO was added and thoroughly mixed. Heat shock was given at 42°C for 7 minute and centrifuged at 13,000 rpm for 10 seconds. Supernatant was removed and pellet was dissolved in 1ml 1X TE. Again it was centrifuged at 13,000 rpm for 10 seconds and supernatant was removed. The pellet was re-dissolved in 50-100µl of TE and spread on SC minimal medium lacking uracil (appendix-VI).

3.10.1 Isolation of Total DNA from Yeast

Transformed yeast cells were grown on SC-U minimal broth for overnight at 30°C under shaking condition at 200 rpm. Cells were harvested by centrifugation for 1 minute at 13,000 rpm. The pellet was washed with distilled water and again, centrifuged for 1 minute at 13,000 rpm. The pellet was re-suspended in lysis buffer (Appendix-XI) and 200mg of acid washed glass beads (Sigma) were added. This mixture was vortexed for 5 minutes. Protein was removed by adding equal volume of phenol: chloroform: IAA (25:24:1) and centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and the DNA was recovered by isopropanol precipitation. The DNA was washed with 70 per cent alcohol and air dried. Pellet was re-dissolved in 50µl TE buffer.

3.10.2 Confirmation of Yeast Clones

Total DNA from yeast was quantified using ethidium bromide spotting method and PCR amplified the chitinase gene using specific primer. The construct having endochitinase gene from pGSK01 was named as pGSY1 and the construct having endochitinase gene from pSUM1C was named as pGSY2.

3.11. INDUCTION AND EXPRESSION OF ENDOCHITINASE GENE IN YEAST

The pYES2/CT contains *GAL* 1 Promoter which is induced by the galactose. Induction of endochitinase gene was carried out as per the protocol mentioned by Song *et al*. (2005) with minor modifications.

The transformed *S. cervisiae* INVSc1 cell harboring the expression plasmid pGSY1 were grown on SC-U minimal medium. Isolated colonies were inoculated in 200ml of SC-U minimal medium having 10mg/l adenine and 2 per cent raffinose. It was grown at 30°C for three days under shaking condition. These cells were harvested by centrifugation at 1,500g for 5 minutes and used for inoculating the 50 ml of SC -U minimal medium having 2 per cent galactose. These cells were grown at 30°C for three days under shaking condition at 200 rpm. The cells were collected by centrifugation at 1,500g for 5 minutes at 4°C. Lysate was prepared by dissolving the pellet in the breaking buffer (Appendix XII) and adding the equal volume of acid washed glass beads. It was vortexed for 30 seconds followed by 30 seconds on ice. This step was repeated for four to five times for a total of four minutes. Then it was centrifuged for 13,000 rpm for one minute at 4°C. The supernatant was collected in clean centrifuge tube which was pre-cooled on ice. The total protein of the supernatant was measured by the Lowry's method (Lowry *et al.,* 1951) and was stored at -20°C for further use.

3.11.1 Measurement of Enzyme Activity

Enzyme activity in the lysate from clone was measured according to Miller (1959) with certain modification. Endochitinase activity was estimated colorimetrically using glycol chitin as substrate. For this experiment yeast with blank pYES2/CT was used as a control. The reaction mixture was prepared mixing 0.4 ml of 0.03 per cent glycol chitin substrate, 0.4ml of appropriately diluted crude enzyme solution and 0.4ml of McIvaline buffer. This mixture was incubated at 50^{O}C for 30 minutes and reaction was stopped by adding 0.5 ml of dinitrosalisylic acid and immediately boiled for 15 minutes. The intensity of the color was measured using spectrophotometer at 540nm. Simultaneously, standard solutions (200µg, 400µg, 600µg, 800µg, and 1000µg,) were prepared using N-acetyl glucose amine. The graph was drawn concentration against the absorbance. Before doing enzymatic assay, total protein was estimated using Lowry's method and the protein was appropriately diluted and equal quantity was used for the enzyme assay.

3.12 POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium Dodecyl Poyacrylamide Gel Electrophoresis was done as per the protocol given by the Sambrook and Russel (2001).

3.12.1 Preparation of the Polyacrylamide Gel

The glass plates were assembled as per the manufacturer's instruction. Seventeen ml of 12 per cent (Appendix VIII) polyacrylamide gel was poured into the gap between the glass plates leaving sufficient space for the stacking gel. The 0.1 per cent SDS was poured on the top of the polyacrylamide gel in order to make the surface smooth and level. The gel was left for 30 minutes or until it solidified. After solidification, the SDS layer was removed by washing with the water for two to three times. About 5 ml of 5 per cent stacking gel was poured on the resolving gel after inserting the comb. After the polymerization the comb was carefully removed and wells were washed with the deionized water for two to three times to remove the unpolymerized acrylamide. While pouring the resolving gel and stacking gel, enough care was taken to avoid air bubble formation in the gel.

3.12.2 Preparation of Sample for the SDS PAGE

The sample for the SDS PAGE was prepared as described in the section 3.11, and it was diluted to get the equal concentration of total protein. To this supernatant, equal volume of the loading dye (Appendix VIII) was added and heated upto 95^{O}C for 10 minutes. To each well about 50 µg of the sample and equal volume of dye (V/V) mixture was loaded.

3.12.3 Running of the Polyacrylamide Gel Electrophoresis

After loading the dye, the apparatus was submerged in the running buffer and rest of the parts was assembled as per the manufacturer's instruction. Initially the voltage of the current was kept at 70 mV, when protein entered the resolving gel the voltage was increased upto 120 mV. The gel was run upto the end of the resolving gel usually four to five hours. After sufficient run the glass plates were carefully removed and gel was transferred to tray containing the staining solution.

3. 12.4 Staining and De-staining of Polyacrylamide Gel

After running the polyacrylamide gel electrophoresis, the gel was carefully removed and placed in the staining solution (Appendix VIII). The gel was stained for 3 hours in shaking condition. After staining with coomassie brilliant blue, the excess dye was removed by de-staining solution (Appendix VIII) under shaking condition. The de-staining solution was replaced for two to three times until the clear blue color bands appeared. The gel was sealed in polyethylene bag and stored at 4^{O}C.

3.12.5 Zymography for Checking the Chitinase Activity

The SDS PAGE was performed as mentioned in the earlier section. In addition to all the ingredients 0.1 per cent glycol chitin was added to the polyacrylamide gel.

Restoration and staining of the SDS PAGE gel was performed as described by the Trudel and Asselin (1989) with minor modifications.

After running the gel, it was carefully removed from the glass plate and immersed in the 0.2M sodium acetate buffer (pH5.0) with 1 per cent TritonX-100 to remove the SDS and kept at 37^{O} C for 4 hours under shaking condition.

After four hours, the gel was rinsed with clean deinonised water for two to three times. The activity of the chitinase was stopped by adding the 0.01 per cent calcofluor white M2R in 0.1M Tris buffer (pH 8.9) for 15 minutes at 37^{O}C under shaking condition.

3.13 PATHOGEN INHIBITION ASSAY

Sclerotium rolfsii was used for the pathogen inhibition assay. Two different concentrations of cell lysate *viz*., 100µg and 200µg total protein were used. First, lysate was spread over the PDA plate uniformly and a single sclerotial body of S*. rolfsii* was placed on the center of the plate. This was incubated at 28^{O}C for three days and observed for the inhibition. In addition, the sclerotial bodies were dipped in the cell lysate for two different time's *viz*., 5 minutes and 10 minutes. Treated sclerotial bodies were then placed on the PDA plate and incubated at 28^{O}C for 72 hours for germination and growth.

IV. EXPERIMENTAL RESULTS

The results of the experiments conducted during the present investigation are presented below.

4.1 ISOLATION OF *Trichoderma* Spp.

Following the serial dilution plate method, 44 *Trichoderma* isolates were obtained from 48 soil samples. Of these, 17 belong to *T. harzianum*. The details of the isolates are given in the Table 1. The isolates were meant to be added to the *Trichoderma* resource base of the Department. No further study was done on these cultures.

4.2 SCREENING OF *Trichoderma* spp. FOR CHITINASE ACTIVITY

Thirty three isolates (already available in the Department) were used for screening which were previously identified as *T. virens* (8 isolates), *T. harzianum* (6 isolates), *T. viride* (11 isolates), *T. koningii* (2 isolates), *T. pseudokoningii* (2 isolates), and *T. polysporum* (4 isolates) (Upendra 2006). The results of this experiment are presented in Table 2 and typical plate with hydrolysis zone is shown in Plate 1. *Trichoderma* isolates were screened using the glycol chitin plate and all tests repeated three times. Differences were observed among species and isolates of the same species with respect to chitinase production after induction with colloidal chitin and chitin degradation. Among all the isolates, *T. virens* IABT 1010, *T. koningii* IABT1016, *T. polysporum* IABT1018 were found to be more efficient chitinase producers. Chitinase production was observed within 24 hours of induction with colloidal chitin containing Mandels and Reese (1965) (Appendix 1C) medium. In all most all the isolates, chitinase production was stable up to 96 hours and in some cases there was decrease in chitinase production (IABT1002, IABT1006, IABT1010, IABT1027) after 72 hours. Of the eight *T. virens* isolates, three gave good dark zone (IABT1001, IABT1002, IABT1010) and the other two (IABT1006, IABT1007) were found to be moderate chitin degraders. Of the four *T. polysporum* isolates tested, two isolates (IABT1004, IABT1018) formed good dark zone, one isolate (IABT1011) formed moderate dark zone and other isolate produced a minor zone of hydrolysis. Among the 11 *T. viride* isolates, three isolates (IABT1013, IABT1014, IABT1022) gave good dark zones, five isolates gave moderate zone of hydrolysis (IABT1012, IABT1026, IABT1027, IABT1028, IABT1029), two isolates (IABT1020, IABT1021) gave very small dark zone in glycol chitin plate and one isolate (IABT1023) failed to form hydrolysis zone on glycol chitin plate. Of the two *T. pseudokoningii*, isolates tested, one isolate (IABT1024) gave good dark zone and the other isolate (IABT1008) gave very small dark zone. *T. koningii* included two isolates; one isolate (IABT1016) showed good dark zone and the other isolate (IABT1030) showed moderate dark zone. Of the six *T. harzianum* isolates tested three isolates (IABT1015, IABT1025, IABT1019) gave good dark zone, two isolates (IABT1031, IABT1032) showed moderate dark zone and one isolate (IABT1033) formed the very small hydrolysis zone. In isolates where chitinase production was not observed, growth of the isolates was very slow. Chitinase production reached maximum with in 48 hours of induction and was stable up to 96 hours. Therefore, culture filtrate taken after 48 hours of induction can be used for routine screening of *Trichoderma* isolates for chitinolytic activity.

