CLONING AND CHARACTERIZATION OF CHITINASE GENE/S FROM NATIVE ISOLATES OF *Serratia marcescens*

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I. INTRODUCTION

One of the main challenges of the first half of this millennium will be the production of food and feed in quantities, large enough to feed the ever-increasing world population. To feed the entire world population and improve their health in the future there is a clear need to improve productivity and nutritional quality of food crops. This may be accomplished by the development of cultivars of crops better adapted to the conditions they are grown in, effective control of insects pests and diseases, and enhanced nutritional quality. Crop diseases cause a yearly loss to the extent of up to 12% of agricultural production, worldwide (James *et al*., 1991).

Unfortunately, the most important crop plants are still susceptible to a number of important fungal pathogens and conventional breeding programs have partly succeeded in introducing suitable traits into these cultivars. Hence, finding new ways to create crop plants resistant to phytopathogenic fungi is a major challenge and genetic engineering of plants provides a possible means to reach this goal (Guido *et al*., 1992).

Investigations studies on the lytic activity among biocontrol agents have focused largely on the characterization of enzyme systems capable of degrading fungal cell wall components, of which chitinases are among the most intensively studied (Chernin *et al*., 1997; Inbar and chet, 1991; Ordentlich *et al*., 1988 ; Zhang and Yuen, 2001).

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine. Chitinases are of great biotechnological interest. Firstly, these enzymes may be used to convert chitin-containing biomass into depolymerised components. Secondly, chitinases may be exploited for the control of fungal and insect pathogens of plants (Kramer and Muthukrishnan, 1997; Roberts and selitrennikoff, 988; Melchers and Stuiver, 2000) as they can inhibit growth of chitin-containing plant-pathogens and plague insects that need chitins for normal development (Spindler and Spindler-barth, 1996).

Genetic engineering offers the potential to improve elite cultivars for individual traits such as disease resistance. Resistance to fungal pathogens is highly desirable. Chitinases from the chitinolytic bacteria such as Serratia *marcescens* are endolytic enzymes that solubilize chitin more rapidly than the exolytic enzymes and could therefore, be more efficient in controlling fungal diseases of crop plants (Monreal and Reese, 1969).

The addition of chitin to soil has been shown to reduce populations of fungal plant pathogens (Mitchell and Alexander, 1962) and plant parasitic nematodes by increasing populations of chitinolytic bacteria, especially actinomycetes and fungi. Increase in such microbes are correlated with reductions in pathogenic fungi and nematodes (Mankau and Das, 1969).

Several chitinolytic bacteria and fungi, viz, *Serratia marcescens, Aeromonas caviae, Enterobacter agglomerans* and *Trichoderma harzianum* have been shown to be potent biological control agents (BCA's) protecting plants against pathogenic fungi (Ordentlich *et al*., 1988). *S. marcescens* is an active producer of chitinases and an excellent model for studying the degradation and utilization of chitin (Ferrer *et al*.,1996; Palomar *et al*., 1990; Regue *et al*., 1991). Transgenic tobacco plants expressing high levels of *S. marcescens ChiA* exhibited increased tolerance to *R. solani* compared to untransformed control plants (Howie *et al*., 1994).

In the light of the above background, this research programme focused mainly on;

1. Cloning of chitinase genes from native *S. marcescens* and
2. Expression of the cloned chitinase in *E. coli* and tobacco.

II. REVIEW OF LITERATURE

The present research envisaged cloning of genes encoding chitinases from native isolates of *Serratia marcescens* and analysis of their expression in *E. coli* and tobacco.

Significant yield losses occur due to fungal diseases attacks occuring in most of the agricultural and horticultural species. In the Indian context, fungal diseases cause major yield losses in cereal, pulse and oilseed crops. On the basis of a recent survey (Grover and Pentel, 2003), one of the challenges facing breeders during the development of improved crop cultivars has been the incorporation of resistance to diseases.

Breeding for disease resistance in crop plants by conventional methods is time consuming and cumbersome. It has to be a continuous process, as new races of pathogens continue to evolve and crops loose resistance. Breeders have been successful in protecting some of the major crops grown around the world from fungal diseases. Although it is shown genes for resistance to disease have been incorporated from wild species, be possible, wide hybridization programmes face numerous difficulties. The sexual crosses are difficult to make and genetic exchange in the hybrids is poor due to low frequency of pairing between chromosomes of crop species and alien species. Problems can also arise due to linkage drag, which lower the yield of the crop variety. For example, *Lr9 R* gene from *Aegilops umbellulata*, which confers resistance to wheat brown rust was shown to be linked with yield depression (Ortelli, *et al*., 1996). Similarly *Wsm1* conferring resistance against wheat streak caused yield reduction of up to 21 percent (Sharp *et al*., 2002). The most significant development in varietal development for disease resistance has been the use of the techniques of gene isolation and genetic transformation to develop transgenics resistant to fungal diseases.

2.1 STRATEGIES TO CONTROL DISEASE

Various methods used in disease control are classified as regulatory, cultural, biological, physical and chemical, depending on the nature of the agents employed. The disease control mechanisms currently used to prevent and control fungal infections mostly involve repeated spraying of chemicals combined with the labor-intensive practices of canopy management. The use of chemicals is environmentally unfriendly and very expensive. Building up of resistance in the pathogen population is always a concern. Moreover, the application of chemicals is becoming increasingly undesirable due to consumer insistance on healthier and more naturally produced foods. Furthermore, the long-term adverse effects of agricultural chemicals on the environment are well-known and it is generally accepted now that the agricultural community should scale down their dependence on these products. In the last decade, there has been increasing interest in transgenic approach to control diseases.

2.1.1 Biocontrol agent

Any disease control involving antagonists is biological control. Baker and Cook (1974) defined biological control as “reduction of inoculum density or disease producing activities of a pathogen or parasite in it’s active or dormant stage by one or more organisms, accomplished naturally or through manipulation of host environment or antagonist or by mass introduction of one or more antagonists.” Further, Cook and Baker (1983) shortened this definition to “biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man.” Several classic reviews with exhaustive literature on biological control of plant pathogens are available (Adams, 2004 ; Datar, 1982).

Pesticides are widely used to control plant diseases. However, most pesticides are hazardous to the health of humans and animals. They also have deleterious effects on ecological systems. Biological control promises to be a useful alternative approach in the control of plant pathogens (Weller *et al*., 1988). Many factors have to be considered in deciding whether a biological system is feasible for control of a particular pathogen.

Availability of a suitable microorganism capable of maintaining itself on the host plant in case of leaf or stem diseases, persistence at an effective population level in the ecosystem where the crop is grown, a highly competitive ability in relation to other soil or plant microflora are some of the essential factors for the biological control agent to be effective against any disease. Mere ability to control pathogen *in vitro* is not enough, it has to be effective against pathogens *in vivo* (Mukerji and Garg, 1988).

2.1.1.1 Bacterial antagonists

The lytic activity of bacteria is one of the mechanisms that has been implicated in biocontrol for several years (Chernin *et al*., 1995; Lim *et al*., 1991; Mavingui and Heulin 1994 ; Mitchell and Hurwitz 1964). Investigative studies on lytic activity among biocontrol agents have focused largely on the characterization of enzyme systems capable of degrading fungal cell wall components, of which chitinases are among the most intensively studied (Chernin *et al*., 1997; Inbar and Chet, 1991; Jones *et al*., 1986; Ordentlich *et al*., 1988 ; Zhang and Yuen, 2000; Zhang and Yuen, 2001). Bacteria are extremely important in biological control of plant pathogens. They grow rapidly and utilize different forms of nutrients (Baker and Cook 1974). Migula (1999) used *P. fluorescens* in sorghum charcoal rot control and concluded that, it was next best to *Trichoderma* spp. Significant inhibition of growth of *M. phasiolina* by B*. subtilis* has been observed under *in vitro* conditions (Mukherjee and Garg, 1988). Kobayashi *et al*., (1995) identified *Stenotrophomonas maltophilia* strain 34S1 was identified as a biocontrol agent for summer patch disease of Kentucky bluegrass (*Poa pratensis*) caused by the root-infecting fungus *Magnaporthe poae*.

Inbar and Chet (1991) isolated a strain of *Aeromonas coviae* from soil, which showed high level of chitinolytic activity and considered it as a potential biological control agent. Sundheim *et al.* (1988) have shown that a chitinase produced by gram-negative bacterium, *S. marcescens* inhibited the growth of a plant pathogenic *Fusarium* spp. and reduced the disease caused by the same fungus.

2.1.1.2 *Serratia marcescens* as biocontrol agent

S. marcescens is known to be a potential agent for the biological control of plant diseases caused by various phytopathogenic fungi, whose cell walls contain chitin as a major structural component (Ordentlich *et al*., 1988). *S. marcescens* chitinases and genes encoding them have been shown to have biocontrol potential in a variety of experiments. A highly chitinolytic strain of *S. marcescens* was found to suppress the growth of *Botrytis* sp. *in vitro* (Someya *et al*., 2000). In the greenhouse setting, *S.marcescens* was shown to control *B. cinerea*, *Rhizoctonia solani*, and *Fusarium oxysporum* (Someya *et al*., 2000). Similarly, S. *marcescens* controlled the growth of *Sclerotinia minor*, the casual agent of basal drop disease of lettuce grown in green house (El Tarabily *et al*., 2000).

The *chiA* and *chiB* genes from *S. marcescens* have been transferred to other bacterial species like *Pseudomonas fluorescens* and *E.coli* in an attempt to improve their ability to control fungal plant pathogens (Sundheim *et al*., 1988; Koby *et al*., 1994 ; Downing and Thomson 2000) or to create new biocontrol agents (Oppenheim and Chet, 1992 ; Shapira *et al*., 1989).

Partially purified *chiA* protein produced in transgenic *E. coli*, was found to reduce disease caused by *Sclerotium rolfsii* in beans and *Rhizoctonia solani* in cotton (Shapira *et al*., 1989). When *ChiA* was combined with *Bacillus thuringiensis* or with low concentration of the *B. thuringiensis* delta-endotoxin, a synergistic toxic effect was seen on insect larvae (Regev *et al*., 1996 ; Sampson and Gooday, 1998). Akutsu *et al.,*(1993) reported that a highly chitinolytic *S. marcescens* B2, isolated from tomato phylloplane, suppressed the growth of *Botrytis* spp. *in vitro* and controlled broad bean chocolate spot, caused by *B. fabae*, in a growth chamber (Iyozumi *et al*., 1993 ; Yamamoto *et al.*,1992) and cyclamen gray mold, caused by *B. cinerea,* in a greenhouse (Iyozumi *et al*., 1996).

Chitin-supplemented application of *B. circulans* GRS 243 and *S. marcescens* GPS resulted in improved control of *LLS* compared with application of bacterial cells alone. Both these strains are chitinolytic and inhibited the germination of conidia of *Cercospora arachidicola*, *P. personata* and urediospores of *Puccinia arachidis* in a controlled environment (Kishore *et al*., 2005). Suppression of damping off in cyclamen

plants was observed when plants were treated with a highly chitinolytic *S. marcescens* strain B2 (Someya *et al*., 2000). Biocontrol of *E. saccharina* involved combining *P. fluorescens* strains producing the Cry1Ac7 protein and a *S. marcescens* chitinase, *ChiA* (Katrina *et al*., 2000).

2.1.2 PR proteins and host plant resistance

Many defense mechanisms are triggered in plants in response to infection by plant pathogens. Among them, the production of pathogenesis-related (PR) proteins is well documented (Datta and Muthukrishnan, 1999) PR proteins in plant is correlated with resistance in a number of plant pathogen interactions (Van Loon, 1997). Hence, several attempts have been made to exploit these PR proteins to develop disease-resistant transgenic plants (Broglie *et al*., 1991 ; Grison *et al*., 1996). Among the PR proteins, chitinases belonging to the PR-3 group appear to be potential candidates for the management of fungal diseases (Anuratha *et al*., 1995 ; Liu *et al*., 1994). Chitinases inhibit fungal growth both directly and indirectly. They hydrolyze fungal cell walls which contain chitin, leading to fungal hyphal lysis and inhibition of fungal growth (Roberts and Selitrennikoff, 1988 ; Schlumbaum *et al*., 1986). The chitinases can also release elicitors from the fungal cell walls and these elicitors could induce various defense responses in plants (Ren and west, 1992). It has been demonstrated that constitutive, high-level expression of chitinases in transgenic plants can enhance resistance to a variety of pathogens (Broglie *et al*., 1991; Lin *et al*., 1995 ; Marchant *et al*., 1998 ; Tabei *et al*., 1997).

2.1.2.1 Breeding for resistance

Intra-specific transfer of genes is easily performed by hybridisation in all plants that can be propagated sexually. Transfer of genes by hybridization becomes more difficult or impossible with increasing phylogenetic distance. Recent developments in recombinant DNA has allowed transfer of DNA from any organism to the plant genomes; nuclear, mitochondrial or chloroplast. There is however, a vast difference between sexual crosses and genetic transformation. In sexual transfer a whole genome, or at least a chromosome or a chromosomal segment is transferred. Conversely, by genetic transformation, only a very short DNA fragment is transferred and integrated in the genome (Galun, 1993). Traditional approaches to control epidemic spread of diseases are no longer sufficient. Hence, the development of pathogen resistant plant genotypes has become an important target in plant biotechnology. Using molecular techniques, many genes for disease resistance genes have been isolated during the last few years. However, their use in the development of disease resistant crop cultivars is still limited.

2.1.2.2 Transgenics technology for disease resistance

Alternative strategies are required to supplement the conventional methods of plant improvement, particularly when the source of effective resistance is available in taxonomically unrelated species. The most significant development in the varietal development is the use of genetic engineering principles to improve elite cultivars for individual traits such as disease resistance. In the last decade, there has been increasing interest in transgenic approach to fungal disease resistance. The state-of the-art technology allows the transfer of individual genes into plants without altering their intrinsic properties (Kikkert *et al*., 1998).

2.1.2.3 Antifungal molecules

Till date, genes encoding many antifungal molecules which can inhibit fungal growth *in vitro* have been exploited to make fungus-resistant transgenic plants. Some of these molecules are: PR proteins, ribosome inactivating proteins, small cystein-rich proteins, lipid transfer proteins, storage albumins, polygalactourodinase inhibitor proteins (PGIPs), phytoalexins and non-plant antifungal proteins, which include the cell wall degrading enzymes like chitinases of biocontrol bacteria (Grover and pentel, 2003; Ordentlich *et al*., 1988; Jones *et al*., 1986).

2.1.2.4 Chitinases

Chitinases (EC 3.2.1.14) hydrolyse the β-1,4-linkages in chitin. Chitin is a highly stable homopolysaccharide of *β*(1→4)-linked *N*-acetyl-D -glucosamine (GlcNAc). The

second most prominent biopolymer in nature next to cellulose, it represents a major structural component of many agronomically important pests including insects, fungi, and nematodes (Bird and Mcclure, 1976). The enzymatic digestion or deformation of the chitin component of these organisms by chitinase could present an effective method for their control (Veldkamp, 1955).

The addition of chitin to soil has been shown to reduce populations of fungal plant pathogens and plant parasitic nematodes (Mankau and Das, 1969). Chitin application leads to increased populations of chitinolytic bacteria, especially actinomycetes and fungi. These increases are correlated with reduction in pathogenic fungi and nematodes, more importantly, they reduce the infectivity and damage to crops (Brown *et al*.,1999). Although the evidence for the role of chitinase in fungal and nematode control is indirect, the correlation is strong and suggestive. Highly purified chitinases are necessary to determine unequivocally determine their efficacy against fungi and nematodes (Mitchell and Alexander, 1962)

Proper selection of chitinase genes is very important for the development of transgenic plants with enhanced disease resistance. Elicitor-inducible antifungal chitinases may have a greater potential for effective protection against pathogens. Through plants do not contain chitin, however, many plants produce chitinases upon infection by fungi, bacteria, and viruses. Purified plant chitinases have been shown to inhibit fungal growth (Jones *et al*., 1986; Schlumbaum *et al*., 1986).