Table 1: *Trichoderma* spp. isolated and the source of soil samples

Sl.No.	Isolate No.	*Trichoderma* spp.	Place
1.	IABT1074	*Trichoderma harzianum* Rifai	Joida
2.	IABT1075	*Trichoderma pseudokoningii* Rifai	Sintheri
3.	IABT1076	*Trichoderma harzianum* Rifai	Sintheri
4.	IABT1077	*Trichoderma* sp.	Joida
5.	IABT1078	*Trichoderma pseudokoningii* Rifai	Joida
6.	IABT1079	*Trichoderma pseudokoningii* Rifai	Kulagi
7.	IABT1080	*Trichoderma pseudokoningii* Rifai	Sintheri
8.	IABT1081	*Trichoderma harzianum* Rifai	Sintheri
9.	IABT1082	*Trichoderma pseudokoningii* Rifai	Sintheri
10	IABT1083	*Trichoderma hamatum* (Bon.) Bain	Perumbandi
11.	IABT1084	*Trichoderma harzianum* Rifai	Yadagiri
12.	IABT1085	*Trichoderma harzianum* Rifai	Kalathgiri
13.	IABT1086	*Trichoderma harzianum* Rifai	Kalathgiri
14.	IABT1087	*Trichoderma harzianum* Rifai	Manikedara
15.	IABT1088	*Trichoderma harzianum* Rifai	Kalathgiri
16.	IABT1089	*Trichoderma* sp.	Kalathgiri
17.	IABT1090	*Trichoderma harzianum* Rifai	Kalathgiri
18.	IABT1091	*Trichoderma harzianum* Rifai	Kalathgiri
19.	IABT1092	*Trichoderma* sp.	Thitimuthi
20.	IABT1093	*Trichoderma* sp.	Yadagei
21.	IABT1094	*Trichoderma* sp.	Dharwad
22.	IABT1095	*Trichoderma* sp.	Kalatgiri
23.	IABT1096	*Trichoderma* sp.	Kaveri dam
24.	IABT1097	*Trichoderma* sp.	Dharwad
25.	IABT1098	*Trichoderma* sp.	Yana
26.	IABT1099	*Trichoderma* sp.	Yana

27.	IABT1100	*Trichoderma* sp.	Yadalli
28.	IABT1101	*Trichoderma* sp.	Dattapeth
29.	IABT1102	*Trichoderma* sp.	Yadageri
30.	IABT1103	*Trichoderma harzianum* Rifai	Yadageri
31.	IABT1104	*Trichoderma viride* Pers.: S.F. Gray	Kalathgiri
32.	IABT1105	*Trichoderma harzianum* Rifai	Kaveri Dam
33.	IABT1106	*Trichoderma* sp.	Yana
34.	IABT1107	*Trichoderma harzianum* Rifai	Yana
35.	IABT1108	*Trichoderma* sp.	Kaveri Dam
36.	IABT1109	*Trichoderma* sp.	Dharwad
37.	IABT1110	*Trichoderma* sp.	Yadalli
38.	IABT1111	*Trichoderma* sp.	Yana
39.	IABT1112	*Trichoderma harzianum* Rifai	Kalathgeri
40.	IABT1113	*Trichoderma* sp.	Kalathgeri
41.	IABT1114	*Trichoderma harzianum* Rifai	Kalathgeri
42.	IABT1115	*Trichoderma harzianum* Rifai	Bharamagiri hills
43.	IABT1116	*Trichoderma* sp.	Bharamagiri hills
44.	IABT1117	*Trichoderma harzianum* Rifai	Bharamagiri hills

Table 2: Screening of different *Trichoderma* spp. for chitinase activity

Sl No.	Isolate No.	*Trichoderma* isolates	Duration of Induction (hr)			
			24	48	72	96
1	IABT1001	*T. virens*	+	++	+++	+++
2	IABT1002	*T. virens*	++	+++	+++	++
3	IABT1003	*T. reesei*	++	+++	+++	+++
4	IABT1004	*T. polysporum*	+	+++	+++	+++
5	IABT1005	*T. virens*	-	-	-	-
6	IABT1006	*T. virens*	+	++	++	+
7	IABT1007	*T. virens*	++	++	++	++
8	IABT1008	*T. pseudokoningii*	+	+	+	+
9	IABT1009	*T. polysporum*	+	+	+	++
10	IABT1010	*T. virens*	+++	+++	++	++
11	IABT1011	*T. polysporum*	-	++	++	+++
12	IABT1012	*T. viride*	+	++	++	++
13	IABT1013	*T. viride*	++	+++	+++	+++
14	IABT1014	*T. viride*	++	+++	+++	+++
15	IABT1015	*T. harzianum*	++	+++	+++	+++
16	IABT1016	*T. koningii*	+++	+++	+++	+++
17	IABT1017	*T. virens*	-	-	-	-
18	IABT1018	*T. polysporum*	+++	+++	+++	+++
19	IABT1019	*T. harzianum*	++	+++	+++	+++
20	IABT1020	*T. viride*	+	+	+	+
21	IABT1021	*T. viride*	-	+	+	+
22	IABT1022	*T. viride*	++	+++	+++	+++
23	IABT1023	*T. viride*	-	-	-	-
24	IABT1024	*T. pseudokoningii*	++	+++	+++	+++
25	IABT1025	*T. harzianum*	++	+++	+++	+++
26	IABT1026	*T. viride*	+	++	++	++

27	IABT1027	*T. viride*	++	+++	++	+
28	IABT1028	*T. viride*	+	+	++	+++
29	IABT1029	*T. viride*	++	++	++	++
30	IABT1030	*T. koningii*	+	++	++	++
31	IABT1031	*T. harzianum*	+	++	++	++
32	IABT1032	*T. harzianum*	+	++	++	++
33	IABT1033	*T. harzianum*	+	+	+	+

Legend

+++: Good zone of hydrolysis - : No hydrolysis

++ : Moderate zone of hydrolysis, **+**: Very small zone of hydrolysis

LEGEND

Plate 1

C Control (No induction)

IABT1016 *T. koningii*

IABT1007 *T. Virens*

IABT1029 *T. Viride*

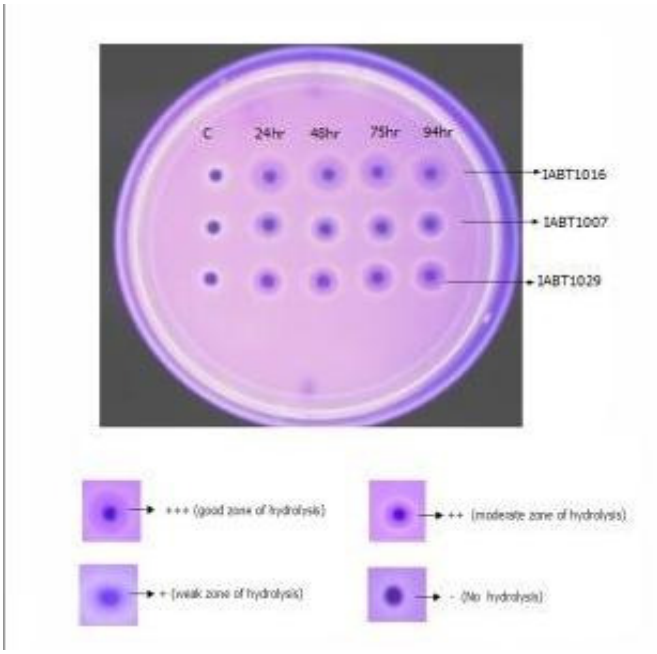

Plate_1_Glycol chitin plate assay to asses the chitinase activity

4.3 CLONING OF GENE ENCODING ENDOCHITINASE

The endochitinase specific primers; TVD-1 and THR-3 were designed for amplification of full length gene (encoding 46 kDa proteins and named as *chit46*) from *T. viride* and *T. harzianum* respectively. Among the different concentrations of respective primers tested (5, 10, 20, 30, 40 and 50 pM/µl), 5 pM/µl concentration was found to give good amplification without leaving primer dimers in the reaction. Therefore, this concentration was used routinely in further PCR amplification in a reaction volume of 20 µl.

The template DNA of *T. viride* isolates gave an amplification of ~1.6kb (Plate 2) and *T. harzianum* gave ~1.5kb amplicon (Plate 3). The large scale amplification of both the gene was achieved using the respective primers and were separated in a low melting agarose gel of 1 per cent and eluted. The eluted fragments were ligated to pTZ57R/T cloning vector, and were transferred to *E.coli* DH5α separately. These recombinant *E.coli* DH5α were maintained on the luria agar having ampicillin as a selection pressure.

To remove the extra length upstream to the coding region of endochitinase gene in pSUM1C, TVR-1 primers were used and large scale amplification was achieved by using the same primer (Plate 4). The 1.5 kb fragment was eluted from 1 per cent agarose gel and ligated to pTZ57R/T then transferred to *E.coli* DH5□. Transformants were selected and maintained on the Luria agar with ampicillin.

4.4 CONFIRMATION OF CLONES

The transformed cells were picked up, streaked on Luria agar ampicillin (100 ppm) containing X-GAL and isopropyl-β-D-thiogalactosidase (IPTG) for clonal selection. Recombinant cells were selected based on blue/white colony assay. Endochitinase gene cloned from *T. viride* isolate IABT1012 in pTZ57R/T was named as pGSK12, out of 7 transformants, 5 were white; from isolate IABT1013 named pGSK13, out of 10 transformants, 8 were white; construct having gene from isolate IABT1015 was named as pGSK15, out of 17 transformants, 15 were white; and the construct having endochitinase gene from pSUM1C was named as pGSK01, out of 9 transformants 7 were white. The confirmation of the clones was done by restriction digestion and PCR amplification. pGSK12, pGSK13 gave the 1.6 kb amplicon by using the specific primer mentioned in the section 3.4.1 and restriction analysis was done using the *Hin*d III and *Eco*R I which released the 1.6 kb fragment from the pGSK12 and pGSK13. In case of pGSK15 and pGSK01 PCR amplification gave approximately 1.5 kb fragment. Restriction analysis was done using the *Kpn* I and *Hin*d III for pGSK15 and for pGSK01 *Xba* I and *Bam*H I were used, which released the fragment size of 1.5 kb from the clone. The restriction analysis of pGSK12, pGSK13, pGSK15 and pGSK01 are presented in the Plate 5, 6 and 7 respectively. PCR amplification of pGSK12, pGSK13, pGSK15 and pGSK01 presented in the Plate 8, 9 and 10 respectively.

4.5 SEQUENCING AND *IN SILICO* ANALYSIS OF THE CLONES

The full length of cloned genes (pGSK12, pGSK13 and pGSK15) of about 1.6 kb was sequenced using M13 primers employing primer walking technique. The nucleotide sequence of pGSK12 and pGSK15 after removing vector sequence through GENE TOOL and VecScreen service of the NCBI website is shown in the Fig 2 and 3, respectively. Among the two clones pGSK12 has a of length 1668 bp and pGSK15 has 1551bp. Clone pGSK12 has unique restriction sites for *Hin*d III, *Bgl* III*, Sal* I, and *Bam* HI at 47, 56, 765 and 990 positions respectively. The restriction map of the sequence (pGSK12 and pGSK15) and clone (pGSK12 and pGSK15) is presented in Fig 4, 5, 6 and 7, respectively. The available sequence information from cloned fragment was subjected to analysis using BLAST algorithm available

at http://www.ncbi.nlm.nih.gov. It showed homology with conserved domain of glycosyl hydrolases family 18 (Fig 8). Both the (pGSK12 and pGSK15) were translated to amino acid using NCBI BLAST using the option CDS feature. The amino acid sequence of gene is shown in Fig 9 and 10. It has four exons and code for 430 and 429 amino acids, respectively. Restriction analysis of the clone pGSK15 showed two unique restriction sites viz., *Sal* I and *Bam*H I at 686 and 911 positions respectively.

The sequence of cloned fragment (pGSK12) was subjected to BLASTn and BLASTx, and the homology results are presented in Table 3 and 4, respectively. It showed 95 per cent and 99 per cent homology with reported *T. harzianum Chit HAR-2* gene for endochitinase (AB041752) at nucleotide and amino acid levels, respectively. The change in amino acid was observed at four positions (Table 5).

The homology results of the Blastn and Blastx for the clone pGSK15 are presented in the Table 6 and 7, respectively. It showed 95 per cent and 99 per cent homology with reported *T. harzianum Chit HAR-2* gene for endochitinase (AB041752) at nucleotide and amino acid levels, respectively. The change in amino acid was observed at four positions (Table 8).

The endochitinase gene in pGSK13 has 99.8 and 99.5 per cent similarity with pGSK12 and pGSK15 at nucleotide level respectively and 99.8 and 99.5 per cent similarity with pGSK12 and pGSK15 at amino acid level respectively.

The protein has a calculated molecular mass of 46 kDa and theoretical pI of 5.90. Analysis using Signal P (Verson 3.0) indicated the presence of a putative eukaryotic N-terminal signal sequence consisting of 21 amino acids. A signal cleavage site was predicted after Ala_{22} Ser_{23}.

The 3D structure of the protein predicted using the protein tools that are accessible on the proteomics service of the Swiss Institute of Bioinformatics and presented as backbone in flat ribbon form and space filled predicted active site are presented in Plate 11 and 12, respectively.

4.5.1 Phylogenetic Analysis of the Chitinase Gene from *Trichoderma*

The various species of Trichoderma were taken for the analysis of phylogeny. The species and the accession numbers are given in Table 9 along with these sequences pGSK12 (*T. viride*) and pGSK15 (*T. harzianum*) are also used to know the phylogenetic position of these two species with respect to chitinase gene. Multiple sequence alignment was done by using Bioedit. For multiple sequence alignment Clustal W algorithm with gap opening penalty of 10.0 and gap extension penalty 1.0 was used. The multiple alignment of different species of *Trichoderma* is given in Fig 11, in which the consensus pattern of active site ([LIVMFY] - [DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-X-E) is highlighted in the box. The phylogenetic analysis was done by neighbor joining method and identity matrix generated is presented in the Table 10 and phylogenetic tree is presented in Fig. 12.

LEGEND

Plate 2

M 1 kb ladder (New England Biolabs)

I PCR amplification from genomic DNA of *T. viride* IABT1012

II PCR amplification from genomic DNA of *T. viride* IABT1013

Plate 3

M 1 kb ladder (New England Biolabs)

I and II PCR amplification from genomic Genomic DNA of *T. harzianum* IABT1015

Plate 4

M λ *Hind* III/*Eco*R I double digest

I and II PCR amplification of pSUMIC (*T. virens*) PCR amplification

Plate 5

M λ *Hind* III/*Eco*R I double digest

I Restriction of pGSK12 clone

II Restriction of pGS 13 clone

Plate 6

M λ *Hind* III/*Eco*R I double digest

I and II Restriction of pGSK15

Plate 7

M 1 kb ladder (New England Biolabs)

I and II Restricted of pGSK01

Plate 8

M λ *Hind* III/*Eco*R I double digest

I PCR amplification of pGSK12 clone

II PCR amplification of pGDK13 clone

Plate 9

M 1 kb ladder (New England Biolabs)

I and II PCR amplification of pGSK15

Plate 10

M 1 kb ladder (New England Biolabs)

I and II PCR amplification of pGSK01

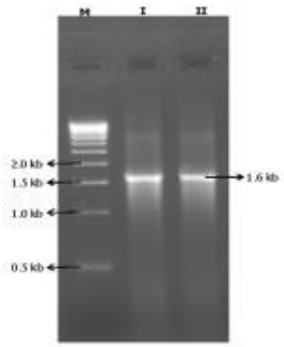

Plate 2. PCR amplification of endochitinase gene from *T. viride* (IABT1012 and IABT1013)

Plate_2_PCR amplification of endochitinase gene from T. viride (IABT1012 and IABT1013)

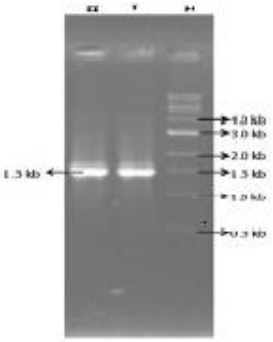

Plate 3. PCR amplification of endochitinase gene from *T. harzianum* IABT1015

Plate_3_PCR amplification of endochitinase gene from T. harzianum IABT1015

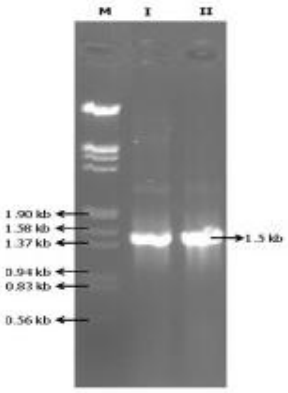

Plate 4. PCR amplification of endochitinase gene from pSUM1C

Plate_4_PCR amplification of endochitinase gene from pSUM1C

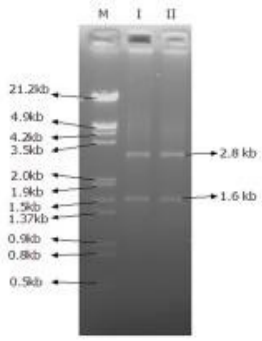

Plate 5. Restriction analysis of endochitinase gene from *T. viride* (pGSK12 and pGSKT13)

Plate_5_Restriction analysis of endochitinase gene from T. viride (pGSK12 and pGSK13)

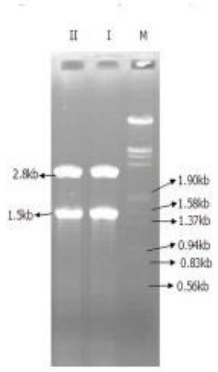

Plate 6. Restriction analysis of endochitinase gene from *T. harzianum* (pGSK15)

Plate_6_Restriction analysis of endochitinase gene from T. harzianum IABT1015

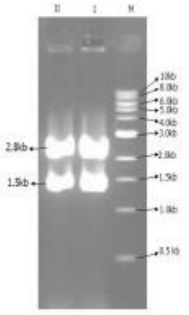

PLate 7. Restriction analysis of endochitinase gene from *T. virens* (pGSK01)

Plate_7_Restriction analysis of endochitinase gene from T. virens (pGSK01)

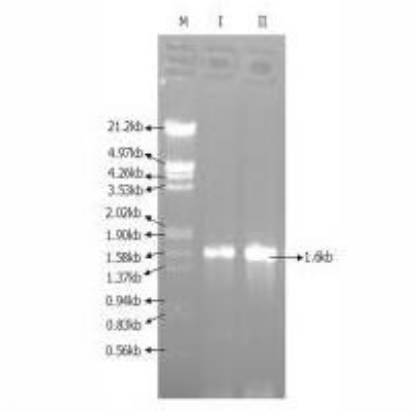

Plate_8_PCR amplification of the gene encoding endochitinase from pGSK12 and pGSK13

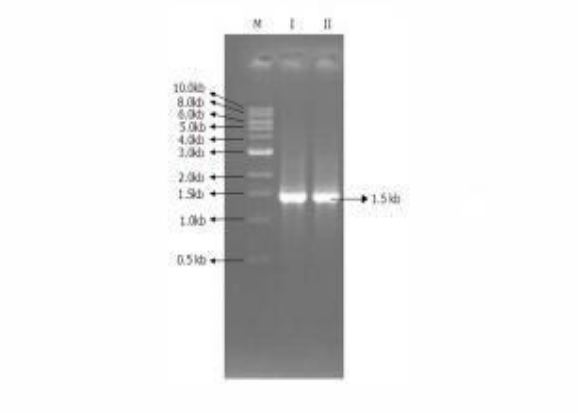

Plate_9_PCR amplification of the gene encoding endochitinase from pGSK15

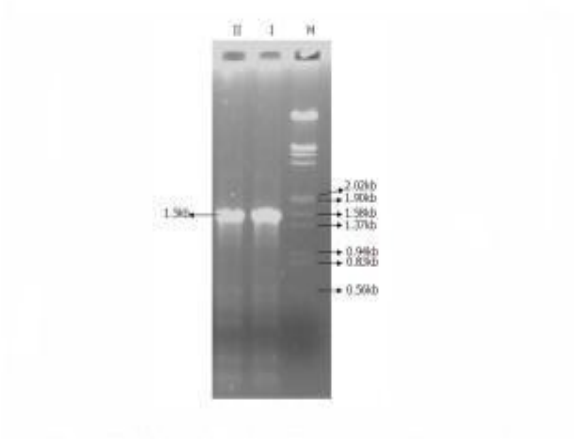

Plate_10_PCR amplification of the gene encoding endochitinase from pGSK01

Fig_2_Complete nucleotide sequence of endochitinase gene(chit46) from T. viride IABT1012

Fig. 2 Complete nucleotide sequence of endochitinase gene (*chit46*) from *T. viride* IABT1012

TGGTTCCCATTCAGCGCTTCAGAAGAAAGTTTAATCCACCATCGGAAAGCTTGAAAGATCTACAACAT
CACAAGCAATTCACCATGTTGAGCTTCCTCGGAAAATCCGTAGCCTTGTTGGCTGCGCTGCAGGCTAC
TCTCAGCTCTGCAAGCCCCCTGGCCACAGAAGAGCGCTCTATTGAGAAGAGAGCTAACGGATACGCAA
ACTCCGTCTATTTCACCAACTGGTGAGTGAAATAAACGAGCTTCCGTAGCTTCACTGCTGACTATAAG
CGATTAAAGGGGCATTTACGACCGCAACTTCCAGCCTGCCGATTTGGTGGCATCAGATGTCACTCATG
TCATCTACTCTTTCATGAACCTCCAGGCAGACGGCACAGTGTAAGCCAGATAACCCAGATAGGAGACA
TCTCCAAAAGCCTCTTTCTCGTTGCTAATCATCTTTCAGTGTCTCTGGCGATACCTACGCTGATTTCG
AGAAGCACTATGCCGATGATTGTACGTTCCCCTCTTATGGCCAGATGCTATGTATACATACTTACACT
CACACTAGCTTGGAATGATGTCGGCACCAATGCCTACGGCTGTGCCAAGCAGCTGTTCAAGGTCAAGA
AGGCCAACCGAAGGCCTCAAGGTTCTGCTCTCCATCGGTGGCTGGACCTGGTCCACCAACTTCCCTTCT
GCAGCAAGCACCGATGCCAACCGAAAGAACTTTGCGAAAACTGCCATTACCTTCATGAAGGATTGGGG
TTTCGATGGCATTGACGTCGACTGGGAGTACCCTGCAGACGCCACCCAGGCCTCCAACATGGTTCTTC
TGCTGAAGGAAGTCCGATCTCAGCTGGATGCTTATGCTGCCCAGTACGCCCCTGGATACCACTTCCTC
CTCACCATTGCCGCCCCAGCTGGCAAGGATAACTACTCCAAGCTGCGCCTGGCTGATCTCGGCCAAGT
CCTCGACTACATCAACCTCATGGCCTACGACTACGCTGGATCCTTCAGCCCCCTCACCGGTCACGACG
CCAACCTGTTTGCCAACCCGTCCAACCCCAATGCCACCCCCTTCAACACCGATTCCGCTGTCAAGGAT
TATATCAAGGGAGGTGTTCCCGCCAACAAGATTGTTCTCGGCATGCCCATCTACGGACGATCATTCCA
GAACACCGCTGGTATTGGCCAGACTTACAACGGAGTTGGAGGTGGCGGTGGTGGCTCCACTGGAAGCT
GGGAGGCCGGTATCTGGGATTACAAGGCTCTTCCCAAGGCGGGCGCCACCATCCAGTACGATTCTGTC
GCAAAGGGTTACTACAGCTACAACGCCGGCACCAAGGAGCTCATCTCTTTCGATACCCCTGACATGAT
CAACACCAAGGTTGCCTACCTCAAGTCTCTCGGCCTGGGAGGTAGCATGTTCTGGGAGGCCTCAGCCG
ACAAGAAGGGAGCTGACTCGCTCATTGGAACAAGCCACAGAGCTCTTGGAGGCCTGGACACGACTCAG
AACCTGCTGAGCTACCCCAACTCCAAGTATGATAACATCAGGAACGGTCTGAACTAAGCTGCCTATTT
TCAGGCGCTTTTTGGACATTGAAGATGTTGCGGGGAAATGCTCT

Legend

5′ upstream sequence : 1.....83

Coding sequence : join (84.....227,283.....380, 448.....473, 553.....1553)

3′ UTR sequence : 1554.....1608.