2.1.2.5 Classification of chitinases

The chitinases so far sequenced are classifed into two different families based on the amino acid sequence similarity of their catalytic domains. They form family-18 and-19 in the family classification system of glycoside hydrolases (Henrissat, 1991). Family 18 contains chitinases from bacteria, fungi, viruses, animals and some plant chitinases. On the other hand, family-19 contains only plant chitinases and the recently identified *Streptomyces griseus* chitinase C (Ohno *et al*., 1996). The chitinases of the two different families do not share amino acid sequence similarity, have completely different three-dimensional (3D) structures (Perrakis *et al*., 1994) and molecular mechanisms. they are therefore likely to have evolved from different ancestors.

Phylogenetic analysis has shown that bacterial family-18 chitinases can be clustered in three subfamilies, which have diverged at an early stage of bacterial chitinase evolution (Harl *et al*., 1995). *S. marcescens* chitinase C1 is found in one subfamily, whereas chitinases A and B of the same bacterium belong to another subfamily. Chitinase C1 is the only *S. marcescens* chitinase that has a B-type catalytic(Fn3) domain (Suzuki *et al*., 1999) and a fibronectin type III domain. Therefore, ChiC1 is structurally unique compared with the other two chitinases.

2.1.2.6 Mode of action of chitinase

Chitinases cleave the glycosidic bonds in the chitin chain by either an endolytic or an exolytic mechanism (Monreal and Reese, 1969). The two families contain both endochitinases, which randomly cleave in the chitin chain. The latter cleave off chitobiose (GlcNAc)2 from the reducing or the nonreducing end of the chitin chain (Monreal and Reese, 1969). In addition to endo and exochitinases, chitin degrading organisms contain chitobiases (*N*acetyl-β-glucosaminidases), a third class of chitinolytic enzymes that convert GlcNAc dimers into monomers (Tews *et al*., 1996).

The family-18 chitinases occur in bacteria, fungi, viruses and in animals. Chitinases of this family are classified as class III and V. They hydrolyze glycosidic bonds with the retention of anomeric configuration at C_1 atom (Kramer and koga, 1986). The catalytic domain of these chitinases have a fold of barrel with a catalytic groove as demonstrated by 3-D structural analysis of Hevamine (Kramer and Muthukrishnan, 1997). These chitinases catalyse the hydrolysis of Glc-N-Ac-Glc-N-Ac and Glc-N-Ac-Glc-N- linkages. These chitinases are inhibited by allosamidine, an isomer of N-acetyl glucosamine.

The family-19 chitinanse occurs in plants and *Streptomyces griseus* (Ohno *et al*., 1996). The chitinases of this family are classified as class I, II, and IV. They hydrolyze glycosidic bond with an inversion of anomeric configuration at C_1 atom (Stinizi *et al*., 1993; Broglie *et al*., 1991). The catalytic domain of these chitinases has a fold of high helical content and structural similarity, including conserved core of the enzyme (Girison *et al*., 1996). They catalyse the hydrolysis of Glu-N-Ac and Gluc-N-Ac linkages only. The activity of these chitinases is insensitive to allosamidine. They catalyse the hydrolysis of chitin similar to acid-base mechanism (Grison *et al*., 1996; DeSouza and murray, 1995). The conserved region of the catalytic domain of this family of chitinases resembles crystal structure of lysozyme. Two amino acid residues in the catalytic groove, Glu and Asp and the general acid base mechanism in chitin hydrolysis are similar in these chitinases (Terwisscha *et al*., 1996).

2.1.2.7 The biological role of chitin and chitin degrading enzymes

The biological role of chitin and chitin degrading enzymes is diverse. In many organisms, chitins play an important structural role, so do the chitinolytic enzymes. In most fungi, chitinases are closely associated with the cell wall and are proposed to have a role in the maintenance of cell wall plasticity (Rast *et al*., 2003; Adams, 2004). The functions of chitin and chitinases in insects are somewhat similar. Chitin is an important part of the exoskeleton and insect growth and morphogenesis depend on the capability of enzymes to remodel chitin-containing structures (Merzendorfer and Zimoch, 2003). Chitin and chitinases are also known to be important in budding of yeast (Kuranda and Robbins, 1991).

Other organisms, especially those not containing chitin themselves, use chitinases for other purposes. Plants use chitinases as part of their defence against chitin containing pathogens (Kasprzewska, 2003) and bacteria use chitin as a source of energy and need chitinases to utilize it. Genes for chitinase have also been identified in several viruses belonging to family baculoviridae, which is restricted to arthropod hosts (Hawtin *et al.,* 1995; Wang *et al.* 2004). Interestingly, chitinases from these viruses have strong homology with chitinases from *S. marcescens*, suggesting common origin (Hawtin *et al*., 1995; Wang *et al.* 2004). The role of chitinases in humans is not clear, but human acidic chitinase has proposed to have a role in the onset of asthma (Zhu *et al.,* 2004).

2.1.2.8 Structural characteristics of bacterial chitinase

Chitinases are members of the enzyme superfamily of glycoside hydrolases, which are characterized by the ability to hydrolyze glycosidic bonds. To date, the amino acid sequences of more than 12600 glycoside hydrolases have been identified. The enzymes are divided into more than 90 families based on the amino acid sequences of their catalytic domain (Henrissat 1991; Henrissat and Davies, 1997). These families are described in the CAZY database (http://afmb.cnrs-mrs.fr/CAZY/).

The catalytic domains of glycoside hydrolases belonging to a family have common folds and similar catalytic mechanisms. Still, family members can be very different with respect to the presence of additional domains, the architecture of the substrate binding-cleft and mode of interaction with the polymeric substrate (exo- or endoactivity) (Davies and Henrissat, 1995; Eijsink *et al*., 2003).

Bacterial chitinases generally consist of multiple functional domains, such as chitin-binding domains (ChBDs) and fibronectin type III-like domains (Fn3 domains), linked to the catalytic domain. The importance of the ChBD in the degradation of insoluble chitin has been demonstrated for some bacterial chitinases (Blaak and Schrevpf, 1995; Watanabe *et al*., 1994).

2.1.2.9 Bacterial chitinases

Some bacteria synthesise and secrete chitinases, following induction with chitin in the surrounding media (Monreal and Reese, 1969). Chitin, a polysaccharide, is particularly an important nutrient source for maintaining the marine ecosystem in the environment (Gooday, 1990). Chitinolytic marine bacteria play a critical role in the process of recycling continous

materials such as the exoskeleton of crustaceans and insects. If the insoluble form of chitin could not be returned to the ecosystem in a biologically usable form, the marine environment would be completely depleted of carbon and nitrogen source in a relatively short time (Yu *et al*., 1991). *Alteromonas* sp. strain O-7 is a gram-negative, flagellated, motile and aerobic rod shaped bacterium of marine origin, which is capable of chitinonlytic activity (Tsujibo *et al*., 1993). Similarly, chitinases were found in other marine bacteria like *Vibrio harveyi* (Svitil and Kirchman, 1998). Species of the genera *Serratia, Bacillus* and *Vibrio* have been reported to secrete several chitinolytic enzymes and chitin-binding proteins, which are thought to degrade chitin synergistically, in the extracellular environment (Bassler *et al*., 1991; Watanabe *et al*., 1997; Watanabe *et al*., 1994).

Nowani and Kapadnis (2003) reported the presence of chitin degrading potential in genera, *Streptomyces, Kitasatosporia, Saccharopolyspora, Nocardioides, Nocardiopsis,Herbidospora, Micromonospora, Microbispora, Actinoplaes, Serratia, Bacillus* and *Pseudomonas*. Their investigation formed a comprehensive base for the study of diversity of chitinolytic systems in bacteria.

2.1.2.10 Significance of bacterial chitinases over plant chitinases

Plants have been transformed with plant chitinase encoding genes as a means to alter their resistance to fungal pathogens, but no single plant chitinase gene has produced an adequate level of resistance. Reasons for this may be that (i) plant chitinases usually affect only the hyphal tip and are unable to effectively degrade harder chitin structures (ii) have weak antifungal activity alone (iii) are inhibitory only to a limited number of fungal species and (iv) have no effect on several important pathogens. Enzymes of *S. marcescens* are strong inhibitors of many important plant pathogens and the chitinases are able to lyse the hard chitin wall of mature hyphae, conidia, chlamydospores and sclerotia. They are more active than corresponding plant enzymes, effective on a much wider range of pathogens and nontoxic to plants at high concentrations. *S. marcescens* has evolved specifically to be capable of using other fungi but not plants as carbon sources, and as such represent a potential source of powerful antifungal genes. In terms of antifungal activity, many bacteria, including *Bacillus circulans*, *Streptomyces, lidans*, *Aeromonas* sp. and *S.marcescens* have been shown to produce multiple chitinases (Alam *et al*., 1996) and the efficient degradation of chitin is assumed to be achieved by the combined action of these chitinases. (Jones *et al*., 1986). *S. marcescens* was found to be the most active organism of the 100 tested for the production of chitinase. *Enterobacter liquefaciens* produced nearly as much enzyme. Under optimal conditions of pH and temperature, yields of chitinase were obtained in 4-6 days (Monreal and Reese,1969; Reid and David, 1981).

2.1.2.11 Chitinase of *Serratia marcescen*

The Gram-negative bacterium *S. marcescens* secretes a variety of extracellular enzymes including chitinases (Hines *et al*., 1988). It is one of the most effective bacteria for degradation of chitin (Monreal and Reese, 1969). When this bacterium is cultivated in the presence of chitin, a variety of chitinolytic enzymes and chitin-binding proteins can be detected (Fuchs *et al*., 1986). The precise number of different enzymes is somewhat difficult to determine on the basis of biochemical studies only, since some of the enzymes occur in multiple forms, on SDS-PAGE gel (Fuchs *et al*., 1986). Further chitinases have a multi-domain structure, which makes them sensitive for partial proteolytic degradation. Detailed studies by a number of groups clearly show that *S. marcescens* produces at least three chitinases (ChiA, ChiB, ChiC), a chitobiase and a putative chitin-binding protein (CBP21) (Brurberg *et al*., 1996) as listed in the following table. They however were not certain that these five proteins represented the complete chitinolytic machinery of the bacterium. The chitinolytic activity of *S. marcescens* is of great interest because it is one of the best-characterized chitinolytic machineries known. Recently determined crystal structures of *ChiA* (Perrakis *et al*.,1994), *ChiB* and the chitobiase (Tews *et al*., 1996) provide detailed insight on how a natural set of chitinolytic enzymes may be built up. *S. marcescens* 2170, an

active producer of chitinase (Ferrer *et al*., 1996; Palomar *et al*., 1990;Regue *et al*., 1991) is an excellent model for studying the degradation and utilization of chitin (Palomar *et al*., 1990).

Chitinases and CBP from *S. marcescens*

SDS-PAGE band (kDa) [1)]	Gene (protein name)	Localization in *S. marcescens*[3)]	N-terminal signal peptide
57 - 58	*chiA* (ChiA)	extracellular	yes
52 - 54	*chiB* (ChiB)	periplasm / extracellular	no
48 - 52	*chiC* (ChiC1)	extracellular	no
35 - 36	*chiC* (ChiC2)	extracellular	no
95	*chb*	periplasm	yes
21 - 22	*cbp* (CBP21)	extracellular	yes

Brurberg *et al*., (1996)

Chitinases of *Serratia* are the most well studied enzymes. *S. marcescens* KCTC 2172, *S. marcescens* 2170, *S .liquifaciens* produces 22-54 kDa chitinases. (Woytowich *et al*., 2000; Suzuki *et al*., 1998; Watanabe *et al*., 1997). In *S. marcescence* ChiA is produced as a 563-residue precursor, which is secreted from the cells with concomitant cleavage of an N-terminal signal peptide. The resulting enzyme has 540 residues and a calculated molecular mass of 58.5 kDa (Brurberg *et al*., 1994; Perrakis *et al*., 1994). Mature *ChiB* contains 498 residues and its calculated molecular mass is 55.4 kDa (Van Aalten *et al*., 2001; Brurberg *et al*., 1994). The *chiC* gene gives rise to variants of *ChiC*, which are exported without cleavage of the N-terminal signal peptide. Instead, an unspecific N-terminal processing of the protein seems to occur, which results in the production of several slightly different ChiC species, lacking the N terminal residues (Suzuki *et al*., 1999; Gal *et al*., 1997).

2.2 SCREENING MICROBES FOR CHITINOLYTIC ACTIVITY

Chitinolytic activity of the microbes can be measured based on substrate hydrolysis, pathogen inhibition, biochemical estimation of the enzymes and by specific PCR techniques to show the presence of the concerned genes.

2.2.1 Substrate Hydrolysis in Media

Soil and aquatic systems harbor chitin degraders. Most fungi and bacteria produce chitinases only when grown on a chitin-containing substrate, making it an inducible enzyme. The substrates commonly employed for enumeration of chitin degraders are mushroom chitin (containing glucan) and shrimp chitin, which are used directly or processed to different forms such as swollen chitin, wiley milled chitin, colloidal chitin etc. Monreal and Reese (1969) found that *S. marcescens* (QMb1466) and a related bacterium, *Enterobacter liquefaciens* produced many times more enzymes on colloidal chitin than on swollen chitin or native (wiley milled) chitin.

S. marcescens was found to be the most active of over 100 organisms tested for the production of chitinase. *E. liquefaciens* produced nearly as much enzyme. Under optimal conditions of pH and temperature, yields of chitinase were obtained in 4-6 days (Monreal and Reese, 1969; Reid and David, 1981). The influence of pH on chitin hydrolysis by *streptomycetes* from a range of acidic and neutral soils was studied *in vitro* and 24 isolates were tested for their ability to hydrolyze chitin by measuring the zone of hydrolysis.

2.2.2 Inhibition of pathogens

S. plymuthica isolate C48, significantly suppressed the growth of *Verticillium longisporum* while a chemically constructed chitinase-deficient mutant C48/3Rif^{r}chi^{-} did not exhibit antifungal activity. A decrease in the percentage of diseased plants following root application of the wild type bacteria in greenhouse, supported their role in antifungal activities (Berg *et al.*,1996).

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

2.2.3 Biochemical Estimation:

Chitinolytic activity was assayed by measuring the release of reducing sugars from colloidal chitin. The absorbance of the reaction mixture at 582 nm was measured using calibration curve for N-acetyl-D -glucosamine to determine the reducing sugars (Tikhonov *et al.*, 2002).

Harman *et al.* (1993) measured and monitored endochitinase activity spectrophotometrically, as the release of the p-nitrophenol (PnP) from p-nitrophenyl-N-acetyl-D-glucosaminide. They also measured endochitinase activity by the reduction in turbidity of a suspension of colloidal chitin by the enzyme solution of *Trichoderma* (Harman *et al.*, 1993). Alternatively, endochitinase activity could be measured by using a microtitre plate assay as described for exochitinase but using p-nitrophenyl-β-D-N,N',N''–acetylchitotriose as the substrate (Harman *et al.*,1993 ; Henrinkson and Meredith,1984).

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

2.2.4 DNA Based Techniques for detection of genes

Whenever information on the entire or conserved domains of the target gene is known, PCR based techniques are used to detect and also pull out the desired sequences from genomic DNA or from clones. PCR primers were designed for chitinase genes in four γ-proteobacteria of family Alteromonacae and Enterobacteriacae (group I chitinases) and used to explore the occurrence and diversity of these chitinase genes in cultured and uncultured marine bacteria. PCR primers were designed based on conserved nucleotide sequences of chitinase genes in cultured bacteria and used to identify the efficient chitinase gene (Cottrell *et al.*, 2000).