Fig_3_Complete nucleotide sequence of endochitinase gene (chit 46) from T. harzianum IABT1015

Fig. 3 Complete nucleotide sequence of endochitinase gene (*chit 46*) from *T. harzianum* IABT1015

CACCATGTTGAGCTTCCTCGGAAAATCCGTAGCCTTGTTGGCTGCGCTGCAGGCTACTCTCAGCTCTG
CAAGCCCCCTGGCCACAGAAGAGCGCTCTATTGAGAAGAGAGCTAACGGATACGCAAACTCCGTCTAT
TTCACCAACTGGTGAGTGAAATAAACGAGCTTCCGTAGCTTCACTGCTGACTATAAGCGATTAAAGGG
GCATTTACGACCGCAACTTCCAGCCTGCCGATTTGGTGGCATCAGATGTCACTCATGTCATCTACTCT
TTCATGAACCTCCAGGCAGACGGCACAGTGTAAGCCAGATAACCCAGATAGGAGACATCTCCAAAAGC
CTCTTTCTCGTTGCTAATCATCTTTCAGTGTCTCTGGCGATACCTACGCTGATTTCGAGAAGCACTAT
GCCGATGATTGTACGTTCCCCTCTTATGGCCAGATGCTATGTATACATACTTACACTCACACTAGCTT
GGAATGATGTCGGCACCAATGCCTACGGCTGTGCCAAGCAGCTGTTCAAGGTCAAGAAGGCCAACCGA
GGCCTCAAGGTTCTGCTCTCCATTGGTGGCTGGACCTGGTCCACCAACTTCCCTTCTGCAGCAAGCAC
CGATGCCAACCGAAAGAACTTTGCGAAAACTGCCATTACCTTCATGAAGGATTGGGGTTTCGATGGCA
TTGACGTCGACTGGGAGTACCCTGCAGACGCCACCCAGGCCTCCAACATGGTTCTTCTGCTGAAGGAA
GTCCGATCTCAGCTGGATGCTTATGCTGCCCAGTACGCCCCTGGATACCACTTCCTCCTCACCATTGC
CGCCCCAGCTGGCAAGGATAACTACTCCAAGCTGCGCCTGGCTGATCTCGGCCAAGTCCTCGACTACA
TCAACCTCATGGCCTACGACTACGCTGGATCCTTCAGCCCCCTCACCGGTCACGACGCCAACCTGTTT
GCCAACCCGTCCAACCCCAATGCCACCCCCTTCAACACCGATTCCGCTGTCAAGGATTATATCAAGGG
AGGTGTTCCCGCCAACAAGATTGTTCTCGGCATGCCCATCTACGGACGATCATTCCAGAACACCGCTG
GTATTGGCCAGACTTACAACGGAGTTGGAGGTGGCGGTGGTGGCTCCACTGGAAGCTGGGAGGCCGGT
ATCTGGGATTACAAGGCTCTTCCCAAGGCGGGCGCCACCATCCAGTACGATTCTGTCGCAAAGGGTTA
CTACAGCTACAACGCCGGCACCAAGGAGCTCATCTCTTTCGATACCCCTGACATGATCAACACCAAGG
TTGCCTACCTCAAGTCTCTCGGCCTGGGAGGTAGCATGTTCTGGGAGGCCTCAGCCGACAAGAAGGGA
GCTGACTCGCTCATTGGAACAAGCCACAGAGCTCTTGGAGGCCTGGACACGACTCAGAACCTGCTGAG
CTACCCCAACTCCAAGTATGATAACATCAGGAACGGTCTGAACTAAGCTGCCTATTTTCAGGCGCTTT
TTGGACATTGAAGATGTCGCAGGGAAATGCTCTTGGAGGAGCAGGGTCATGAAGT

Legend

5′ upstream sequence : 1.....4

Coding sequence : join (5.....148,205.....301, 369.....394, 474.....1474)

3′ UTR sequence : 1475.....1551.

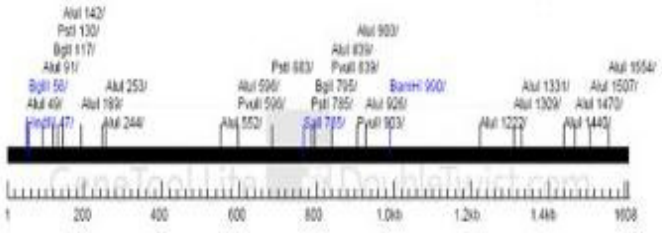

Fig 4: Restriction map of endochitinase (chit 46) sequence from T. viride IABT1012 with common enzyme

Fig_4_Restriction map of endochitinase (chit 46) sequence from T. viride IABT1012 with common enzyme

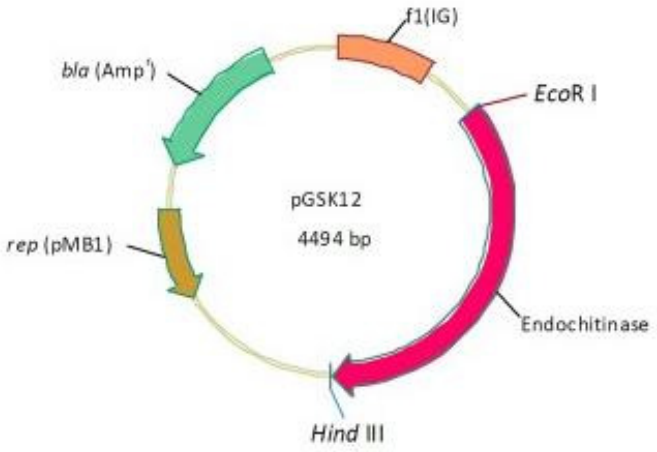

Fig 5: Construct map of pGSK12

Fig_5_Construct map of pGSK12

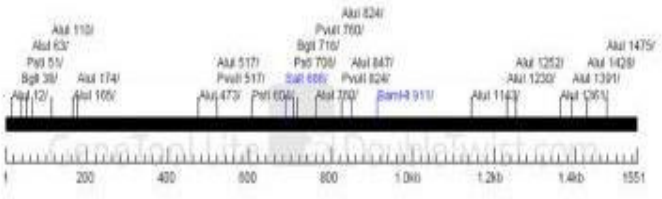

Fig_6_Restriction map of endochitinase (chit 46) sequence from T. harzianum IABT1015 with common enzyme

Fig 7: Construct map of pGSK15

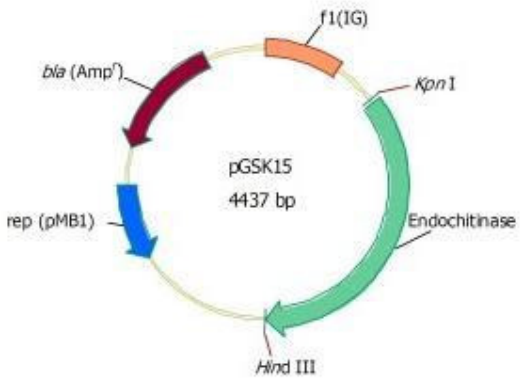

Fig_7_Construct map of pGSK15

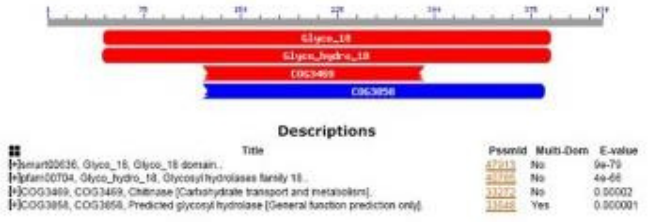

Fig_8_rpsBLAST result of endochitinase gene showing conserved domain

Fig. 9 Deduced amino acid sequence of endochitinase (chit 46) gene cloned from T. viride IABT1012

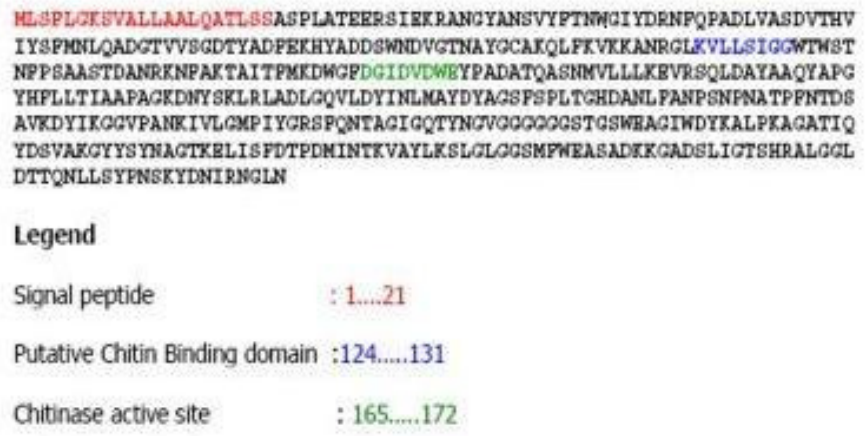

Fig_9_Deduced amino acid sequence of endochitinase (chit 46) gene cloned from T. viride IABT1012

Fig. 10 Deduced amino acid sequence of endochitinase gene (chit 46) cloned from T. harzianum IABT1015

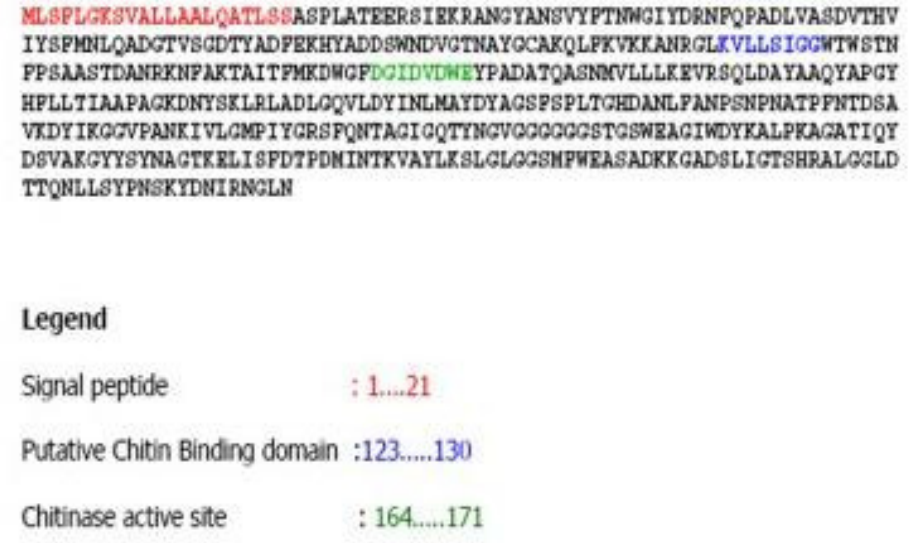

Fig_10_Deduced amino acid sequence of endochitinase gene (chit 46) cloned from T. harzianum IABT1015

Table 3: Nucleotide- Nucleotide blast (BLASTn) result of cloned endochitinase gene from *T. viride* IABT-1012

Accession No.	Homology Sequence	Homology (%)	Score bit	E-value
AB041752.1	*T. harzianum* chit-HAR2 gene for endochitinase	95	2635	0.0
AB041751.1	*T. harzianum* chit-HAR1 gene for endochitinase	95	2534	0.0
AB041755.1	*T. viride* chit-VIRI gene for endochitinase-VIR	97	2603	0.0
AB041754.1	*T. hamatum* chit-HAM gene for endochitinase	94	2213	0.0
AY758408.1	*H. lixii* strain T12 bacterial-type endochitinase	97	1906	0.0
AB041749.1	*T. virens* chit-G1 gene for endochitinase-G1	98	1910	0.0

Table 4: Protein- protein blast (BLASTx) result of cloned endochitinase gene from *T. harzianum* IABT1012

Accession No.	Homology Sequence	Homology (%)	Score bit	E-value
BAB40590.1	*T. harzianum* endochitinase-HAR2	99.3	724	0.0
BAB40592.1	*T. hamatam* endochitinase-HAM	99	714	0.0
BAB40593.1	*T. viride* endochitinase-VIRI	98	709	0.0
BAB40587.1	*H. virens*, endochitinase-G1	96	709	0.0
BAB40589.1	*T. harzianum* endochitinase-HAR1	95	708	0.0
CAJ34422.1	*H. virens* endochitinase-G1	91	704	0.0

Table 5: Amino acid changes in the cloned endochitinase gene in BLASTx search for pGSK12

Changed from	Changed to	Positions
V	I	32
V	A	110
Q	K	279
S	A	394

Table 6: Nucleotide-Nucleotide blast (BLASTn) result of cloned endochitinase gene from *T. harzianum* IABT-1015

Accession No.	Homology Sequence	Homology (%)	Score bit	E-value
AB041752.1	*T. harzianum* chit-HAR2 gene for endochitinase	95	2547	0.0
AB041751.1	*T. harzianum* chit-HAR1 gene for endochitinase	95	2486	0.0
AB041755.1	*T. viride* chit-VIRI gene for endochitinase-VI	97	2518	0.0
AB041754.1	*T. hamatum* chit-HAM gene for endochitinase	94	2203	0.0
AY758408.1	*H. lixii* strain T12 bacterial-type endochitinase	97	1898	0.0
AB041749.1	*T. virens* chit-G1 gene for endochitinase-G1	98	1888	0.0

Table 7: Protein- protein blast (BLASTx) result of cloned endochitinase gene from *T. harzianum* IABT1015

Accession No.	Homology Sequence	Homology (%)	Score bit	e-value
BAB40590.1	endochitinase-HAR2 *T. harzianum*	99%	759	0.0
BAB40593.1	endochitinase-VIRI *T. viride*	97%	755	0.0
BAB40589.1	endochitinase-HAR1 *T. harzianum*	97%	753	0.0
BAB40592.1	endochitinase-HAM *T.hamatatum*	99%	750	0.0
BAB40587.1	endochitinase-G1 *H. virens*	98%	744	0.0
AL78813.1	class V chitinase *H. virens*	98%	742	0.0

Table 8: Amino acid changes in the cloned endochitinase gene in BLASTx search for pGSK15

Changed from	Changed to	Positions
V	I	32
V	A	110
Q	K	279
S	A	394

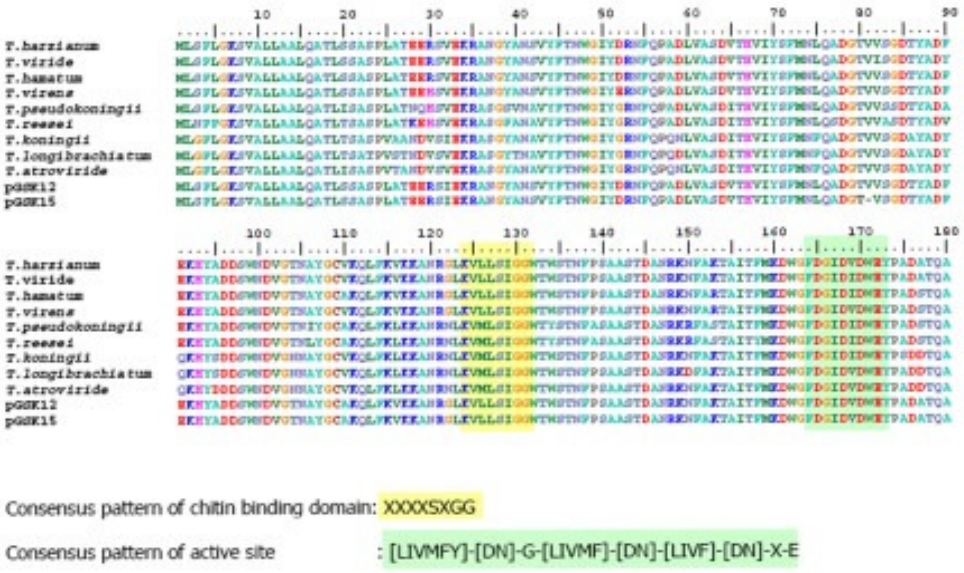

Fig.11 Multiple sequence alignment showing the chitinase active site

Fig_11A_Multiple sequence alignment showing the active site

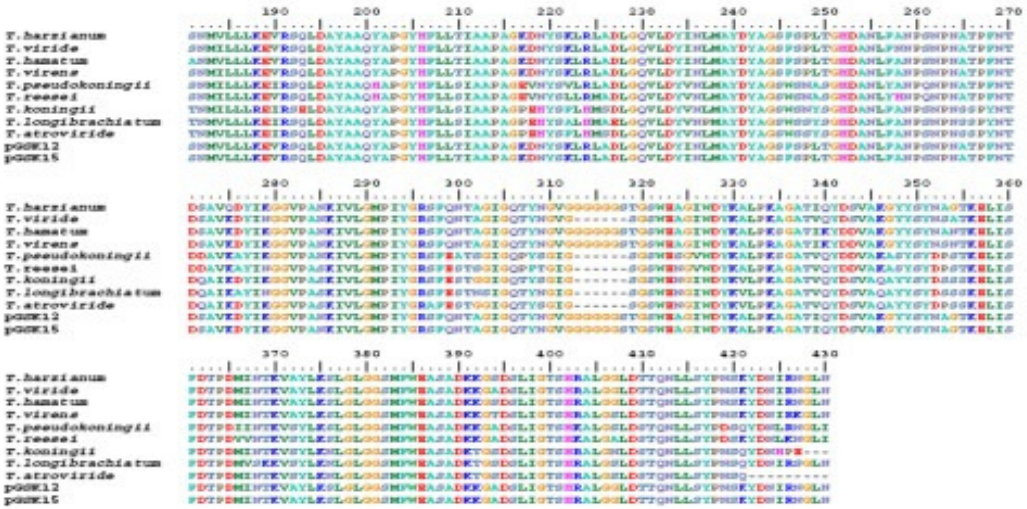

Fig.11 Multiple sequence alignment showing the chitinase active site (Contd.)

Fig_11B_Multiple sequence alignment showing the active site

Table 9: The accession numbers of *Trichoderma* chitinase gene used for multiple alignment and phylogeny

Acc. No.	*Trichoderma* spp.	Description of chitinase gene	Author
BAB40590	*T. harzianum*	endochitinase-HAR2	Nakahara *et al*., (2000)
BAB40593	*T. viride*	endochitinase-viri	Nakahara *et al.,*(2000)
BAB40592	*T. hamatum*	endochitinase-HAM	Nakahara *et al.,* (2000)
BAB40587	*T. virens*	endochitinase-G1	Nakahara *et al*.,(2000)
BAB40594	*T. pseudokoningii*	endochitinase-P	Nakahara *et al.,*(2000)
BAD44715	*T. reesei*	46 kDa chitinase	Ike *et al.,* (2006)
AAF19612	*T. koningii*	42 kDa endochitinase	Lieckfeldt *et al*.,(2000)
ABD42921	*T. longibrachiatum*	endochitinase	Bhunchoth *et al.,*(2006)
AAF19614	*T. atroviride*	42 kDa endochitinase	Lieckfeldt *et al*.,(2000)

Fig .12 Phylogenetic trees showing the position of cloned endochitinase

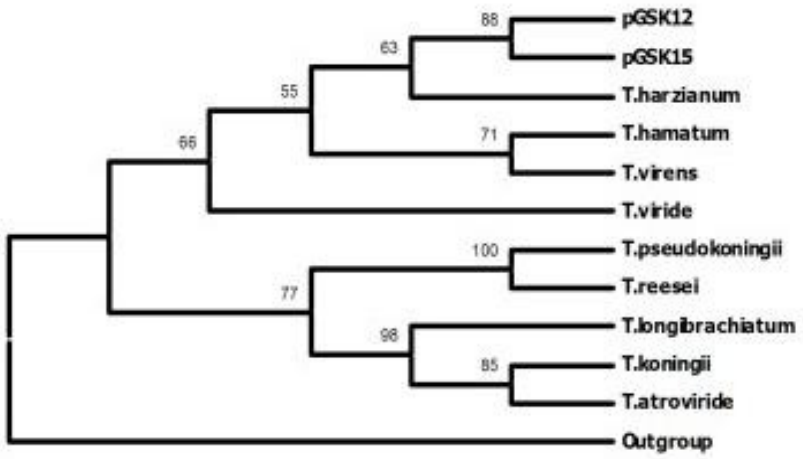

Fig_12_Phylogenetic tree showing the position of cloned endochitinase

4.6 CLONING OF THE CHITINASE GENE INTO THE YEAST EXPRESSION VECTOR.

For directional cloning of endochitinase gene into pYES2/CT, pSUM1C pYES2/CT and pGSK01 were cut with the *Xba* I and *Bam*H I, ligated and *E. coli* DH5α cells were transformed. The constructs were named as pGSY1 having endochitinase gene from pGSK01 (Fig 13) and pGSY2 for pSUM1C (Fig 14). The presence of gene was confirmed through PCR (Plate 13 and 14 respectively) and restriction analysis (Plate 15 and 16 respectively). The pGSY1 and pGSY2 were isolated from the *E. coli* and transferred to yeast. The transformants were picked and streaked on SC-U minimal medium lacking uracil. Total DNA isolated from these clones was confirmed through PCR amplification using specific primers (section 3.4.1.) (Upendra, 2006). About 1.7 and 1.5 kb fragments were amplified from the total DNA of yeast clones having pGSY1 and pGSY2, respectively and are shown in Plate 17 and 18, respectively.

4.7 EXPRESSION OF ENDOCHITINASE GENE IN YEAST

4.7.1 Measurement of Enzyme Activity

The pGSY1 and pGSY2 having chitinase gene in yeast was induced by galactose in SC-U minimal medium. The cell lysate was prepared as mentioned in the section 3.11. The total protein was estimated by Lowry's method and total protein per µl was presented in the Table 11. About 100 µg of the protein was used for the sugar conversion assay. The reducing sugar was estimated using the DNS method. The amount of reducing sugar generated after 30 minutes induction at 50^{O} C is given in Table 11. There was 9.8 times increase in the reducing sugar than the control. This indicated the expression of endochitinase gene in yeast. The color change in the solution due to production of reducing sugar is shown in Plate 19.

4.7.2 Sodium Dodecyl Sulphate Polyacryl Amide Gel Electrophoresis (SDS PAGE)

The SDS PAGE was conducted by loading 50µg of total protein into each well and gel was run at 70 mV initially, when it passed the staking gel, voltage was increased upto 120mV. The clear 46 kDa band and approximately 44 kDa intense band (after cleavage of signal peptide) formed in the yeast having pGSY2 construct and in control a very weak band was formed (Plate 20).

4.7.3 Zymograph Analysis for Chitinase Activity

To further confirm the expression, chitinase activity in the gel was studied. The gel preparation was done as described in the section 3.12.1. After the reactivation of protein in the 0.2M sodium acetate buffer (pH5.0) with 1 percent TritonX-100, it was strained using the calcofluor white M2R for 15 minutes and documented in UV DOC system. The clear dark color band was formed on the gel, indicating degradation of the glycol chitin in this region. And in the control, this band was not observed.

4.7.4 Pathogen Inhibition Assay

Of the two concentrations (100 and 200µg) of proteins tested, inhibition of *Sclerotium rolfsii* was observed on plate with 200µg total protein compared to control (Plate 21). In order to check the influence of cell lysate on germination of the sclerotial bodies, they were dipped in cell lysate and kept for germination. Dipping of sclerotial bodies for 10 min had more inhibition compared to control (yeast with pYES2/CT).

Table 10: Similarity matrix used for construction of phylogenetic tree

Seq->	*T. har*	*T. vir*	*T. ham*	*T. vire*	*T. psk*	*T. res*	*T. kon*	*T. lon*	*T. atr*	pGSK12	pGSK15	Outgroup
T. har	ID	95.0	97.0	96.5	83.0	81.3	80.4	81.1	80.6	99.0	98.6	11.9
T. vir		ID	94.0	94.1	84.9	83.9	82.3	82.5	81.3	95.8	95.3	11.5
T. ham			ID	97.2	84.1	82.0	80.0	80.2	79.7	97.4	96.9	11.6
T. vire				ID	83.9	82.0	80.2	80.6	80.0	96.2	95.8	11.4
T. psk					ID	93.3	83.0	82.7	82.7	83.4	83.0	11.9
T. res						ID	81.8	82.0	80.8	81.8	81.3	11.3
T. kon							ID	92.4	95.4	80.4	80.0	10.4
T. lon								ID	92.6	80.6	80.2	10.8
T. atr									ID	80.2	79.7	10.8
pGSK12										ID	99.5	11.9
pGSK15											ID	11.6
Outgroup												ID