2.3 CLONING AND EXPRESSION

In order to generate transgenics, gene for chitinase could be from diverse sources. May *et al.* (1995) cloned a gene encoding a chitinase from S. marcescenns BJL200 and expressed it in *E. coli*. (May *et al.*, 1995). The cosmid library of *S. marcescens* DNA made in pLAFRI and functional assay for degradation of chitin was done for the clones in the library (Fuchs *et al.*,1986). A DNA fragment (*Pchi5422*) containing two genes encoding a 54 kDa and a 22 kDa chitinases were isolated from cosmid DNA library of *S. marcescen* KCTC2172, seperately subcloned in *E. coli* and the individual chitinases were expressed and purified from the culture broth using chitin affinity chromatography (Sang Wan gal *et al.,*1997). Kenji morimoto *et al.* (1997) described cloned sequenced and expressed a gene (chiB) encoding a major chitinase, in the culture fluid of *C. paraputrificum* M21 and the characterized its translated product. They also analysed the respective domains constituting the modular chitinase ChiB.

Sitrit *et al.* (1993) expressed *chiA* gene from *S. marcescens* in *R. meliloti* and demonstrated that the nodule extracts from *chiA* expressing alfalfa plants caused lysis of *Rhizoctonia solani* hyphal tips. Co-application of *B. thuringiensis* □-endotoxin and bacterial chitinases significantly increased the insecticidal effect of the former against insect larvae (Regev *et al.* 1996 and Smirnoff, 1971)

Joshi *et al.* (1988) cloned two *S. liquefaciens* chitinases (*chiA* and *chiB*) genes and a chitobiase (*chiC*) gene and described the identification and cloning of two other genes (*chiD* and *chiE*) through transposon mutagenesis and deletion analysis. chiA and chiE were shown to be concerned with the regulation of expression of chitin-degradative enzymes. Leonid *et al*.. (1997) cloned *chiA*, from a soil borne*E. agglomerans*. Its complete sequence was determined and expressed in *E. coli* JM109 carrying the *E. agglomerans chiA* gene. The antifungal activity of the secreted endochitinase was demonstrated *in vitro* by inhibition of *Fusarium oxysporum* spore germination, and *R. solani* on plates and the root rot disease caused by this fungus in cotton seedlings under greenhouse conditions (Leonid *et al*.,1997).

The majority of bacterial chitinase genes have been cloned by screening plasmid-based genomic libraries in *E. coli* plated on media incorporating colloidal chitin. Such an approach has proven to be effective in cloning chitinases from Enterobacteriaceae such as *S. marcescens* (Fuchs *et al*., 1986) and other gram-negative bacteria viz., *Aeromonas hydrophila* (Roffey and Pemberton, 1990) and *Vibrio vulnificus* (Wortman *et al*., 1986).

*chi*A of *Ewingella americana* (EMBL/Genbank/DDBJ accession number X90562), was cloned by expression screening of a plasmid-based *E. americana Hin*dIII genomic library in *E. coli* using remazol brilliant violet-stained carboxymethylated chitin incorporated into selective medium. The *chi*A gene had a 918-bp ORF, terminated by a TAA codon, with a calculated polypeptide size of 33.2 kDa, corresponding to a previously purified and characterised 33-kDa endochitinase from *E. americana.* The deduced amino acid sequence shared 33% identity with chitinase II of *Aeromonas* sp. No. 10S-24 and 7.8% identity with a chitinase from *Saccharopolyspora erythraeus* (Inglis *et al*., 2000*).* Chitinase A of *Vibrio carchariae* was expressed in *E. coli* M15 cells as a 575-amino-acid fragment with full enzymatic activity using the pQE60 expression vector. The yield of the highly purified recombinant protein was approximately 70 mg per litre of bacterial culture (Songsiriritthigul *et al*., 2005).

The gene encoding an extracellular chitinase from marine *Alteromonas sp*. O-7, was cloned in *E. coli* JM109 by using pUC18 (Tsujibo *et al*.,1993). The *chiA* gene of S. marcescens was cloned into a shuttle vector pαHY300. The recombinant enzyme in transfermed *B. subtilis* was analyzed for chitinase activity and the highest activity occurred at the sixth day. The hyphal tips of *Botrytis elliptica* and *Penicilliu italicum* showed abnormal swelling when treated with *ChiA* culture broth (Hsiu-Yun Chang *et al*., 2005).

The gene coding for the major chitinase of *S. marcescens*, *chiA*, was cloned under the control of the *tac* promoter into the broad-host-range plasmid pKT240 and the integration vector pJFF350. *Pseudomonas fluorescens* carrying *chiA* either on the plasmid or integrated into the chromosome is an effective biocontrol agent of the phytopathogenic fungus *R. solani* on bean seedlings under plant growth chamber conditions. (Katrina *et.al*., 2000).

The *chiA* and *chiB* genes, encoding chitinases A and B of four different strains of *S. marcescens*, QMB1466 (Harpster and Dunsmuir, 1989; Jones *et al*., 1986), BJL200 (Brurberg *et al*., 1995 ; Brurberg *et al*., 1994), KCTC2172 (Gal *et al*., 1997) and 21709 (Watanabe *et al*., 1997), have been cloned and sequenced. Nucleotide and deduced amino acid sequences of the *chiA* and *chiB* genes of these strains were found to be very similar to each other.

2.4 TRANSGENIC PLANTS

Induction of bacterial chitinase gene (*Chi A*) from *S. marcescens* in transgenic tobacco plants conferred increased resistance to *Alternaria longipes*. This increase in resistance, however, appeared to decrease with the age of the plants (Suslow *et al*., 1988). Broglie *et al*.,(1991) were the first to show enhanced fungal resistance in transgenic plants brought about by the expression of a single chitinase gene. A *Chi-a1* gene from bean under

the control of the CaMV 35S into both tobacco and canola resulted in better resistance to *R. solani*. Later, enhanced protection against *R. solani* was also observed in transgenic rice plants constitutively expressing a rice *Chi-a1* chitinase gene in the transgenic *Nicotaina sylvestris* plants (Vierheilig *et al*., 1993).

Transgenic tobacco plants expressing high levels of *S. marcescens ChiA* exhibited increased tolerance to *R. solani*a compared to untransformed plants (Howie *et al*., 1994). Asao *et al.* (1997) have also shown that transgenic plants (tobacco, strawberry and cucumber) constitutively expressing rice chitinase gene showed increased resistance to fungal diseases.

Chitinases from the biocontrol fungus *T harzianum* are known to inhibit spore gerermination and hyphal elongation of the grapevine pathogens *Botrytis cinerea* (bunch rot) and *Uncinula necator* (powdery mildew) in *in vitro* assays. Embryogenic cultures of *Vitis vinifera* L. cultivars Merlot and Chardonnay were biolistically transformed with the *Trichoderma* endochitinase gene *ThEn42* under the control of a double CaMV 35S promoter and alfalfa mosaic virus leader sequence. A total of 101 'Merlot' and 93 'Chardonnay' putatively transformed plants were regenerated and evaluated for expression of chitinase using a fluorometric assay. About 41% of the 'Merlot' and 55% of the 'Chardonnay' selections had 10 to 100-fold higher chitinase activity than non-transformed plants (Kikkert *et al*., 1998).

Transfer of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) chitinase gene to *Nicotiana tabacum* cultivars (CF80, K326 and Xanthi-nc) resulted in expression of chitinase and it was confirmed using immunoblotting, and enzyme activity using a fluorometric assay(Shi *et al* ., 2000). The *Saccharomyces cerevisiae* chitinase, encoded by the CTS1-2 gene has been evaluated for it's *in planta* antifungal activity by constitutive over expression in tobacco plants to assess its potential to increase the plant's defence against fungal pathogens. Leaf extracts from transgenic tobacco plants inhibited *Botrytis cinerea* spore germination and hyphal growth by up to 70% in a quantitative *in vitro* assay, leading to severe physical damage on the hyphae (Carstens *et al*., 2003).

There is evidence that chitinase are effective against several plant pathogens. In recent years, considerable progress has been made in producing disease-resistant and high-yielding transgenic plants In modol plants. It may be necessary to integrate different resistance genes together in order to broaden the host defense against different fungi.

III. MATERIAL AND METHODS

The present study was carried out to clone chitinase genes from *Serratia marcescens*, study its expression in *E. coli* and subclone into plant transformation vector and study expression in tobacco a model system. The materials used and methods employed are as follows

3.1 BACTERIA AND VECTORS USED TO ISOLATE CHITINASE GENEs

Six strains of *Serratia marcescens* (5,29,63,74,91,141) were obtained from the culture collection of the Department of Agricultural microbiology, UAS Dharwad. Some of the isolates were from soils of Western Ghats of Uttara Kannda district of Karnataka.

The vectors used in this study include:

Vector	Source/Reference
pTZ57R/T	MBI, Fermantas, USA
pET28a(+)	Novagen, Germany
pHS100	Dr. Hari Misra, BARC, Mumbai

3.2 SCREENING THE ISOLATES FOR CHITINOLYTIC ACTIVITY

The chitinolytic activity of the available *S. marcescens* isolates were scored by their ability to produce a halo of clearing zone on colloidal chitin plate after incubation at 28±2℃ for 48 hrs.

3.2.1 Preparation of Colloidal Chitin

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

5g of chitin powder (Himedia Chemicals Co. Mumbai) was added slowly into 60ml of concentrated HCl (Sd. Fine Chemical) and left overnight at 4℃ with vigorous stirring. The mixture was added to 2.0L of ice-cold 95% ethanol with rapid stirring and kept overnight at room temperature (25℃). The precipitate was collected by centrifugation at 5000xg for 20 minutes at 4℃ and was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Colloidal chitin solution (5%) was prepared and stored at 4℃ until further use.

3.3 IDENTIFICATION OF *chiA, chiB* and *chiC*

3.3.1 Total DNA isolation from *Serratia marcescens*

The total DNA was isolated from *S. marcescens* isolates by following the protocol of Sambrook and Russell (2001) with some modification as described below.

Twenty five ml of overnight grown culture in Luria broth grown at 28℃ was centrifuged at 10,000 rpm at 4° C for 10 minutes. The pellet was re-suspended in 10 mM Tris, 100 mM sodium chloride solution and centrifuged at 10,000 rpm for 10min at 4℃. After discarding the supernatant, the pellet was re-suspended in 2.5 ml of T_{50} E_{20} buffer containing 25µl of RNAse (10mg/ml). The solution was incubated at room temperature for 10 minutes and after adding 2.5ml of Sarkosyl (2% in $T_{50}E_{20}$), it was incubated at 50° C for 45 minutes. 25 µl of proteinase K (10mg/ml) was added to it and further incubated at 55℃ for 10 minutes. Equal volume of phenol was later added, mixed gently and centrifuged at 10,000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and extraction was repeated

twice. Equal volume of phenol: chloroform (1:1) was added, centrifuged and the aqueous phase was separated. Later, equal volume of chloroform: isoamylalcohol (24:1) was added, centrifuged and the aqueous phase was separated. To the supernatant, 1/10th volume of 3M sodium acetate (pH 5.5) was added and incubated on ice for 20 minutes. Two volumes of cold, absolute ethanol was added and centrifuged. The supernatant was discarded and the pellet was washed with 70 percent alcohol, dried and dissolved in 250 µl of $T_{10}E_1$. The components of reagents are listed in Appendix I. Total DNA isolated was quantified by following the ethidium bromide spotting method as given by Sambrook and Russel (2001).

Appendix I. Reagents for total DNA isolation

3.3.2 Primers for PCR amplification of chitinase genes

The primers designed from reported *S. marcescens* sequence using Gene Tool software were as follows.

chiA

F: 5' GCCCATGGAAGGAATCAGTTATGCGCAAAT 3' (30mer, with *Nco*I site)

R: 5' GCGGATCCCAACGCACTGCAACCGATTAT 3' (29 mer, with *Bam* HI site)

chiB for cloning in pET28a (+):

F: 5' GCCCATGGCGGCAACCCCACTCCTCATG 3' (28mer, with *Nco*I site)

R: 5' GCGAATTCGCCCATCATCTTCTCCTACGTCAG 3' (32 mer, with *Bam* HI site)

Chi B cloning in plant transformation vector pHS100:

F : 5' GCTCTAGACGGCAACCCCACTCCTCATG 3' (28 mer,with XbaI site)

R : 5' GCGGATCCGCCCATCATCTTCTCCTACGTCAG 3' (32 mer, with *Bam* HI site)

chi C

F: 5' GCCCATGGAGGCCACCATGAGCACAAATAAC 3' (31mer, with *Nco*I site)

R: 5' GCGGATCCCGATTAGCGATTAGGCGATGAG 3' (30 mer, with *Bam* HI site)

3.3.3 PCR amplification

Different concentrations of primers 2.5, 5, and 10 pM were used to optimise amplification using total DNA of S. marcescens. Primers at Five pM concentration were found optimum and used in all further studies. Taq DNA polymerase, 10x assay buffer and individual dNTPs were obtained form M/s Bangalore Genei Private Ltd., Bangalore. Eppendorf Master Cycler (5331) was used to run the PCR programme. The master mix (Appendix III) required was prepared and 20 µl was distributed into each PCR tubes.

Appendix III. Conversion table for the amount of a PCR fragment required per ligation reaction

The following PCR amplification conditions were employed for amplification of *chi A* ,*chi B, chi* C genes

Stage	Step	Temperature (°C)	Duration (min)	No. of cycles
I	Initial denaturation	94	5	1
II	Denaturation	94	1	

Contd….

III	Annealing			
	Chi A	55	1	40
	Chi B	64	1	40
	Chi C	60	1	40
IV	Extension	72	1	
	Final extension	72	20	1
V	Hold	4	-	-

3.3.4 Electrophoresis

About 20 µl of the *chiA ,chiB and chiC* amplicons from each tube along with 3 µl of loading dye were electrophoresed in 1.0 per cent agarose gel along with λ-DNA *EcoR1* and *HindIII* double digest as DNA marker, at 50V for the initial 30 min and then at 70V for 1 hour. The buffer used was 1X TAE at pH=8.0. The DNA bands in the gel were visualised on a UV-transilluminator and documented using a gel documentation system (Uvitec Cambridge, England).

3.4 PCR BASED CLONING

The cloning of *chiA, chiB* and *chiC* was done by T/A cloning method following user's manual (MBI Fermentas)

3.4.1 Gel elution of the PCR amplicon

The single sharp amplicon at 1.7 kb in *chiA*, 1.5 kb in *chiB* and 1.45 kb in *chiC* was cut out using a sharp sterile scalpel by keeping the gel at low intensity UV transilluminator and collected in a sterile pre-weighed 2.0 ml microcentrifuge tube. The Qiagen gel extraction kit was used to elute the DNA from the agarose block as described in user's manual.

3.4.2 Cloning of PCR product

The purified PCR amplicon of *chiA*, *chiB* and *chiC* were ligated to pT257R/T cloning vector (2868 bp), separately, as described in InsT/A clone™ PCR product cloning kit (K1214) of MBI, Fermentas, USA. For ligation, an optimal molar ratio of 1:3 vector : insert was calculated. The ligation mixture along with linerised vector and amplicon DNA were mixed in 0.5 ml microcentrifuge tubes and incubated at 16 C for 16 hrs for ligation.

3.4.3 Preparation of competent cells

The component cells of *E. coli* DH5α were prepared following the protocol mentioned by Sambrook and Russell (2001) with minor modifications.

An isolated colony from *E. coli* DH5 α 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 - 3 hours till it attained a OD of 0.3 to 0.4 at 600 nm. The culture was chilled in ice for 30 min and 25 ml of culture was dispensed into two centrifuge tubes of capacity 50 ml. The cells were pelleted at 6000 rpm for 5 min. The supernatant was discarded and pellet was suspended in 12.5 ml of ice-cold 0.1 M calcium chloride. The centrifuge tubes were again kept in ice for 45 min and later centrifuged at 4000 rpm for 10 min. The pellet was dispensed in 1 ml of 0.1M $CaCl_2$ and to this 88 µl of dimethyl sulfoxide (DMSO) was added if intended for later use. About 200 µl of cells were distributed to each chilled 1.5 ml micro centrifuge tubes and immediately used.

3.4.4 Transformation of *E. coli* DH5α

About 100 µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 µl of ligation mixture was added and mixed gently. The mixture was chilled in ice for 45 min and heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2min.It was Immediately transferred to ice 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 min and about 700 µl of supernatant was discarded and the pellet was dissolved in remaining supernatant and spread on the plates having Luria agar with Amp_{50}, X-gal, IPTG and incubated overnight at 37°C.

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

3.5 CONFIRMATION OF CLONES

The Confirmation of the presence of cloned insert was done by PCR amplification of recombinant vectors with respective primers. The total DNA and cloning vector were used as positive and negative controls in the process.

The confirmation was also done through comparative restriction analysis of selected clones and the control vector to ensure the presence of insert with *Nco*I and *Bam*HI for *chiA, chiB* and *chiC* genes.

3.5.1 Sequencing of clones

The full length *chiA* 1.7 kb, 1442 bp *chiC* and 600bp *ChiB* amplicon cloned in pTZ257R/T was sequenced using M13 primers walking technique at Bangalore Genei Private Ltd., Bangalore. The sequences were subjected to analysis using BLAST algorithm available at http://www.ncbi.nim.nih.gov.

3.6 SUB CLONING OF THE CLONED *chiA chiB* AND *chiC* GENES INTO A PROKARYOTIC EXPRESSION VECTOR

3.6.1 Vector and clone isolation

For analysing the expression of the cloned *chi* genes, prokaryotic expression vector pET28a (+) was used and the *chi* genes were digested with respective restriction enzyme to release the insert.

The alkaline lysis protocol of Brimbion and Dolly (1979) and Brimbion (1983) with certain modifications was used for isolation of plasmids.

About 10ml of overnight grown culture was centrifuged at 5000 rpm for 2 min at 4°C in 2.0 ml microcentrifuge tubes. The supernatant was removed and pellet was washed with STET (0.25 volume of original culture). It was centrifuged at 5000 rpm for 2 min. The pellet was resuspended in 200 µl of ice-cold alkaline lysis solution-I by vigorous vertexing. Later, 400 µl of freshly prepared alkaline lysis solution-II was added to each tube and the contents were mixed by inverting the tubes 4 to 5 times and kept in ice for about 5 min. To this suspension, 300 µl of alkaline lysis solution-III was added and again mixed thoroughly by gently inverting the tubes for 4-5 times. The tubes were stored on ice for 5 minutes and centrifuged at 13,000 for 8 min. The supernatant was transferred to fresh tubes and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to precipitate proteins, mixed well and centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous layer was transferred to a fresh tube and two volumes of isopropanol were added. The contents were mixed and allowed to stand for 2 minutes at room temperature. The solution was later centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 70 per cent ethanol and spun for 1 min at 13,000 rpm to recover the plasmid. The supernatant was discarded, pellet dried completely and dispensed into 25 µl of

$T_{10}E_1$ (pH=8.0) containing 3 µl of RNase A (10 mg/ml). The solution was kept at 50°C for 15 min and then stored at -20°C.

3.6.2 Electrophoresis

About 2 µl of the isolated DNA along with 1 µl of loading dye was electrophoresed in 0.7per cent agarose gel along with λ-DNA *Hind* III double digest as DNA molecular weight marker. Electrophoresis was done at 70 V for 1 hour. The buffer used was 1x TAE (pH,8.0). After separation, the DNA bands were visualised on a UV-transilluminator and documented using gel documentation system (Uvitec Cambridge, England).

3.6.3 Linearizing the vector and clone

Recombinant pTZ57R/T containing *chiA, chiB* and *chiC* amplicons were cut with *Nco* I and *Eco*R I restriction enzymes. Accordingly expression vector pET28 (a+) was also digested with the same restriction enzymes to have compatible cohesive ends. The complete restriction was confirmed by electrophoresis.

3.6.4 Ligation and transformation of *E. coli* BL21 (pLysS)

The single sharp bands corresponding to vector 5.3 kb, *chiA* insert of 1.7 kb, *ChiB* of 1.5 kb and chiC of 1.450 kb were cut out from agarose gels, eluted as described previously (section 3.4.1) and quantified by ethidium bromide spotting method.

The ligation reaction was carried out with molar ratio of 1:3 (ends of vector : insert). The components of the ligation mixture was taken in a 0.5 ml microcentrifuge tube and incubated at 16°C for 16 hrs.

Competent cells of *E. coli* BL 21 (pLysS) were prepared following the protocol described earlier (3.4.3) and transformed with the ligation mixture as described in 3.4.4. The pellet containing transformed cells was dispensed in about 200 µl of supernatant and plated on Luria agar containing kanamycin (25 µg/ml) and incubated at 37°C for 12-16 hrs. The colonies obtained were further streaked on Luria agar with kanamycin (50 µg/ml).

3.6.5 Confirmation of transformation

Transformation was confirmed in individual colonies by setting PCR using respective primers. PCR amplification of recombinant pTZ57R/T plasmids extracted from clones and that of expression vector pET28(a+) were used as positive and negative controls, respectively. Further confirmation of clones was done through restriction analysis of plasmids of selected clones and control vector with a pair of restriction enzymes (*Nco*l and *BamH1)* to release the inserts.

3.7 EXPRESSION OF *chiA, chiB* and *chiC genes*

The expression of cloned genes analysed was using the procedure outlined in Sambrook and Russell (2001).

About 5 ml of Luria broth with kanamycin (50 µg/ml) was incubated with 50 µl of overnight culture and kept at 37°C with shaking. One ml of culture was taken and induced by adding 1mM IPTG. It was again incubated for 3 to 4 hours at 37°C under shaking condition. After induction, the protein was extracted and analyzed by SDS-PAGE.

For extraction of proteins, the cell culture was centrifuged at 13,000 rpm for 1 min at room temperature. The pellet was resuspended in 100 µl of $T_{10}E_1$ and 100 µl of 2X SDS gel loading buffer added to it. The mixture was heated at 95°C for 10 minutes and centrifuged at 5000 rpm for 5 minutes at room temperature. About 60 µl of each such suspension was loaded on 10 per cent polyacrylamide gel using the suspension of cells containing vector alone as control. Electrophoresis was done at 50 V for one hrs. The gells were stained with coomasie brilliant blue and documented.

3.8 SUBCLONING OF THE *chiA, chiB* and *chiC* INTO PLANT TRANSFORMATION VECTOR

3.8.1 Vector and clone isolation

For subcloning of *chiA, chiB* and *chiC*, a plant expression vector (pHS100), was used. The vector, pHS100 and plasmids from (pNKK0901, pNKK2502 and pNKK1202) clones were isolated using protocol described in 3.6.1.

Sequential digestion of pHS100 was done with two restriction enzymes – *Xba1 and* Bam HI. The vector pHS 100 was first restricted with *Bam* HI and the linearlized vector was eluted using Qiagen gel extraction kit and again restricted with *Xba* I enzyme. The vector was eluted as per the protocol given in user's manual.

ChiA and *chiC* inserts were obtained by restricting pNKK0901 and pNKK1202by *Xba* I and *Bam*HI, simultaneously. Respective 1.7 kb and 1.45kb size were eluted as described earlier. As *chiB* gene has an internal *Bam*HI site, the primers having *XbaI* and *Bam*HI sites were used to amplify pNKK2502 and it was completely restricted with *XbaI* enzyme. The DNA was again extracted using Quiagen PCR purification kit and then extracted DNA was now partially restricted with *Bam* HI for 30 seconds. The intact 1442 bp insert was separated, eluted and extracted using Qiagen gel extraction kit.

3.8.2 Ligation and Transformation

The ligation reaction was carried out with an optimal molar ratio of 1:3 vector : insert. The components of the ligation mixture was mixed into a 0.5 ml microcentrifuge tube and incubated at 16°C for 16 hours. The component cells of *E. coli* DH5α were prepared as described in section 3.3.5, were transformed with the ligation mixture as described earlier (3.6.4).

Recombinant clones were identified and the plasmid was isolated and confirmed as described earlier using gene specific PCR. Further confirmation was done by complete restriction of clones using *Xba* I and *Bam* H I enzymes.

3.9 *AGROBACTERIUM* TRANSFORMATION

The confirmed recombinant clones of pHS100 were transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating technique. The vector pHS100 is capable of replicating in both *E. coli* and *Agrobacterium* and carries unique cloning sites and a plant selectable marker between its disarmed T-DNA borders. The chromosomal selection is by rifampicin (25 μg/ml) and it contains disarmed Ti-plasmid called pAL4404 which has streptomycin (100 μg/ml) as selectable marker.

The recombinant plasmids with *chiA, chiB* and *chiC* i.e., pNKK0205, pNKK1006 and pNKK2505 were propagated in *E. coli* DH5α cells in Luria both containing 50 μg/ml of kanamycin overnight at 37°C. The *A. tumefaciens* LBA4404 was grown for 16-22 hours at 28°C in yeast extract mannitol agar containing rifampicin (25 μg/ml) and streptomycin (100 μg/ml). The *E. coli* helper strain containing pRK2013 was grown in LB containing kanamycin (50 μg/ml) overnight at 37°C.

The overnight grown cultures were centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the pellet was washed with 0.01 $MgSO_4$ for 2-3 times to remove traces of antibiotics. It was again centrifuged at 13,000 rpm for 1 min and pellet was dispensed in 50 μl of 0.01 M $MgSO_4$. *A. tumefaciens* LBA4404, *E. coli* DH5 (pRK2013) and *E. coli* containing constructs; pNKK0205, pNKK1006 and pNKK2505 were mixed in 1:1:1 ratio separately. The mixture was spotted on LA (plain) and incubated overnight at 28°C. The

spotted culture was scraped and dissolved in 200 µl of 0.01 M $MgSO_4$ and re-spotted on YEMA medium containing streptomycin (100 µg/ml), rifampicin (25 µg/ml) and kanamycin (50 µg/ml) along with *A. tumefaciens* LBA4404, *E. coli* helper strain and *E. coli* with recombinant vectors as negative controls. On this medium, only *Agrobacterium* containing the recombinant transformation vector were expected to grow.

The presence of recombinant plasmid in the *Agrobacterium* was confirmed by PCR amplification.

3.9 TOBACCO TRANSFORMATION

The *Agrobaterium* containing recombinant plasmid pNKK0205 was used for tobacco transformation by using protocol mentioned by Hooykaas and Schilperoopt, (1992) with some modification.

A. tumefaciens LBA4404 transformed with construct pNKK0205 was grown in YEMA with streptomycin (100 µg/ml), rifampicin (25 µg/ml) kanamycin (50 µg/ml) at 28℃ for 24 hours. Leaves of tobacco (from 5-6 week old plants) were surface sterilized by rinsing them for 1 min in 0.1 per cent mercuric chloride, and then rinsed 3-4 times with sterile water to remove the trace of mercuric chloride. The surface sterlized leaves were cut into 1-2 cm^2 disks and cocultivated in acetosyringone (200 µM) treated *Agrobacterium* culture and left for 20 minutes for infection. Cocultivated explants were dried by blotting with the sterile filter paper and placed on solid Murashige and Skoog (MS) medium without any hormones and kept in dark for 2 days. Each leaf disk was transferred to test tubes containing MS medium with 0.5 mg/L NAA and 1mg/L BA for callus induction and cephotaxiime (200 mg/L) to avoid *Agrobacterium* growth. After 10 days, the disks were transferred to MS medium containing NAA (0.5 mg/L), BAP (1 mg/L)¸ cephotaxmine (200 mg/L) and kanamycin (100 mg/L) and cultured for 20 days. The excised calli were placed on shooting medium containing MS with NAA (0.05 mg/L), BAP (1 mg/L), cephotaxmine (200 mg/L) and kanamycin (100 mg/L). After a month in culture, healthy shoots were transferred to MS medium without hormones for rooting. The putative transformants were transferred to pots containing peat and acclimatized in green house.

DNA was extracted from putative transgenic tobacco plants by rapid method and checked by PCR with gene specific primers.

3.10 TRANSGENE EXPRESSION ANALYSIS

Transgene expression analysis of PCR positive plants was done by a single step reverse transcriptase-polymerase chain reaction (RT-PCR).

Total RNA from transgenic plants and control tobacco plants were isolated using RNeasy plant mini kit (Qiagen company, GmbH, Germany) and one step RT-PCR using cMaster™ RT_{plus} PCR System and cMaster™ RT Kit (Eppendorf Hamburg-Germany). Total RNA isolation and RT-PCR reaction were done as per the manufacturer's instructions. RNA so isolated was quantified using a spectrophotometer at 260nm. The RNA samples were diluted to 1µg/µl concentration using DEPC treated water. Equal amount of total RNA from both transgenics and control tobacco plants were used for a one step RT-PCR reaction separately. The procedure does not allow sample cross contamination and all cDNA is utilized as a template for the PCR step. For one step RT-PCR, specific primers for *chi A* were used for amplification of *chiA* transcript. The PCR amplicon intensity was taken as a measure of transgene expression at transcription level.

Components of one-step RT-PCR mixture

The following components were added to prepare reaction mixture in the order mentioned below.

Components	Concentration (µl)
Template RNA (1µg/µl)	2µl
RT_{plus}PCR Buffer with Mg^{2+}	2 µl

dNTP mix (10mM each)	0.4µl
cMaster RT Enzyme	0.25µl
cMaster PCR Enzyme mix	0.2µl
Prime RNase Inhibitor Solution	0.2µl
Forward primer (5 pm/ µl)	1µl
Reverse primer (5 pm/ µl)	1µl
RNase free H_20	13.95µl
Total reaction volume	20 µl

Amplification conditions for One-step RT-PCR as follows

Stage	Step	Temperature (℃)	Duration (min)	No. of cycles
I	Reverse transcription	42	90	1
II	Initial denaturation	93	2	1
III	Denaturation	93	0.25	
	Annealing	55	0.33	39
	Extension	72	0.50	
IV	Final extension	72	20	1
	Hold	4	Forever	-

One step RT-PCR product was fractionated by 1% agarose gel electrophoresis and visualized in gel documentation system.

IV. EXPERIMENTAL RESULTS

In this study, efforts were made to clone and express genes encoding *chiA, chiB* and *chiC* from *S. marcescens* in *E. coli*, clone the isolated genes into a plant transformation vector and analyse their expression in tobacco. The results obtained from various experiments done to achieve the said objectives are presented here.

4.1 SCREENING THE ISOLATES FOR CHITINOLYTIC ACTIVITY

S. marcescens 141 was found to be more effective in releasing chitinase enzyme when different strains of *S. marcescens* were grown on colloidal chitin plate in which had chitin as a sole carbon source (Plate 1).