T. har : *T. harzianum*

T. vir : *T. viride*

T. ham : *T. hamatum*

T. vire : *T. virens*

T. psk : *T. pseudokoningii*

T. res : *T. ressei*

T. kon : *T. koningii*

T. lon : *T. longibrachiatum*

T. atr : *T. atroviride*

Fig. 13 The construct pGSY1 with endochitinase gene from pGSK01

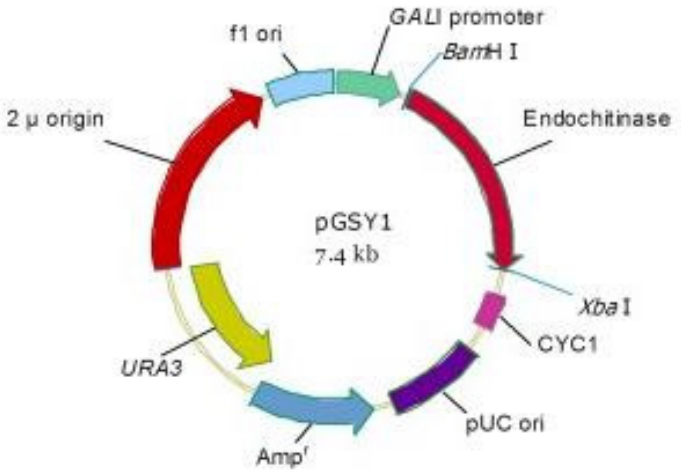

Fig_13_The construct pGSY1 with endochitinase gene from pGSK01

Fig. 14 The construct pGSY2 with endochitinase gene from pSUM1C

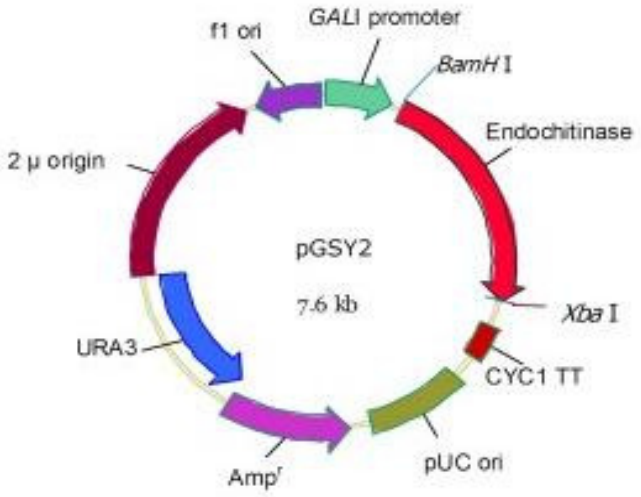

Fig_14_The construct pGSY2 with endochitinase gene from pSUM1C

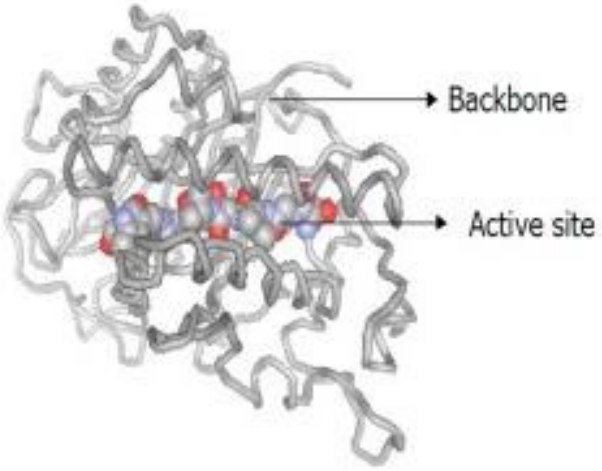

Plate 11. Predicted protein structure of cloned endochitinase gene from *T. viride* IABT1012

Plate_11_Predicted protein structure of cloned endochitinase gene from T. viride IABT1012

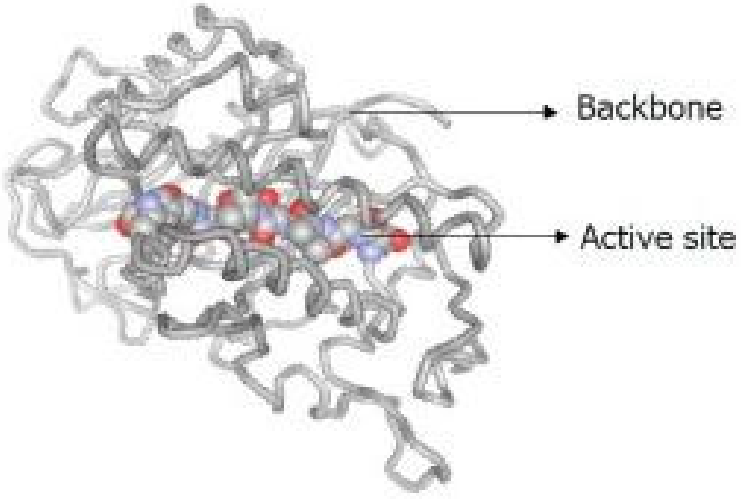

Plate_12_Predicted protein structure of cloned endochitinase gene from T. harzianum IABT1015

Plate 13

M λ *Hind* III/*Eco*R I double digest

I and II PCR amplification of pGSY1 in *E.coli*

Plate 14

M λ *Hind* III/*Eco*R I double digest

I and II PCR amplification of pGSY2 in *E.coli*

Plate 15

M 1 kb ladder (New England Biolabs)

I and II Restriction of pGSY1 in *E. coli*

Plate 16

M 1 kb ladder (New England Biolabs)

I and II Restriction of pGSY2 in *E. coli*

Plate 17

M 1 kb ladder (New England Biolabs)

I and II PCR amplification of pGSY1 in yeast

Plate 18

M 1 kb ladder (New England Biolabs)

I and II PCR amplification of pGSY2 in yeast

Plate 20

M Molecular weight marker (Bangalore Genei Pvt. Ltd.)

1-5 Yeast with pYES2/CT

6-10 Yeast with pGSY2

Plate 21

C No lysate

A Lysate from pGSY2

B Lysate from Yeast with pYES2/CT

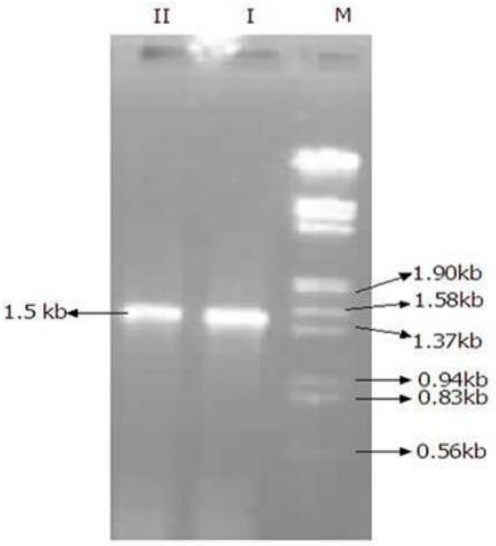

Plate_13_PCR amplification of gene encoding endochitinase in pGSY1 in E. coli DH5α

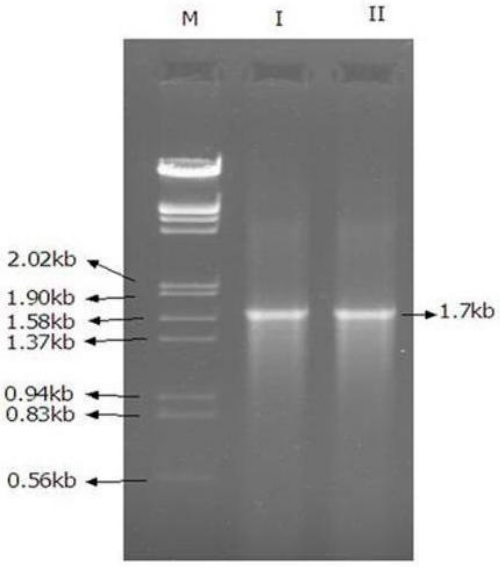

Plate_14_PCR amplification of gene encoding endochitinase in pGSY2 in E. coli DH5α

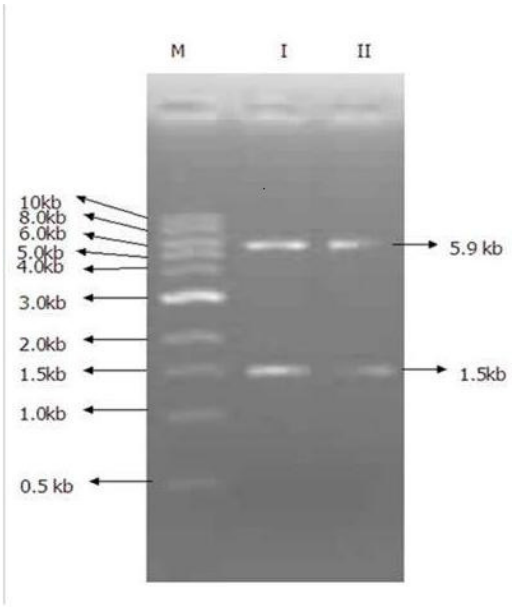

Plate_15_Restriction analysis of pGSY1 in E. coli DH5α

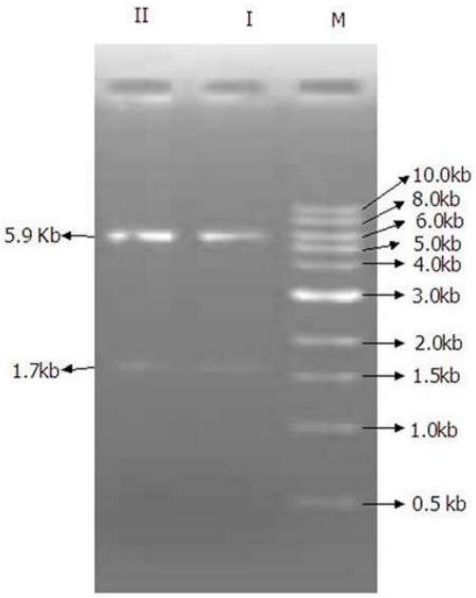

Plate_16_Restriction analysis of pGSY2 in E. coli DH5α

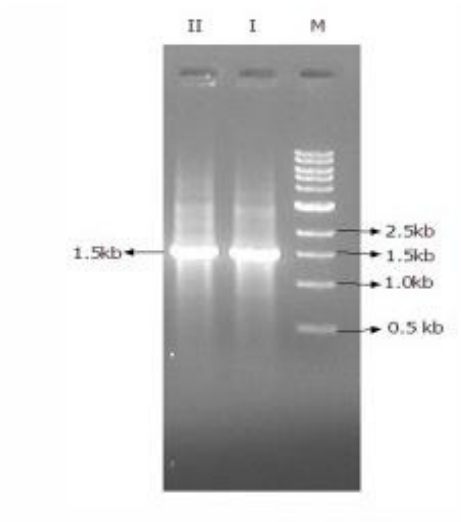

Plate_17_PCR confirmation of gene encoding endochitinase in pGSY1 In yeast

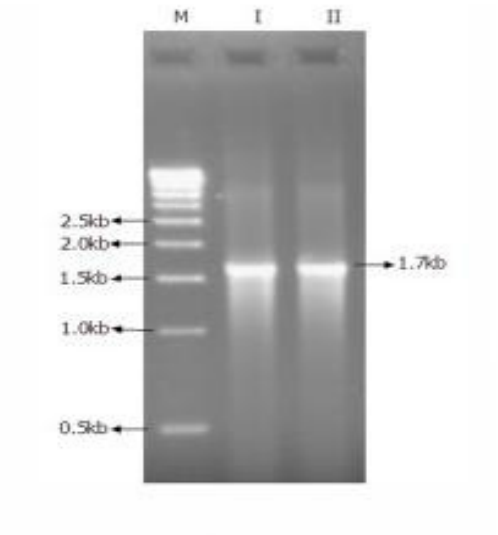

Plate_18_PCR confirmation of gene encoding endochitinase in pGSY2 in in yeast

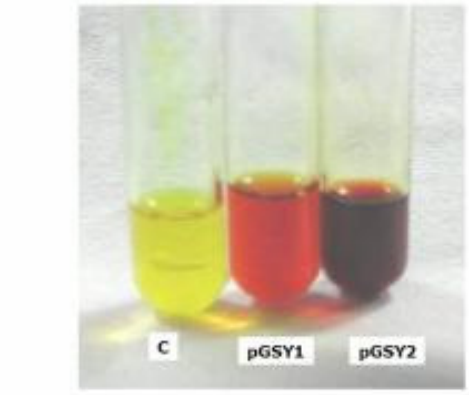

Plate_19_Confirmation of expression of cloned endochitinase gene using DNSA method