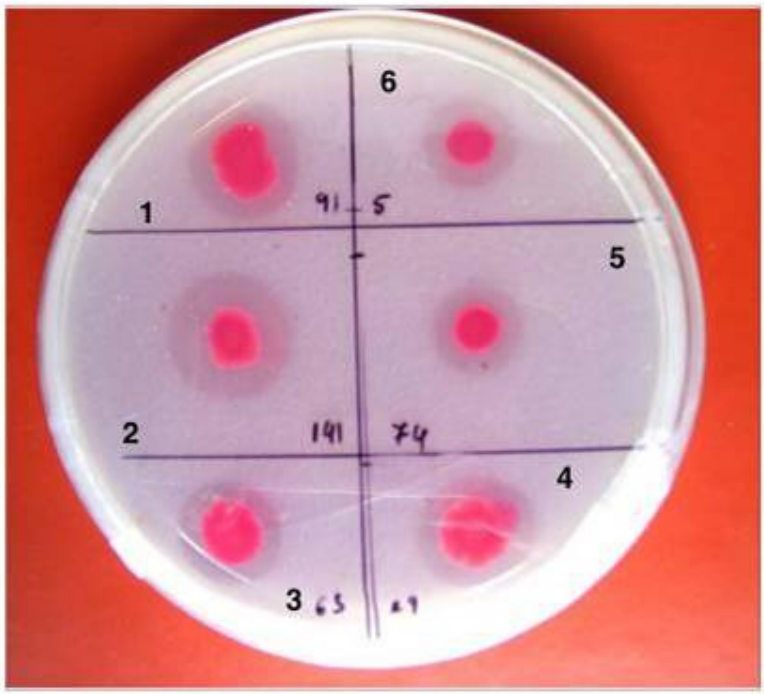

Plate 1. Chitinolytic activity of native isolates of *Serratia marcescens* on colloidal chitin media

Plate 1. Chitinolytic activity of native isolates of *S. marcescens* on colloidal chitin media

1. *S. marcescens* 91
2. *S. marcescens* 141
3. *S. marcescens* 63
4. *S. marcescens* 29
5. *S. marcescens* 74
6. *S. marcescens* 6

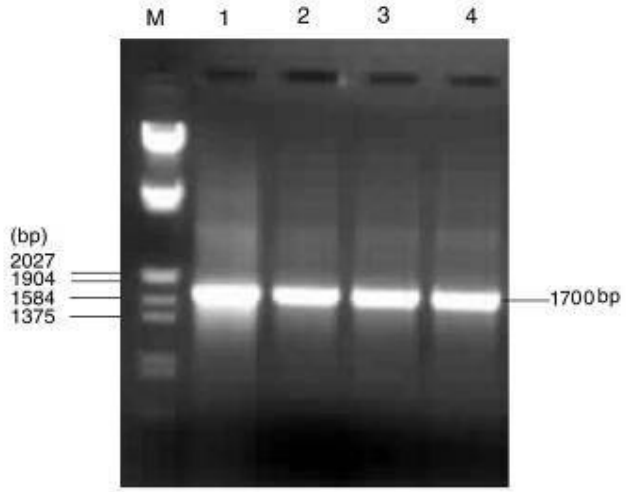

Plate 2. PCR amplification of *chiA* gene from genomic DNA

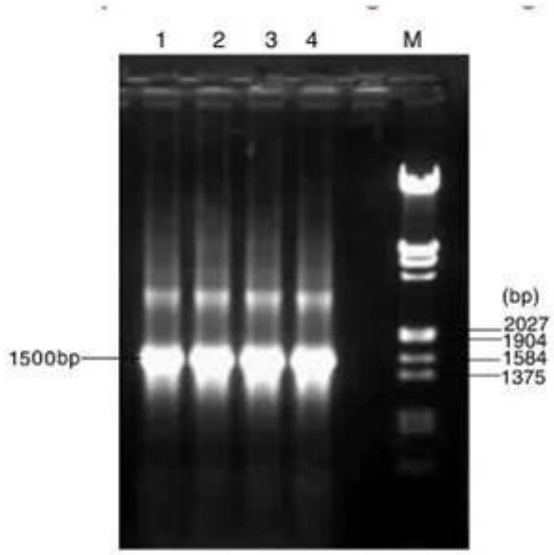

Plate 3. PCR amplification of *chiB* gene from genomic DNA

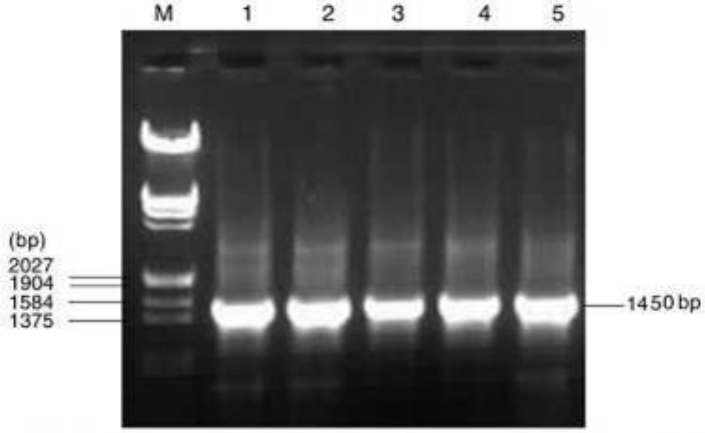

Plate 4. PCR amplification of *chiC* gene from genomic DNA

Plate 2. PCR amplification of *chiA* gene from genomic DNA

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. *S. marcescens ChiA* specific primer (54^0C)
2. *S. marcescens ChiA* specific primer (55^0C)
3. *S. marcescens ChiA* specific primer (55^0C)
4. *S. marcescens ChiA* specific primer (55^0C)

Plate 3. PCR amplification of *chiB* gene from genomic DNA

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. *S. marcescens ChiB* specific primer (63^0C)
2. *S. marcescens ChiB* specific primer (63^0C)
3. *S. marcescens ChiB* specific primer (63^0C)
4. *S. marcescens ChiB* specific primer (63^0C)

Plate 4. PCR amplification of *chiC* gene from genomic DNA

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. *S. marcescens ChiC* specific primer (50^0C)
2. *S. marcescens ChiC* specific primer (60^0C)
3. *S. marcescens ChiC* specific primer (60^0C)
4. *S. marcescens ChiC* specific primer (60^0C)
5. *S. marcescens ChiC* specific primer (60^0C)

4.2 PCR AMPLIFICATION OF *chiA, chiB* AND *chiC* GENES

The PCR was carried out using specific primers designed using reported *S. marcescens chiA, chiB* and *chiC* nucleotide sequence from the database. The amplicons so obtained and separated on 1.0 per cent agarose gel are presented in Plate 2, plate 3 and 4, respectively. From the gels, it is clear that an amplicon of around 1691 bp, 1499bp and 1442 bp was obtained from *chiA, chiB* and *chiC*, respectively. These amplicons were used for further cloning studies.

4.3 CLONING THE AMPLICONS

4.3.1 Ligation and transformation of *E.coli* DH5α

The 1691 bp *chiA,* 1499 bp *chiB* and 1442 bp *chiC* were eluted from preparative gels. pTZ57R/T was used as cloning vector for cloning amplified fragments. The recombinant molecules *i.e.*, pNKK0901, pNKK2502 and pNKK1202 were transferred into *E. coli* DH5α separately using 5 µl of ligation mixture each. Super coiled plasmid DNA of pT257R was used as positive control. The transformation efficiency was found to be 0.25×10^3 CFU/µg and 0.52×10^4 CFU/µg, respectively.

4.3.2 Confirmation of the clones

The transformed cells were picked up and streaked on Luria agar containing amplicillin (100µg/ml), X-gal and isopropyl β-D-thiogalactosidase (IPTG). The clones containing recombinant molecules were selected based on blue-white assay. Plasmids were isolated from clones containing of *chiA, chiB* and *chiC* and the clones were confirmed through PCR amplification by using specific primers (Plate 5, 7 and 6). Further, confirmations was also done by restriction analysis using *Nco1* and *BamH1*, which released 1.7kb, 1.5kb and 1.45kb insert, respectively. (Plate 5, 7 and 6).

The confirmed recombinant vectors with *chiA, chiB* and *chiC* were named pNKK0901, pNKK2502and pNKK1202, respectively.

4.4 SEQUENCE ANALYSIS

The constructs pNKK0901 and pNKK1202 were sequenced completely and pNKK2502 was sequenced partially using M13 primers employing primer walking technique. Fig 1, 2 and 3 represent the maps of pNKK0901, pNKK2502 and pNKK1202, respectively. The complete sequence of nucleotide and amino acid sequences of *chiA, chiC* and partial sequence of nucleotide and amino acid sequences of *chiB* are presented in Fig. 4, 5 and 6.

The nucleotide sequences were analysed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. *chiA* had 99 per cent homology with reported *chiA* (AB015996), (AF454462), 98 per cent with *chiA* of *S. marcescens* strain ATCC 990, 97 per cent with *chiA* of *S. marcescens* (BJL200), 97 per cent homology with *Burkholderia cepacia* chitinase (*chi60*) gene, 96 per cent homology with *Enterobacter sp.*(AY040610) NRG-4 chitinase (*chiA*) gene and 87 per cent homology with *Serratia plymuthica* (CAD32933) *chit60* gene for chitinase.

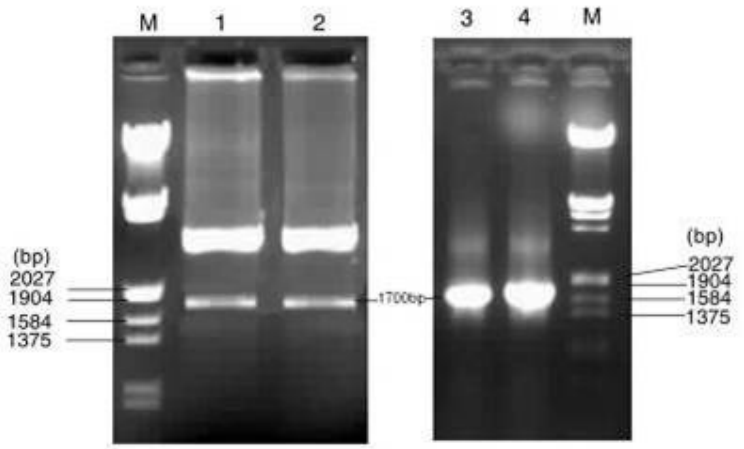

Plate 5. PCR and restriction analysis of pNKK0109

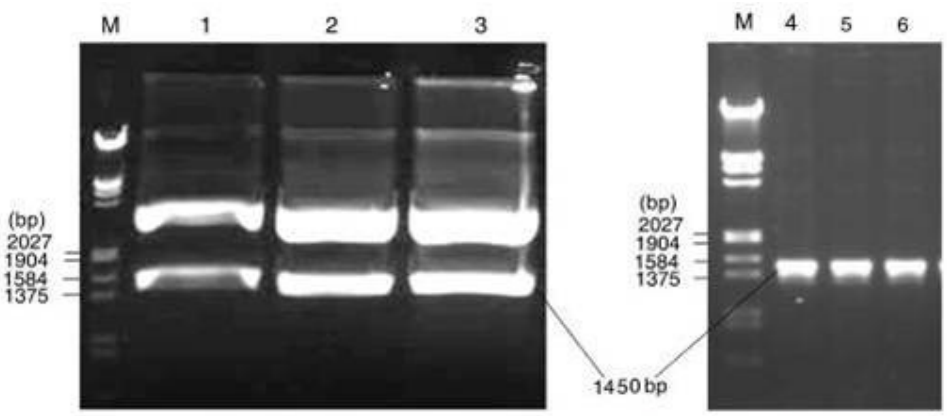

Plate 6. PCR and restriction analysis of pNKK1202

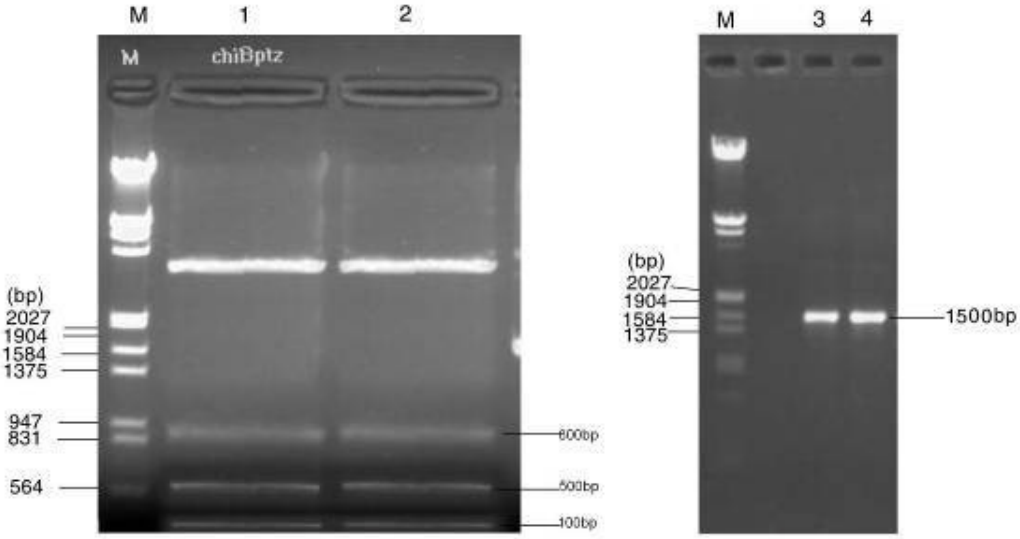

Plate 7. PCR and restriction analysis of pNKK2502

Plate 5. PCR and Restriction analysis of pNKK0109

M. Lambada DNA/ *EcoRI* + *HindIII* marker

Restriction

1. pNKK0109 A
3. pNKK0109 B

PCR

4. pNKK0109 A
5. pNKK0109 B

Plate 6. PCR and Restriction analysis of pNKK1202

M. Lambada DNA/ *EcoRI* + *HindIII* marker

Restriction

1. pNKK1202 A
2. pNKK1202 B
3. pNKK1202 C

PCR

4. pNKK1202 A
5. pNKK1202 B
6. pNKK1202 C
7.

Plate 7. PCR and Restriction analysis of pNKK2502

M. Lambada DNA/ *EcoRI* + *HindIII* marker

Restriction

2. pNKK2502 A
6. pNKK2502 B

PCR

7. pNKK2502 A
8. pNKK2502 B

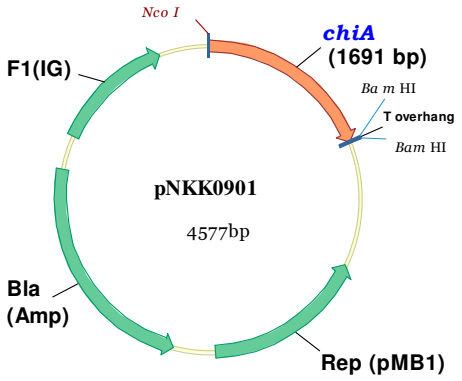

Fig 1. Construct map of pNKK0901 containing full length *chiA* gene in pTZ57R/T

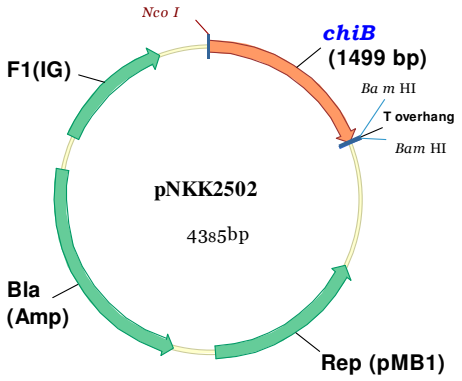

Fig 2. Construct map of pNKK2502 containing full length *chiB* gene in pTZ57R/T

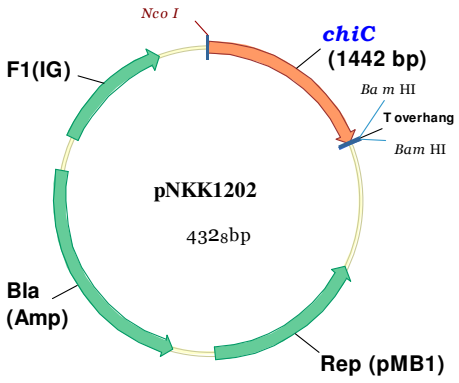

Fig 3. Construct map of pNKK1202 containing full length *chiC* gene in pTZ57R/T

Nucleotide sequence

AAGGAATCAGTTATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCG
CGGCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATTGTTGAAG
TTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCCGATGTTTCCGTCTCCTG
GAATTTATGGAATGGCGACACCGGCACGACGGCAAAAGTTTTATTAAATGGCAAAGAGGCGTGGAGTGGT
CCTTCAACCGGATCTTCCGGTACGGCGAATTTTAAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGG
CATTGTGCAATGCCGACGGCTGCACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCA
GCCATTTGGCGCCGTTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGT
GGTCGGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCGGCGCAA
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AGATTGAAGGCAGCTTCCAGGCGTTGCAGCGCTCCTGCCAGGGCCGCGAGGACTTCAAAGTCTCGATCCA
CGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAGGGCGTGACCGCCTGGGATGACCCCTACAAGGGCAA
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GAGTTCCTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAAGGC
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GACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAACTCGATGGATCACATCTTCCTGATGAGCTACG
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GGACACCGCCTACACCACGGTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAAAT
CGTGGTCGGCACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAATATTCCGTT
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CGGCCAGTTCATGAGCGGCGAGTGGCAGTATACCTACGACGCCACGGCGGAAGCGCCTTACGTGTTCAAA
CCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCGGTGCAGGCCAAAGGCAAGTACGTGTTGG
ATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAGATCGACGCGGATAACGGCGATATTCTCAACAGCATGAA
CGCCAGCCTGGGCAACAGCGCCGGCGTTCAATAATCGGTTGCAGTGCGTTG

Protein sequences

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNNLVKVKNAADVSVSWNLWNGDTGTTA

KVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIVVADTDGSHLAPLKEPLLEKNKPYK

QNSGKVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDP

FAALQKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEFLQTWKFF

DGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRTMLDQLSAETGRKYELTSAISAGKDKIDKVAYNVAQNSMD

HIFLMSYDFYGAFDLKNLGHQTALNAPAWKPDTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIP

FTGTATGPVKGTWENGIVDYRQIAGQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGG

LFSWEIDADNGDILNSMNASLGNSAGVQ*

Fig 4. Complete nucleotide and protein sequence of pNKK0901

Nucleotide sequence

AGGCCACCATGAGCACAAATAACATTATTAATGCCGTCGCCGCCGATGACGCGGCCAT
TATGCCGTCTATTGCCAATAAAAAGATCCTGATGGGTTTCTGGCACAACTGGGCCGCC
GGCGCCAGTGACGGTTATCAACAAGGCCAGTTCGCCAATATGAATCTGACCGACATTC
CCGCCGAGTACAACGTAGTGGCCGTCGCCTTTATGAAAGGCCAGGGCATCCCGACCT
TCAAACCTTACAACCTGTCCGACGCCGAGTTTCGCCGCCAGGTGGGCGTGCTGAACA
GCCAGGGCCGCGCGGTGCTGATCTCCCTCGGCGGCGCAGACGCGCATATCGAGCTGA
AGACCGGCGACGAAGACAGGCTGAAAGACGAGATTATTCGCCTGGTGGAAGTCTATG
GCTTCGACGGCCTGGATATCGATCTGGAACAGGCGGCGATCGGCGCCGCCAATAATA
AAACCGTCTTGCCTGCGGCATTGAAAAAAGTAAAAGACTATTACGCCGCGCAGGGGA
AAAACTTTATTATCAGCATGGCGCCGGAATTCCCGTATTTGCGCACCAACGGCACCTA
TCTGGATTATATCAACGCCCTTGAAGGCTATTACGACTTTATTGCGCCGCAATATTACA
ACCAGGGCGGCGACGGTATTTGGGTGGATGAACTCAACGCCTGGATCACGCAGAATA
ACGACGCCATGAAAGAGGACTTCCTCTACTATCTGACGGAAAGCCTGGTCACCGGCA
CCCGCGGCTATGCGAAAATCCCGGCGGCGAAATTCGTCATCGGCCTGCCGAGCAACA
ACGATGCCGCCGCCACCGGCTACGTGATCGACAAACAGGCGGTGTATAACGCCTTCG
CGCGTCTCGACGCCAAAAGCCTGTCGATCAAGGGCCTGATGACCTGGTCGATCAACT
GGGATAACGGCAAGAGCAAAGCCGGCGTGGCCTACAACTGGGAATTCAAAACCCGCT
ATGCGCCGCTGATTCAGGGCGGCGTCACCCCGCCGCCGGGAAAGCCTAATGCGCCGA
CGGCGCTGACGGTCGCCGAGCTGGGCGCCACCTCGCTGAAACTGAGCTGGGCCGCC
GCCACCGGCGCGTTACCGATCGCCAGCTACACCGTTTACCGCAACGGCAACCCGATC
GGCCAGACCGCCGGCCTGTCGCTGACCGACAGCGGCCTGACCCCGGCCACCCAGTA
CAGCTACTTCGTTACCGCAACCGACAGCCAGGGCAATACCTCGCTGCCGAGCAGCGC
GCTGGCGGTCAAAACCGCCAACGACGGCACGCCGCCCGATCCGGGTGCGCCCGAGT
GGCAGAACAACCGCAGCTACAAAGCCGGCGACGTGGTGAGCTATAAAGGCAAGAAAT
ACACCTGTATCCAGGCGCACACTTCCAACGCCGGCTGGACGCCGGACGCCGCCTTCA
CCCTGTGGCAGCTCATCGCCTAATCGCTAATCG

Protein sequences

MSTNNIINAVAADDAAIMPSIANKKILMGFWHNWAAGASDGYQQGQFANMNLTDIPAEYNVV
AVAFMKGQGIPTFKPYNLSDAEFRRQVGVLNSQGRAVLISLGGADAHIELKTGDEDRLKDEII
RLVEVYGFDGLDIDLEQAAIGAANNKTVLPAALKKVKDYYAAQGKNFIISMAPEFPYLRTNGT
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AKIPAAKFVIGLPSNNDAAATGYVIDKQAVYNAFARLDAKSLSIKGLMTWSINWDNGKSKAGV
AYNWEFKTRYAPLIQGGVTPPPGKPNAPTALTVAELGATSLKLSWAAATGALPIASYTVYRN
GNPIGQTAGLSLTDSGLTPATQYSYFVTATDSQGNTSLPSSALAVKTANDGTPPDPGAPEW
QNNRSYKAGDVVSYKGKKYTCIQAHTSNAGWTPDAAFTLWQLIA*SL

Fig 5. Complete nucleotide and protein sequence of pNKK1202

GCCCTCCTATCCCCAGTGGAGCAGATGTTGCAGGGCAACTACGGTATCAGCGGTTGGGAACGATAAGACCAAA

ACCCCGTATCTGTATCATGCGCAGAACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGC

GAAGTACATCAAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGCGAT

CTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTGGATATGGGCACCGGCC

TGCGATACACCGGCGTCGGTCCCGGCAACCTGCCGATCATGACCGCGCCGGCCTATGTGCCGGGCACCACTTA

CGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCAAGTGGGGTTACATCACCTCGGCGCC

CGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGCCTGGCGTAAGCCGTAAAAAAACCCGGTAGCCGAATGCT

GCGGGGTTTTCAGGATCCGCAATCTAGATGCATTCGCGAGGTACCGAGCTCGATTCCCC.

Fig 6. Partial nucleotide sequence of *chiB* gene

The rps BLAST results of aminoacid sequence of cloned *chiA* showed 99 per cent homology with reported *chiA* of *S. marcescens* (BAA31567.1), 98 per cent with Chitinase A precursor of *S. marcescens* (SP07254), 99 per cent with amino acid sequence endochitinase of *S. marcescens* (AAZ86539), 99 per cent with chitinase of *Enterobacter* sp. (AY040610) NRG-4 and 92 per cent homology with chitinase of *S. plymuthica* (CAD32933).

The length of amplicon was 1721 for c*hiA* sequence and analysis using NCBI tool BLASTn revealed 1691bp open reading frame (ORF) from 13 to 1704 bp in *chiA*. The deduced aminoacid sequence of the protein was 58.0 kDa. The ORF map of pNKK0901 is presented in Fig 8.

Search for conserved domains revealed *chiA* domain of 386 amino acids similar to glyco-domain and glycosyl hydrolase's domain (Fig 7).

gnl|CDD|25596 pfam00704, Glyco_hydro_18, Glycosyl hydrolases family 18..

```
             CD-Length = 323 residues, 100.0% aligned
             Score =  255 bits (651), Expect = 1e-68

Query:  158  KVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRS  217
Sbjct:  1    GRIVGYYTQWGNYGEGFLLEDIPTDKLTHIIYAFANIDGNGTTG---------------  44

Query:  218  CQGREDFKVSIHDPFAALQKAQKGVTAWDDPYKGNFGQLMALK-QAHPDLKILPSIGGWT  276
Sbjct:  45   ----------------------YLADTEDDGSKGCFEQLKDLKKCQNPGVKVLLSIGGWT  82

Query:  277  LSDPFFFM-GDKVKRDRFVGSVKEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETY  335
Sbjct:  83   FSGGFSLLLSDDAKRKTFADSIIDFLKKYG-FDGIDIDWEYPGARG---------DKDNY  132

Query:  336  VLLMKELRTMLDQLSAETGRKYELTSAISAGKDKIDKVAYNVA--QNSMDHIFLMSYDFY  393
Sbjct:  133  TLLLKELREALKKEAK---AGYLLSAAVPAGPIKLDGL-YDIAKIGKYLDFINVMTYDFH  188

Query:  394  GAFDLKNLGHQTALNAPAWKPDTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNG  453
Sbjct:  189  GW--SNITGPNAPLYDGSN-------VDYTVQYYLKAGVPASKLVLGIPFYGRGWTLVNG  239

Query:  454  YQNNIPFTGTATGPVKGTWENGIVDYRQIAGQFMSGEWQYTYDATAEAPYVFKPSTGDLI  513
Sbjct:  240  SGNGGGAPAG-----PGTKAGGILSYKELC-ALVKSGATPTYDDTAKAPYIYKGDT-HFV  292

Query:  514  TFDDARSVQAKGKYVLDKQLGGLFSWEIDAD  544
Sbjct:  293  SYDDPRSIKAKAKYVKDKNLGGVMIWSLDQD  323
```

Fig 7. Conserved domain of *chiA* gene

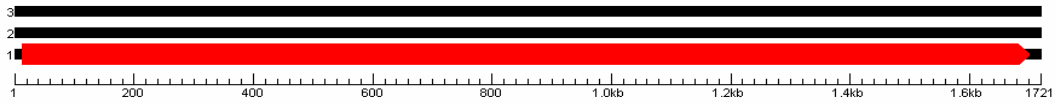

Fig 8. Open reading frame *chiA* gene

The sequence information was analysed to find restriction sites using BTI Gene tool software which revealed the presence of *Sal*1 at 530, *Pst1* at 906, and *Sma*1 site at 955th position in pNKK0901 and their complete restriction maps are shown in Fig 9.

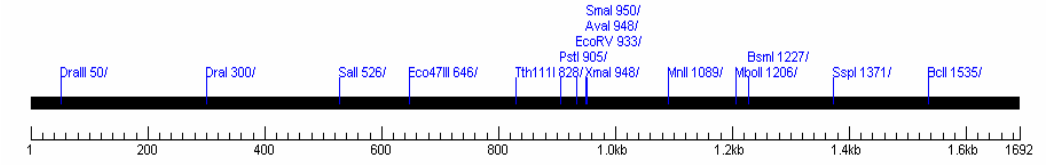

Fig 9. Restriction sites present in *chiA* gene for commercial enzymes

BLAST search for cloned *chiC* showed that it had 99 per cent homology with reported *chiC* of *S. marcescens* (AB019238), 98 per cent homology with *chiC* of *S. marcescens* (L41660.1) and 97% with *chiC* of *S. marcescens* (AF454464). 97 per cent with ChiA precursor of *Vibrio cholerae* (AF097314), 91 per cent with *chiD* gene precursor of *Bacillus circulans* (BACCHIDA) and 87 per cent with c*hiC* gene of *Pseudomonas aeruginosa* (AF279793). The translated BLAST results showed 98 per cent amino acid homology with reported *chiC* of *S. marcescens* of (BAA76623.1), 97 percent homology with reported *chiC* of *S. marcescens* (CAF74787) and 98 per cent homology with chitinase A of *Vibrio harveyi* (AAC4683).

Analysis of cloned 1461 bp for c*hiC* sequence analysis using NCBI tool BLASTn revealed 1442bp open reading frame (ORF) from 9 to 1451 bp in *chiC*. The ORF map of pNKK1202 is presented in Fig 11.

Search for conserved domains revealed *chiC* domain of 234 amino acids is similar to glyco-domain and 323 amino acids similar to glycosyl hydrolase domain . Other domains identified were 93 amino acid stretch similar to Fibronectin type-III domain, 312 amino acid stretch to chitinase (carbohydrate transport and metabolism) domain and 138 amino acids similar to chitin binding domain type-III (fig 10). *chiC* sequence has no restriction sites for common enzymes when it was subject to analyse the restriction sites using BT1 gene tool software. The complete restriction maps are shown in Fig 12.

gnl|CDD|25596 pfam00704, Glyco_hydro_18, Glycosyl hydrolases family 18

CD-Length = 323 residues, 100.0% aligned
Score = 76.3 bits (187), Expect = 8e-15

```
Query:  25   KILMGFWHNWAAGASDGYQQGQFANMNLTDIPAEYNVVAVAFMKGQGIPTFKPYNLSDAE  84
Sbjct:  1    GRIVGYYTQWGNY-GEGFLLEDIPTDKLTHIIYAFANIDGNGTTGYLADTEDDGSKGCFE  59

Query:  85   FRRQVGVLNSQGRAVLISLGGAD-----AHIELKTGDEDRLKDEIIRLVEVYGFDGLDID  139
Sbjct:  60   QLKDLKKCQNPGVKVLLSIGGWTFSGGFSLLLSDDAKRKTFADSIIDFLKKYGFDGIDID  119

Query:  140  LEQ-AAIGAANNKTVLPAALKKVKDYYAAQGKNFIISMAP------EFPYLRTNGTYLDY  192
Sbjct:  120  WEYPGARGDKDNYTLLLKELREALKKEAKAGYLLSAAVPAGPIKLDGLYDIAKIGKYLDF  179

Query:  193  INAL-------EGYYDFIAPQYYNQGGDGIWVDE-LNAWITQNNDAMK-----------  232
Sbjct:  180  INVMTYDFHGWSNITGPNAPLY-----DGSNVDYTVQYYLKAGVPASKLVLGIPFYGRGW  234

Query:  233  --------EDFLYYLTESLVTGTRGYAKIPAAKFVIGLPSNNDAAATGYVIDKQAVYNAF  284
Sbjct:  235  TLVNGSGNGGGAPAGPGTKAGGILSYKELCALVKSGATPTYDDTAKAPYIYKGDTHFVSY  294

Query:  285  ARLD--------AKSLSIKGLMTWSINWD  305
Sbjct:  295  DDPRSIKAKAKYVKDKNLGGVMIWSLDQD  323
```

gnl|CDD|12797 COG3469, COG3469, Chitinase [Carbohydrate transport and metabolism]