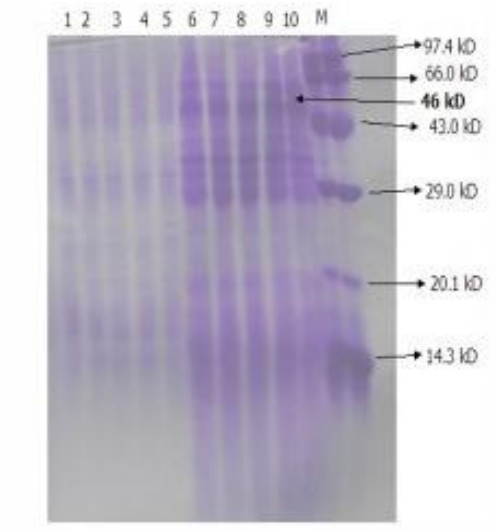

Plate_20_SDS-PAPE analysis of cloned endochitinase gene in yeast

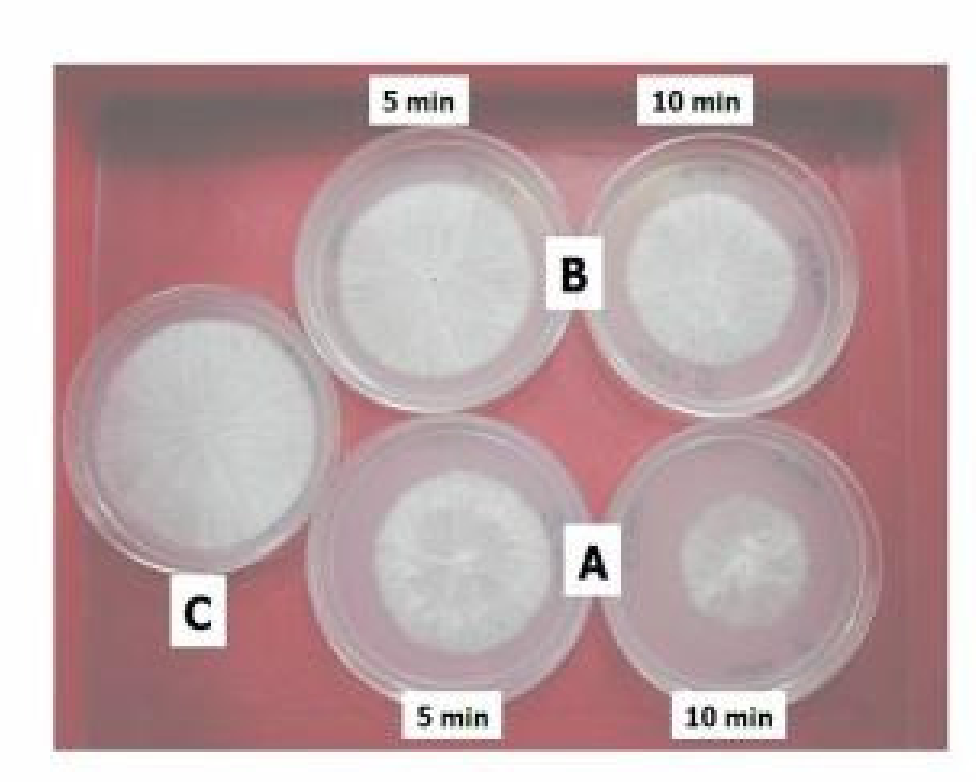

Plate_21_Growth inhibition of S. rolfsii on medium with cell lysate of recombinant S. cerevisiae

Table 11: Total protein and reducing sugar estimated from the cell lysate from pGSY1 and pGSY2

Clones	Protein concentration	Amount of reducing sugar (µg /100µg of total protein)
Control	0.69 µg/ µl	122.9
pGSY1	0.58 µg/µl	401.5
pGSY2	0.79 µg/ µl	1201.5

V. DISCUSSION

For the control of plant diseases, various chemicals are being used for many years. Indiscriminate use of such chemicals leads to the accumulation of toxic compounds which are potentially hazardous to human health and environment. Among these fungicides take major share. In addition, extensive use of chemicals including fungicides resulted in build up of resistance. To tackle the problem of diseases, integrated pest management (IPM) is being employed. IPM includes various cultural, biological and need based chemical practices to control the pathogens. The biological control has now become the heart of IPM for the control of plant diseases. The genus *Trichoderma* has become an integral part of IPM which helps to control the phytopathogenic fungi (Mrinalini and Lalithakumari, 1996). Since, direct use of *Trichoderma* as biocontrol agent has some of the drawbacks like low field performance (Graham and Sticklen, 1994) and adverse effect on VAM fungi (Wyss *et al.,* 1992; McAllister *et al*., 1994; Siddiqui and Mohamood, 1996), alternative approaches for exploiting mechanisms employed by *Trichoderma* were thought of. With the development of various genetic engineering techniques, genes encoding hydrolytic enzymes were cloned, characterized and transferred to plants to impart resistance against plant pathogens (Lorito *et al.,* 1998; Liu *et al*., 2004; Jyoti et *al.,* 2000; Shah *et al*., 2005; Upendra, 2006). Since the different species of *Trichoderma* and isolates of same species differ in their biocontrol potential and chitinase activity, isolation and characterization of *Trichoderma* from different places is important. Therefore, in the present investigation an attempt was made to isolate *Trichoderma* spp. from different soil samples, to screen them for chitinolytic activity and to clone full length endochitinase genes. Further attempts were also made to study the expression of cloned gene in yeast.

In the present study, 44 isolates were from 48 soil samples collected from Western Ghat. Of these 17 belong to *T. harzianum*, five belongs to *T. pseudokoningii,* one belongs to *T. hamatum,* one *T. viride* and 20 were unidentified *Trichoderma* spp. These isolates were added to the culture collection at the Department of Biotechnology. Instead another set of isolates were used for the study.

One of the ways to screen *Trichoderma* species for their biocontrol potential is to screen for their chytinolytic activity. Among the various tests, estimation of reducing sugar released and fluorescence assay has widely been used for screening *Trichoderma* for chitinolytic activity. Luis and Ray (2004) used the glycol chitin plate assay to detect the chitinase produced after induction with acibenzolar-S-methyl at the rate of 20 ppm in cucumber. In this study, this technique was used for thirty three isolates. Differences were observed among different isolates with respect to chytinolytic activity. Similar differences among the *Trichoderma* isolates for chytinolytic activity have been reported by others (Kovacs *et al*., 2004; Aishwarya, 2004; Umamaheswari and Sankaralingam, 2005). For screening, culture filtrate was taken 24, 48, 72 and 96 hours after induction with colloidal chitin. In all the isolates chitinase production started within 24 hours of induction, but maximum production reached within 48 hours. Therefore, culture filtrate taken after 48 hours of induction can be used for routine screening of the *Trichoderma* isolates using glycol chitin plate assay. In most of the isolates, it remained stable up to 96 hours of induction. However in few isolates (IABT1010, IABT1027, IABT1002, IABT1006) chitinase production decreased after 72 hours. In addition, three isolates (IABT1005, IABT1017, and IABT1023) did not show any hydrolysis zone on glycol chitin plate and they were also very slow in their growth. Upendra (2006) screened the same set of isolates against three different pathogens *Rhizoctonia bataticola, Fusarium solani* and *S. rolfsii.* In this study glycol chitin plate assay was compared with the dual culture assay. In general, isolates which produced good zone of hydrolysis on glycol chitin plate assay also inhibited growth of the *R. bataticola, F. solani* completely. However, in some of the isolates though complete growth inhibition of the pathogen was observed, in

glycol chitin plate assay they were found to be moderate chitin degraders. This may be because, *Trichoderma* uses different mechanisms for the control of the plant pathogens (Wafaa, 2002, Howell, 2000).

Further, a full length endochitinase gene from *T. viride* (IABT12 and IABT13) and *T. harzianum* IABT15 was amplified using a pair of specific primers designed for *ech46* gene. The resulting amplicon (1.6 from *T. viride* and 1.5kb from *T. harzianum*) from them were separately cloned into pTZ57R/T. The pGSK12 and pGSK15 had 95 per cent homology with reported endochitinase genes from *T. harzianum* (AB041752.1) at nucleotide level, while 99.3 per cent and 99 per cent at amino acid level respectively.

Both the genes are almost similar and the amino acid sequence differed only at four amino acids at four positions, viz., 32, 110, 279, 394 compared to reported endochitinase gene (Nakahara *et al*., 2002). Differences in amino acids at non critical positions were observed in many of the genes cloned so far.

There was only one amino acid difference between the translated protein sequence of pGSK12 and pGSK15 at 80^{th} position and three differences were observed at nucleotide level and all the three were at non-critical region. The percent similarity was 98.1 between pGSK12 and pSUM1C (previously cloned endochitinase in the laboratory by Upendra, 2006) and 97.9 between pGSK15 and pSUM1C. The endochitinase gene in pGSK13 and pGSK12 has 99.8 per cent identity at amino acid level but *T. viride* IABT1013 is more efficient chitin degrader compared to *T. viride* IABT1012 but there is no significant difference in protein level. This may be because *Trichoderma* has many forms of chitinases other than *chit46.* Further expression of genes encoding chitinase is regulated by different factors.

The sequence pGSK12 and pGSK15 have four exons with three small introns and code for 430 and 429 amino acids with a molecular weight of 46379Da and 46280Da respectively, and theoretical pI of 5.78. Both have a conserved domain of glycosyl hydrolases family 18. The cloned endochitinase gene appears to be an extra cellular chitinase as it has a predicted N-terminal signal peptide and signal cleavage site (after Ala_{22} Ser_{23}).

Both the genes, showed high homology with *T. harzianum* endochitinase gene, though they are cloned from two different species one from *T. viride* and another from *T. harzianum*. Similar observations were made by Latha *et al*. (2002), wherein *T. koningii* was exactly identical to the *T. hamatum* isolates present in Indian type culture collection when tested through RAPD primers. The cloned genes have active site (FDGIDVDWE) and a putative chitin binding domain (XXXSXGG).

In order to validate the expression of the endochitinase gene cloned from *T. virens* (with and without upstream sequence) (pSUM1C and pGSK01) was transferred to yeast expression vector pYES2/CT, under inducible *GAL*1 promoter. The gene was cloned into pYES2/CT at *Bam*H I and *Xba* I sites and then transferred to yeast. To induce the expression of cloned gene, protocol given in the supplier's manual was not effective. The protocol of Song *et al*. (2005) with minor modifications gave good expression.

Four different tests such as substrate conversion SDS PAGE, assay for the chitinase activity in the gel and bioassay against *S. rolfsii* were carried out. Initially, the cell lysate was used as a crude enzyme source and assayed for the reducing sugar released by degradation of glycol chitin. There was 9.8 and 3.3 times higher activity in the pGSY2 and pGSY1 (in which extra length was removed) respectively, than the control. It indicated the expression of functional protein.

Bioassay against the target pathogens provides the final proof for the activity of the protein and decides the usefulness of the cloned gene for the development of transgenics. Bioassay with transformed yeast cell lysate against *S. rolfsii* was observed up to 72 hours in both the test than the control. However, even in the control (yeast with pYES2/CT) inhibition was observed to certain extent. This may be because yeast host also has genes for chitinase, which is required for cell separation (Michael and Phillips, 1991). The higher expression in pGSY2 can be attributed to the length of 5' UTR. The pGSY2 has 173 bp extra in the 5' UTR which is absent in pGSY1. In similar study Akio *et al*. (2004) reported increased *gus* gene activity when length of 5'UTR region was increased. However increase in the GUS activity relatively decreased when 5'UTR length was increased from 69 to 174 bp. Further, Tomoko *et al*. (2003) inserted different length of *Arabidopsis thaliana* heat shock protein (HSP) 5' UTR between CaMV 35S promoter and *gus* gene. Among different lengths tried, 5'UTR with 116bp showed 10 fold higher activities than the control. Akio *et al*. (2004) also reported that presence of A at -3 position (upstream to AUG) and C at -4 position were important for recognition of AUG codon by the ribosome. In this study, clone pGSY1 has A at -3 position but T at -4 position in stead of C. This might have caused the reduction in the expression of the gene.

In this study, three efficient chitin degrading *Trichoderma* isolates were identified which can be used themselves as biocontrol agent and for isolating efficient chitinase genes. Three genes were cloned from two different *Trichoderma* spp. and it is necessary to check their expression in yeast and then in plant. Endochitinase gene which was expressed in the yeast need to be transferred to model plant for validation and to generate transgenics in the target crop plant.

VI. SUMMARY

Several species of *Trichoderma* have been extensively studied for their ability to control different fungal plant pathogens. Endochitinase gene present in *Trichoderma* spp. is well known for its antifungal activity and proved effective for the control of plant pathogens. In the present study, an attempt was made to isolate *Trichoderma* spp. from different soil samples, to screen different *Trichoderma* isolates for chitinolytic activity, to clone full length endochitinase gene from a *Trichoderma* spp. and to study their expression in yeast. The results obtained are summarized below:

- The 44 *Trichoderma* isolates were obtained from 48 soil samples collected from Western Ghat. Of these 17 belongs to *T. harzianum*, other isolates include *T. pseudokoningii, T. hamatum* and 20 unidentified *Trichoderma* species, which were given IABT accession numbers.