CD-Length = 332 residues, 94.3% aligned
Score = 447 bits (1152), Expect = 1e-126

```
Query:  18   MPSIANKKILMGFWHNWAAGASDGYQQGQFANMNLTDIPAEYNVVAVAFMKGQG-IPTFK  76
Sbjct:  20   MPDISNR-VLVGYWHNWKSGAADGYQQGSSADIALADTPRNYKVVTVSFMKGAGDIPTFK  78

Query:  77   PYNLSDAEFRRQVGVLNSQGRAVLISLGGADAHIELKTGDEDRLKDEIIRLVEVYGFDGL  136
Sbjct:  79   PYNDPDAEFRAQVGALNAEGKAVLLSLGGADGHIELKAGQEQAFVNEIIRLIETYGFDGL  138

Query:  137  DIDLEQAAIGAANNKTVLPAALKKVKDYYAAQGKNFIISMAPEFPYLRTNGTYLDYINAL  196
Sbjct:  139  DIDLEQSAILAADNQTVIPAALKAVKDHYKNQGKNFFITMAPEFPYLQGWGAYIPYINEL  198

Query:  197  EGYYDFIAPQYYNQGGDGIWVDELNAWITQNNDAMKEDFLYYLTESLVTGTRGYAKIPAA  256
Sbjct:  199  RDYYDFIAPQLYNQGGDGNWVTESNAWIAQNNDMVKESFLYYLTFSLANGTRGFEKIPAD  258

Query:  257  KFVIGLPSNNDAAATGYVIDKQAVYNAFARLDAKSLSIKGLMTWSINWDNGKSKAGVAYN  316
Sbjct:  259  KFAIGLPSNVDAAATGYVKDPNIVDNAFNRLKATGCNIKGVMTWSVNWDAGKNSDGENYN  318

Query:  317  WEFKTRYAPLIQGG  330
Sbjct:  319  NPYGNKYAPMNNAQ  332
```

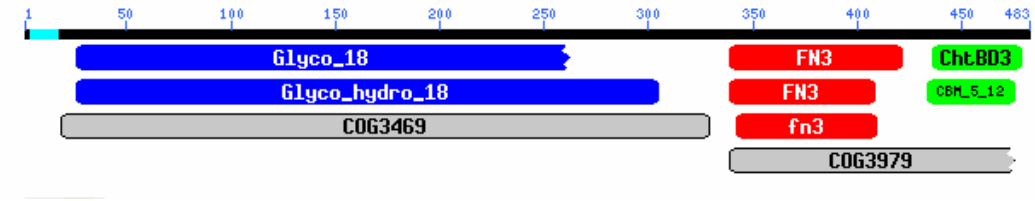

Fig 10. Conserved domain of *chiC* gene

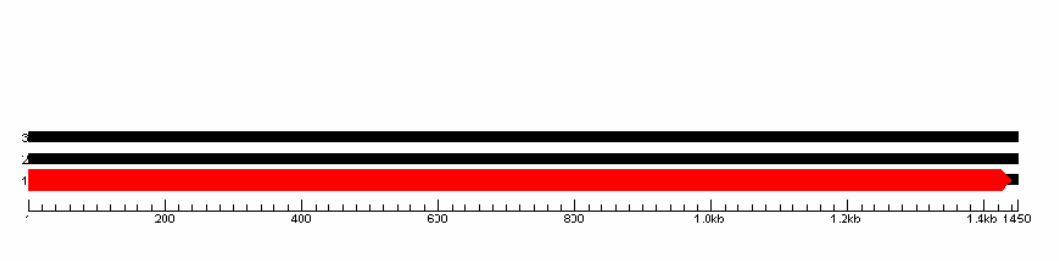

Fig 11. Open reading frame *chiC* gene

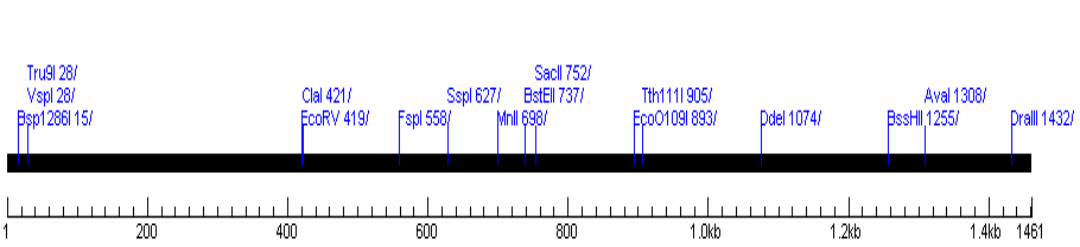

Fig 12. Restriction sites present in *chiC* gene for commercial enzymes

4.5 CLONING INTO PROKARYOTIC EXPRESSION VECTOR

The *chiA, chiB* and *chiC* gene were released by restriction of pNKK0901, pNKK2502and pNKK1202 and subcloned into *Nco1* and *BamH1* sites of the expression vector pET28a (+). The vector and insert from pNKK0901, pNKK2502and pNKK1202 were ligated at 1:3 molar ratio, were used.

The transformants in *E.coli* BL 21 were picked and streaked on Luria agar plates containing 50 µl/ml kanamycin. The pNKK2603, pNKK0207 and pNKK1004 clones containing *chiA*, *chiB* and *chiC*, respectively, were confirmed by PCR with *S. marcescens* total DNA as positive control and pET28a(+) as negative control. The recombinants and *S. marcescens* total DNA gave amplicon of 1691bp, 1499bp and 1442bp respectively, while in pET28a(+) vector, there was no amplification (Plate 7). These clones were also confirmed by restriction digestion with *Nco1* and *BamH1* (Plate 8, 9 and 10).

The maps of pNKK2603, pNKK0207 and pNKK1004 are presented in Fig 13, 14 and 15 respectivly.

4.6 EXPRESSION STUDIES

To check the expression of the cloned *chiA ,chiB and chiC* genes in *E. coli* BL21, the total protein from IPTG induced *E. coli* BL21, BL-21 having pET28a(+) and BL21 with recombinant vectors were subjected to SDS-PAGE. Differential banding pattern in all *chiA* recombinants was observed. The protein band corresponding to approximately 58.0 kDa indicated that the *chiA* gene expressed in *E. coli* (plate 11).

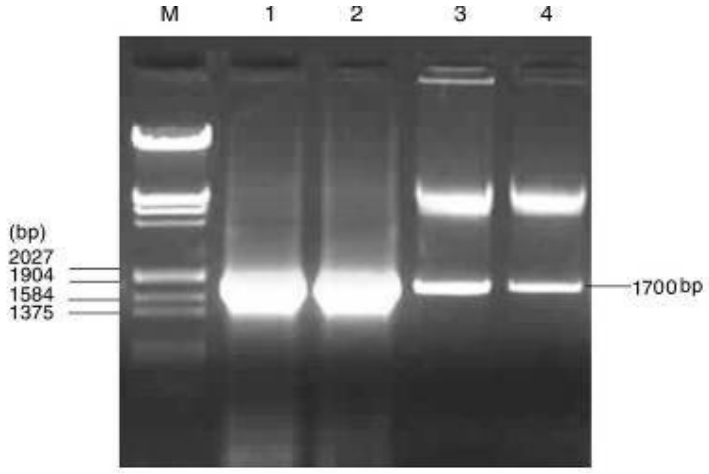

Plate 08. PCR and restriction analysis of pNKK2603

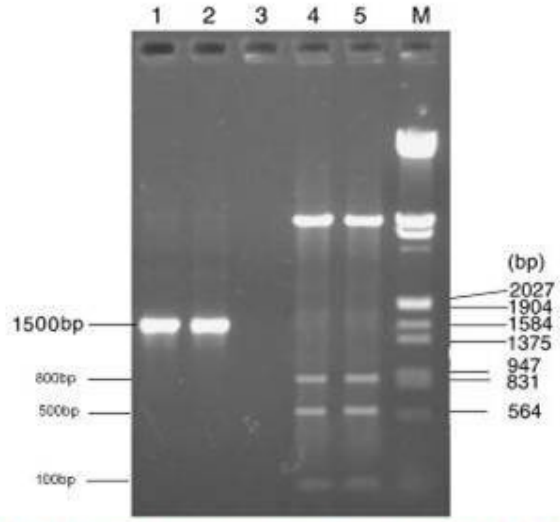

Plate 09. PCR and restriction analysis of pNKK0207

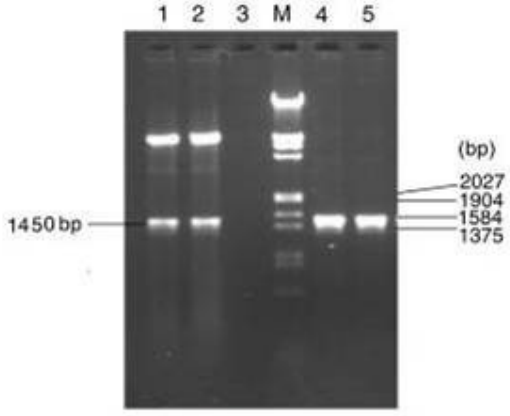

Plate 10. PCR and restriction analysis of pNKK1004

Plate 8. PCR and Restriction analysis of pNKK2603

M. Lambada DNA/ *EcoRI* + *HindIII* marker

PCR

1. pNKK2603 A
2. pNKK2603 B

Restriction

3. pNKK2603 A
4. pNKK2603 B

Plate 9. PCR and Restriction analysis of pNKK0207

M. Lambada DNA/ *EcoRI* + *HindIII* marker

PCR

1. pNKK0207 A
2. pNKK0207 B
3. pET28(+) control vector

Restriction

4. pNKK0207 A
5. pNKK0207 B

Plate 10. PCR and Restriction analysis of pNKK1004

M. Lambada DNA/ *EcoRI* + *HindIII* marker

Restriction

1. pNKK1004 A
2. pNKK1004 B
3. pET28a(+) control vector

PCR

1. pNKK1004 A
2. pNKK1004 B

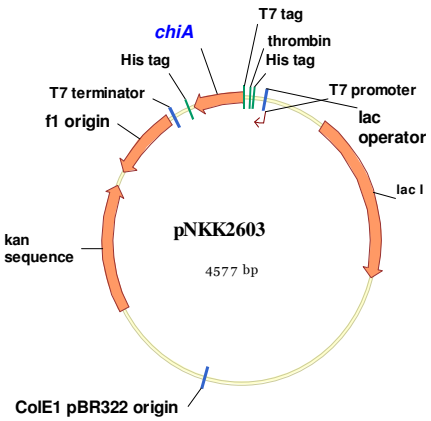

Fig 13. Construct map of pNKK2603 containing full length *chiA* gene in pET28a(+)

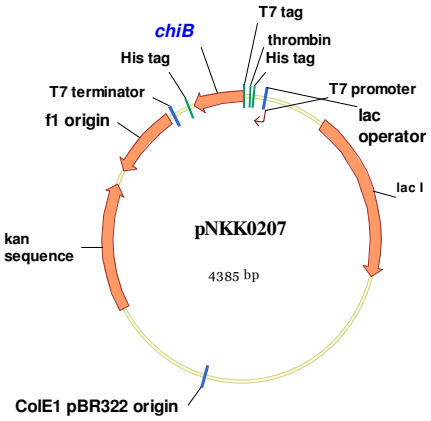

Fig 14. Construct map of pNKK0207 containing full length *chiB* gene in pET28a(+)

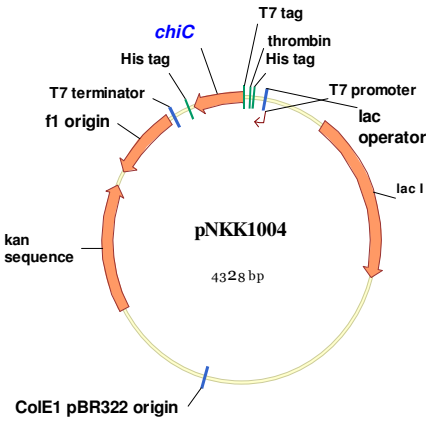

Fig 15. Construct map of pNKK1004 containing full length *chiC* gene in pET28a(+)

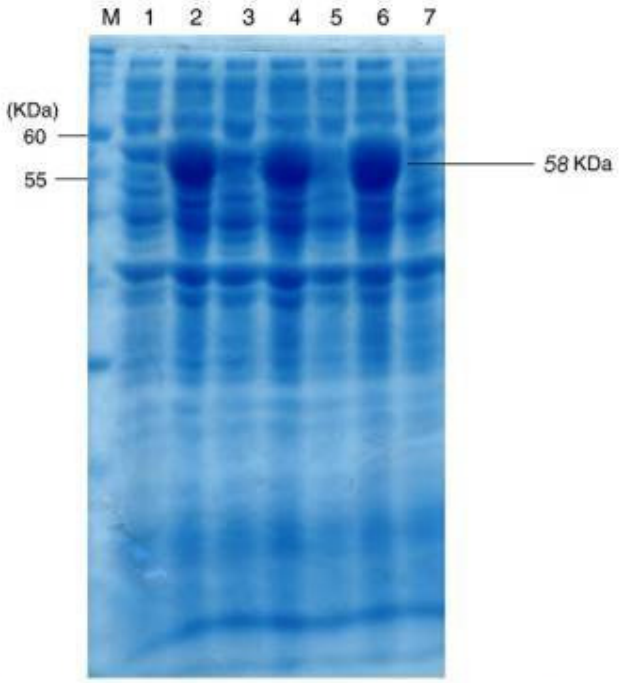

Plate 11. SDS - PAGE analysis for expression of *chiA* gene

Plate 12. Plate assay for expression of *chiA* gene

Plate 11. SDS-PAGE analysis for expression of *chiA* gene

M. Broad range protein marker

1. Induced BL21 (pET28a(+1)
2. Induced pNKK02603 A
3. UnInduced pNKK02603 A
4. Induced pNKK02603 B
5. BL21
6. Induced pNKK02603 C
7. UnInduced BL21 (pET28a(+))

Plate 12. Plate assay for expression of *chiA* gene

1. Induced pNKK02603
2. Induced BL21 (pET28a(+))
3. Induced BL21

4.6.1 Expression studies by chitin plate assay

After confirming the expression through SDS-PAGE the *chiA*, *chiB* and *chiC* clones were grown on media containing 0.5% colloidal chitin as carbon source. The clearing zone was formed around the *chiA* clone (pNKK2603). But there was no such zone around the control (*E.coli, E. coli* with pET-28), *chiB* and in *chiC* clones, when incubated at 37^{o} C up to three days (Plate 12).

4.7 SUBCLONING INTO PLANT TRANSFORMATION VECTOR

The plasmid DNA of pNKK0901, pNKK1202 and the plant transformation vector pHS100 was isolated in quantities and restricted with *Xba*I and *Bam* HI to facilitate directional cloning. The linearized vector was ligated with inserts from pNKK0901 and pNKK1202 at 1:3 molar concentration and transformed into *E. coli* DH5α cells. The transformants were picked and streaked on Luria agar containing kanamycin (50 μg/ml). Plasmid DNA isolated from these clones were confirmed through PCR and restriction analysis using *Xba*I and *Bam*HI enzyme (Plate 13 and 15) and named as pNKK0205 and pNKK2505 clones.

For cloning *chiB* into pHS100, a different strategy was used, as *chiB* contained a *Bam*HI internal site. Plasmid of pHS100 was restricted completely with *Xba*I and *Bam*HI and the pNKK2502 was amplified with a set of new primers having flanking *Xba*I and *Bam*HI sites. The amplicon was purified and restricted with *Xba*I enzyme completely and then partially with *Bam*HI and the 1499 bp band was eluted and ligated with pHS100 vector. The transformants were identified as described before and confirmed (plate 14). The confirmed clone was named as pNKK1008. The restriction maps of pNKK0205, pNKK1006 and pNKK2505 are presented in Fig 16, 17 and 18 respectivly.