- Among the *Trichoderma* spp. tested for chitinolytic activity, *T. virens* IABT 1010, *T. koningii* IABT1016 and *T. polysporum* IABT1018 were found to be more efficient producers of chitinase enzyme with in 24 hours when induced with colloidal chitin.

- The endochitinase gene was amplified from two *T. viride* isolates (IABT-1012 and 1013) and T*. harzianum* IABT1015 using specific primers, and the amplicons were cloned in pTZ57R/T and transferred into *E. coli* DH5α.

- Recombinant plasmids pGSK12, pGSK13 and pGSK15 had the insert of endochitinase gene from *T. virens* isolates IABT1012, 1013 and *T. harzianum* (IABT1015), respectively. Genes in their clone were sequenced using primer walking method.

- The extra length present upstream to the coding region of endochitinase present in pSUM1C was removed using specific primer and was cloned into pTZ57R/T and named as pGSK01.

- Cloned endochitinase gene from *T. viride* isolate IABT1012 (pGSK12) and *T. harzianum* isolate IABT1015 (pGSK15) have a length of 1668bp and 1551 bp respectively. Both the gene contains four exons and three internal introns. Endochitinase from IABT1012 showed 95 per cent and 99.3 per cent homology with reported *T. harzianum* chit-*HAR2* gene for endochitinase (AB041752.1) at nucleotide and amino acid levels, respectively. It codes for 430 amino acids with a molecular weight of 46379Da and theoretical pI of 5.78. Endochitinase from IABT1015 showed 95 per cent and 99 per cent homology with reported *T. harzianum* chit-*HAR2* gene for endochitinase (AB041752.1) at nucleotide and amino acid levels, respectively. It codes for 429 amino acids with a molecular weight of 46280Da and theoretical pI of 5.78. Both the genes have conserved domain of glycosyl hydrolases family 18, conserved catalytic and potential chitin binding domains.

- Clones pGSK12 and pGSK15 showed 99.8 per cent similarity and differed only for one amino acid at 80th position.
- Cell lysate from recombinant yeast (taken after three days of induction with galactose) was used for substrate conversion assay. The pGSY2 clone showed 9.8 times more conversion compared to control. Another clone pGSY1, where upstream sequences were removed, showed 3.3 times more conversion than the control.
- Cell lysate from the yeast clones showed the inhibition of growth of test organism, *S. rolfsii* up to 72 hours.

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Appendix I

Media recipes

A) 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

B) Luria agar

Ingredients	Concentration (g/l)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	18.0
pH	7.2

C) Mandels and Reese (1965)medium:

COMPONENTS	ml/l	(mg/l)
(NH)2SO4(10%)	14	0.14
KH2PO4(1M)	15	0.02
UREA(10%)	3	0.03
CaCl2(10%)	3	0.03
MgSO4.7H2O(10%)	3	0.03
TRACEMETAL STOCK	1	
TWEN 80(OPTIONAL)	2	0.02
DW	1000	

TRACE METAL STOCK

FeSO4(2.5 g)	1.0mg
MnSO4.H2O	0.5mg
ZnSO4(0.83mg)	0.80mg
CoCl2(1.0mg)	0.50mg
HCl concentrated(5ml)	5ml
DW	495ml

pH of the media adjusted to 5.5.

APPENDIX-II

Agarose gel electrophoresis

A) Loading dye composition(6X)

0.25% Bromophenol blue (BPB)

0.25% xylene cyanol (optional)

30% glycerol in water

B) Ethidium bromide

10 mg/ml in distilled water. Stored at 4℃ in dark bottle.

C) Recipe for 1 per cent Agarose gel (40 ml)

Agarose	400 mg
1x TAE	40 ml
EtBr (10 mg/ml)	2 µl

D) 50x TAE composition

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Total volume 1000 ml with double distilled water.

APPENDIX-III

Ligation recipes

A) Conversion table for the amount of a PCR fragment required per ligation reaction

Length of DNA fragment (bp)	picomoles of ends per 1 µg of DNA	Quantity of PCR fragments for ligation reacting in µg (0.54 pmol ends)
100	30.0	0.018
300	10.0	0.054
500	6.0	0.090
1000	3.0	0.180
2000	1.5	0.360
3000	1.0	0.540

B) Ligation reaction recipe

Plasmid vector pTZ57R/T DNA (0.165 µg, 0.18 pmol ends)	3.0 µl
Purified PCR fragment, (Approx. 0.54 pmol ends)	10.0 µl
10x ligation buffer	3.0 µl
PEG 4000 solution	3.0 µl
Deionized water	10.0 µl
T4 DNA ligase, 5U	1.0 µl
Total	30 µl

C) Control ligation reaction recipe

PTZ57R/T DNA (0.165 µg, 0.18 pmol ends)	3.0 µl
Purified PCR fragment (Approx. 0.54 pmol ends)	12.3 µl
10x ligation buffer	3.0 µl
PEG 4000 solution	3.0 µl
Deionized water upto	29 µl
T4 DNA ligase, 5 U	1.0 µl
Total	30 µl

APPENDIX-IV

Selection Plate recipes

Luria agar Amp_{50}	To 100 ml Luria agar 50 µl of Amp_{100} (antibiotic) was added at 50℃
Luria agar Amp_{100}	To 100 ml Luria agar 100 µl of Amp100 (antibiotic) was added at 50℃.
IPTG (200mg/ml)	200mg of IPTG dissolved in 1 ml of sterile water, filter sterilized and stored at 0^0C 5ul/ plate was used.
X-gal solution (20mg/ml)	20mg of X-gal dissolved in 1ml of N,N-dimethyl formamide. Stored at 00C. 40ul/plate was used.

APPENDIX-V

Reagents for plasmid isolation

STET buffer

Tris-Cl (pH 8.0)	10 mm
NaCl	0.1 M
EDTA (pH 8.0)	1.0 mM

Autoclaved and stored at 4℃

Alkaline lysis solution I

Glucose	50 mM
Tris-Cl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM

Autoclaved and stored at 4℃

Alkaline lysis solution II

NaOH	0.2 N
SDS	1% (w/v)

(Prepared fresh and used at room temperature)

Alkaline lysis solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Double distilled water	28.5 ml

Autoclaved and stored at 4℃

APPENDIX- VI

Yeast growing media

A) YPD medium for yeast.

Yeast extract	10 g
Peptone	20 g
Dextrose	20 g
Water	1000ml

If making the plate, Dextrose is added while pouring the plate, otherwise heating the agar with dextrose causes the dextrose to caramelize. Prepare the 20 per cent dextrose stock and add 100 ml to a liter of the medium.

B) Synthetic minimal medium for the yeast

Yeast nitrogen Base with ammonium sulphate	6.7g
Carbon source (Dextrose or Raffinose or Galactose)	20g
Amino acids	
A. Adenine, arginine , cysteine, leucine, lysine, Threonine, Tryptophan,	0.1 g
B. Aspartic acid, histidine, isolucine, methionine, phynylalanine, proline, serine, tyrosine, valine	0.05g
Agar agar Type I	20g
Water	Made up to 100 ml

APPENDIX-VII

Yeast transformation

A) 10X TE buffer

Tris (pH 7.5)	100mM
EDTA	10mM

B) 10X Lithium acetate (LiAc)

For 100 ml, dissolve 10.2g of lithium acetate in 90ml of deionized water. Then adjust the pH to 7.5 with dilute glacial acetic acid and made up the volume to 100 ml. Filter sterilize it and stored at room temperature

C)1X LiAc/ 0.5 X TE

Lithium acetate (pH 7.5)	100mM
Tris Cl (pH 7.5)	5mM
EDTA	0.5mM

Filter sterilized and stored at room temperature

D) 1X LiAc/ 40 % PEG-3350/1X TE

Lithium acetate (pH 7.5)	100mM
PEG 3350	40%
TrisCl (pH 7.5)	10mM
EDTA	1mM

Filter sterilized and stored at room temperature

APPENDIX-VIII

Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS PAGE)

A) Acrylamide solution (30 per cent)

Acrylamide	29 g
Bis acrylamide	1g

Made up the volume to 100 ml using the distilled water and stored at $4^{O}C$ (Do not autoclave).

B) 4X resolving gel buffer (pH 8.8)

Tris Cl (1.5 M)	182.0 g of Tris base was dissolved in little amount of water and pH was adjusted to 8.8 using 1M HCl and final volume was made up to 1000ml.

C) 4X stacking gel buffer (pH 6.8)

Tris Cl (1.5 M)	182.0 g of Tris base was dissolved in little amount of water and pH was adjusted to 6.8 using 1M HCl and final volume was made up to 1000ml.

D) Ammonium per sulphate (APS) 10%

(Add the preparation) It has to be prepared fresh, Don't autoclave.

E) Tank Buffer

Tris Base	25 mM
Glycin pH 8.3	250 m M
SDS	0.1 per cent

F) SDS PAGE Gel

	Resolving gel	Stacking Gel
	12 %	5 %
Water	13.2 ml	6.8 ml
30 % Acrylamide mix	16.0 ml	1.7 ml
1.5 M Tris (pH 8.8)	10.0 ml	1.25 ml
10% SDS	0.4 ml	0.1 ml
10 % APS	0.4 ml	0.1 ml
TEMED	0.016 ml	0.01 ml
Total	40 ml	10 ml

G) Sample loading dye for SDS PAGE (for 2 ml)	
Tris-Cl (100mM, pH 6.8)	200µl
DTT (200mM)	100 µl
Glycerol (20 %)	400µl
Bromo Phenol Blue	4mg
SDS (4 %)	800µl
Deionized water	Made up to 2ml

H) Staining solution (For one liter)	
Commasine Brilliant blue	2.5mg
Methenol	500ml
Acetic acid	100ml
Distilled water	400ml

I) Destainig solution (for one liter)	
Methenol	300ml
Acetic acid	100ml
Distilled water	600ml

APPENDIX- IX

Preparation of calcofluor white M2R

Fifty mg of calcofluor white M2R was dissolved in the sodium citrate buffer and it gives the concentration of 0.1 %. This was diluted to 10 times using Tris-Cl (pH 8.9) for stopping the chitinase mediated reaction

APPENDIX-X

Fungus DNA Extraction Buffer

Tris-Cl	200mM
Sodium chloride	250mM
EDTA	25mM
SDS	0.5 %

APPENDIX-XI

Yeast Total DNA extraction buffer

Tris-Cl	10mM
Sodium Chloride	100mM
Triton X-100	2 %
EDTA	1mM
SDS	1%

APPENDIX-XII

Breaking buffer for yeast cell lysis

Sodium phosphate buffer (pH 7.4)	50mM
EDTA	1mM
Glycerol	5%
PMSF	1mM

CLONING AND FUNCTIONAL CHARACTERIZATION OF ENDOCHITINASE GENE FROM *TRICHODERMA* SPP.

Ganapati Bhat 2007 Major Advisor
Sumangala Bhat

ABSTRACT

The present study was conducted to isolate *Trichoderma* spp. from different soil samples, to screen them for chitinase activity using glycol chitin plate assay, to clone full-length genes encoding endochitinase from *Trichoderma* species and expression of cloned endochitinase gene in *Saccharomyces cerevisiae.* During the study 44 *Trichoderma* isolates were obtained from 48 soil sample collected. Among the thirty three isolates tested for chitinase activity (glycol chitin plate assay), isolates *T. virens* IABT 1010, *T. koningii* IABT1016 and *T. polysporum* IABT1018 were efficient producers of chitinase enzyme. Further, using specific primers, genes encoding endochitinase *chit46* (1.6kb) from *T. viride* and *T. harzianum* (1.5kb) were cloned into pTZ57R/T vector. The clones were confirmed through PCR amplification and restriction analysis. The clones were sequenced and analyzed for homology at nucleotide and protein level to find out conserved domain of protein. Genes encoding endochitinase from both the species have 95 % and 99.5% homology with reported sequence both at nucleotide and protein level. Phylogenetic analysis of cloned genes indicated that both the genes fall in same cluster and closely related to *T. harzianum* endochitinase gene. Endochitinase gene cloned from *T. virens* (cloned earlier) was expressed in *S. cerevisiae* using pYES2/CT vector. Substrate conversion assay using cell lysate indicated 9.8 times more activity in recombinant clones compared to control. Further, cell lysates of recombinant clones significantly inhibited the growth of *Sclerotium rolfsii.* In addition, reduced expression of the same gene was observed when 173bp upstream sequence was removed.