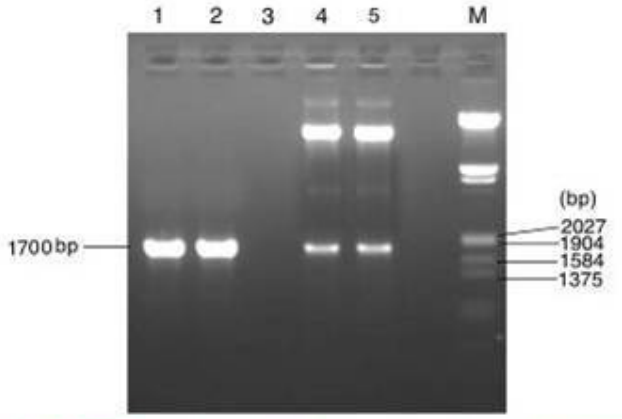

Plate 13. PCR and restriction analysis of pNKK0205

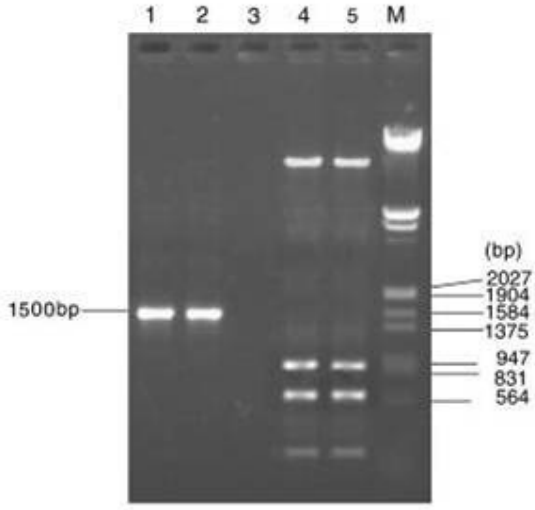

Plate 14. PCR and restriction analysis of pNKK1006

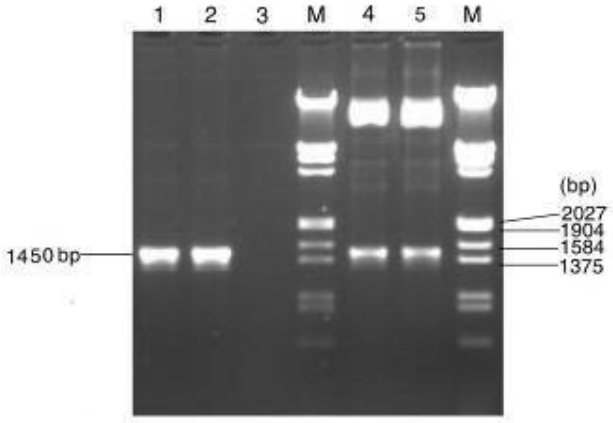

Plate 15. PCR and restriction analysis of pNKK2505

Plate 13. PCR and Restriction analysis of pNKK0205

M. Lambada DNA/ *EcoRI* + *HindIII* marker

PCR

1. pNKK0205 A
2. pNKK0205 B
1. pET28(+) control vector

Restriction

3. pNKK0205 A
4. pNKK0205 B

Plate 14. PCR and Restriction analysis of pNKK1006

M. Lambada DNA/ *EcoRI* + *HindIII* marker

PCR

1. pNKK1006 A
2. pNKK1006 B
3. pHS100

Restriction

4. pNKK1006 A
5. pNKK1006 B

Plate 15. PCR and Restriction analysis of pNKK2505

M. Lambada DNA/ *EcoRI* + *HindIII* marker

PCR

1. pNKK2505 A
2. pNKK2505 B
3. pET28(+) control vector

Restriction

4. pNKK2505 A
5. pNKK2505 B

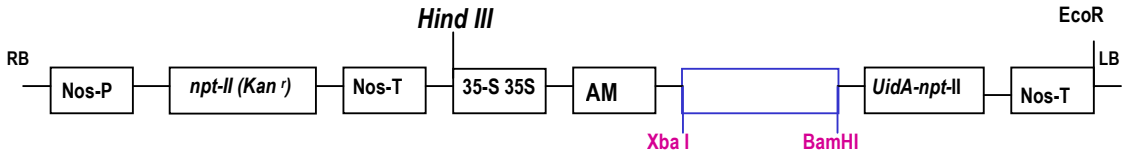

Fig. 16. T-DNA cassette of pNKK0205 containing *chiA* in plant transformation vector pHS100

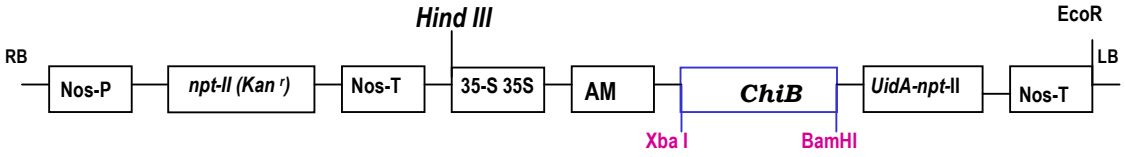

Fig. 17. T-DNA cassette of pNKK1006 containing *chiB* in plant transformation vector pHS100

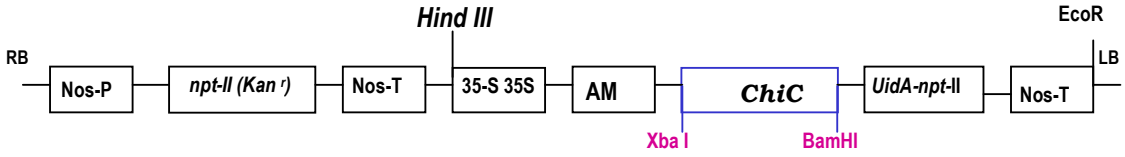

Fig. 18. T-DNA cassette of pNKK2505 containing *chiC* in plant transformation vector pHS100

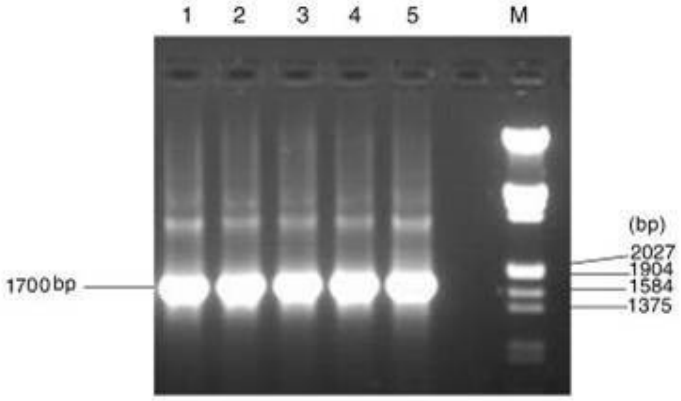

Plate 16. PCR confirmation of pNKK0205 in *Agrobacterium*

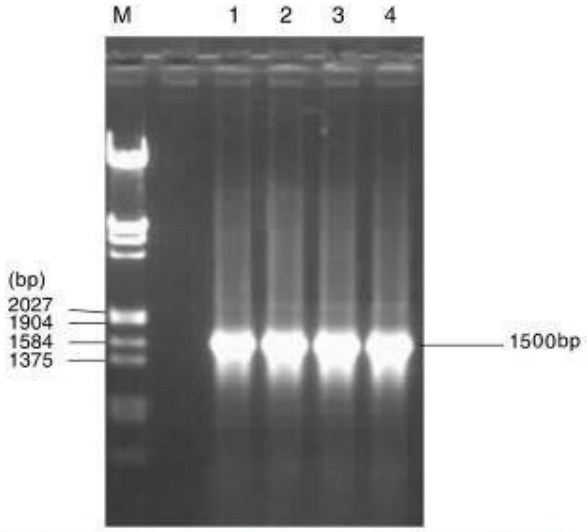

Plate 17. PCR confirmation of pNKK1006 in *Agrobacterium*

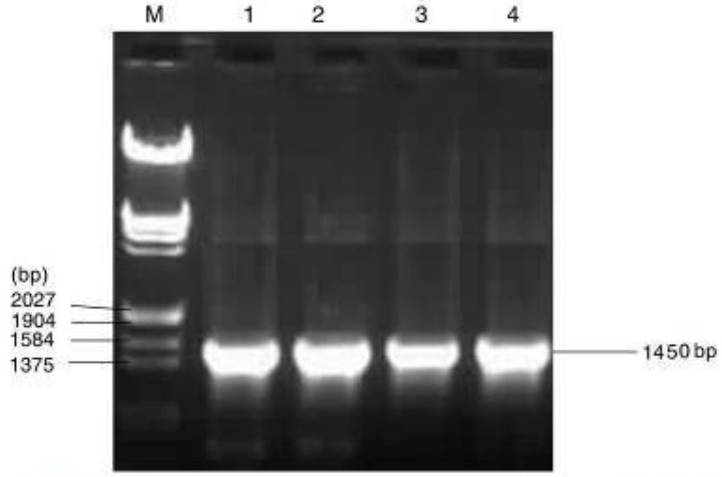

Plate 18. PCR confirmation of pNKK2505 in *Agrobacterium*

Plate 16. PCR confirmation of pNKK0205

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. pNKK0205 A
2. pNKK0205 B
3. pNKK0205 C
4. pNKK0205 D
5. pNKK0205 E
6. Negative control (*Agrobacterium*

Plate 17. PCR confirmation of pNKK1006

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. Negative control (*Agrobacterium*
2. pNKK1006 A
3. pNKK1006 B
4. pNKK1006 C
5. pNKK1006 D

Plate 18. PCR confirmation of pNKK2505

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. pNKK0205 A
2. pNKK0205 B
3. pNKK0205 C
4. pNKK0205 D

4.8 TRANSFER OF RECOMBINANT PLANT TRANSFORMATION VECTOR TO *Agrobacterium tumefaciens* STRAIN LBA4404

pHS100 constructs of *chiA* (pNKK0205), *chiB* (pNKK1006) and *chiC* (pNKK2505) were transferred to *A. tumefaciens* LBA4404 by tri-parental mating using *E. coli* (pRK2013) as helper strain. Patch mating in the ratios 1:1:1, 1:2:1 and 1:2:2 of donor: helper: recipient was done and all were found successful. The transconjugants were picked on YEMA containing kanamycin (50 µg/ml), stereptomycin (1200 µg/ml) and rifampicin (25 µg/ml). The recombinant clones *A. tumefaciens* (pNKK0205, pNKK1006 and pNKK2505) were confirmed through PCR amplification of the plasmids obtained from recombinant *A. tumefaciens* (Plate 16, 17 and 18).

4.8 TOBACCO TRANSFORMATION

A. tumefaciens LBA4404 (pNKK0205) was grown on YEMA with streptomycin (100 µg/ml), rifampicin (25 µg/ml) and kanamycin (50 µg/ml) at 28℃ for 24 hours. Surface sterilized tobacco leaf explants were infected with *Agrobacterium* and co-cultivated on MS medium. The treated explants produced callus and direct shoots within 3 weeks (Plate 19). Majority of the shoots and calli transferred to shooting medium turned albino on both callus induction and shooting medium. Surviving green shoots having well developed root system (Plate 20) were transferred to sterilized peat and shifted to green house (Plate 21).

DNA was isolated from putative transformants and checked for the presence of insert (Plate 22). Two of the four plants were PCR positive for *chi A* gene.

4.9 TRANSGENE EXPRESSION ANALYSIS

Total RNA isolated from both transgenic and a control tobacco plant was quantified using a spectrophotometer at 260 nm. Equal amount of RNA was used for amplification of transgene by using specific primers for *chiA* gene. One step RT-PCR amplification standard schedule was performed on both transgenic and control tobacco plants. RT-PCR amplicon of 1700 bp was observed in transgenics and it was absent in control plants (plate 23). This implies expression of transgene at transcription level. The PCR positive plants are being maintained for further studies.

Plate 19. Callus and direct shoot regeneration

Plate 20. Plants in rooting medium

Plate 21. Putative transgenic tobacco plants with *chiA* gene

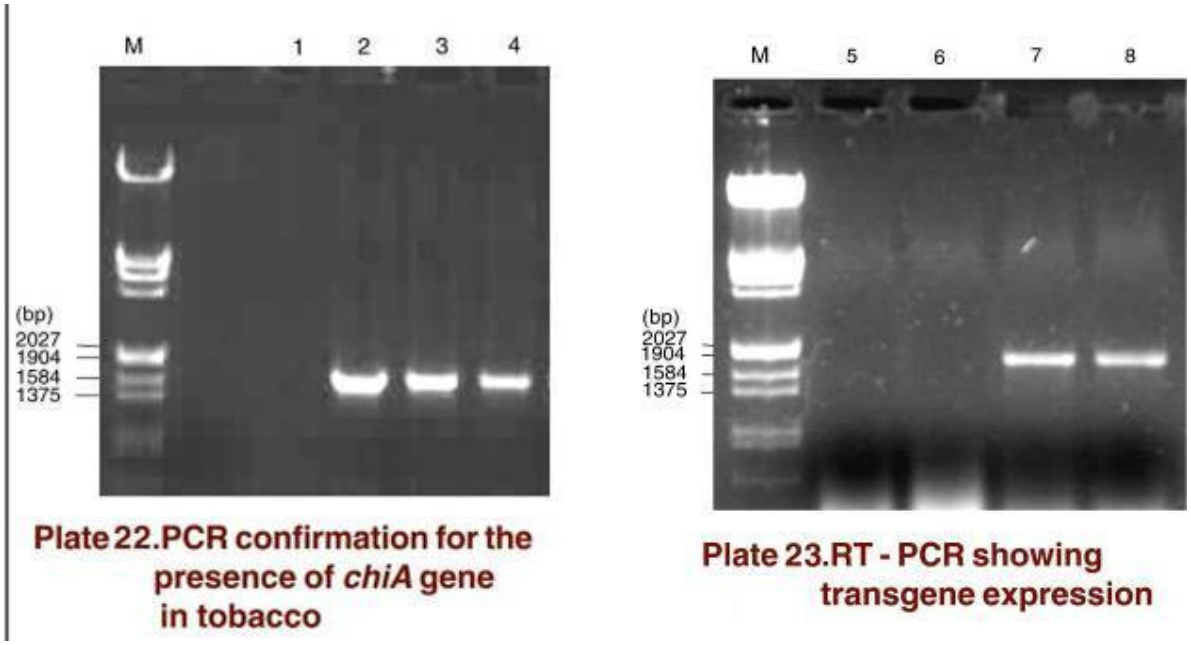

Plate 22.PCR confirmation for the presence of *chiA* gene in tobacco

Plate 23.RT - PCR showing transgene expression

Plate 19. Callus and direct shoot regeneration

Plate 20. Plants in rooting medium

Plate 21. Putitive transgenic tobacco plants with *chiA* gene

Plate 22. PCR confirmation for the presence of *chiA* gene in tobacco

M. Lambada DNA/ *EcoRI* + *HindIII* marker

1. Negative control (Untransformed tobacco plant)
2. S. marcescens Genomic DNA
3. Putitive tobacco plant 1
4. Putitive tobacco plant 2

Plate 23. RT-PCR showing transgene expression.

M. Lambada DNA/ *EcoRI* + *HindIII* marker

5. Negative control (Untransformed tobacco plant)
6. Negative control (Untransformed tobacco plant)
7. Transgenic plant 1
8. Transgenic plant 2

V. DISCUSSION

Plant diseases are important since they damage plants and plant products on which we depend for food, clothing, furniture, housing and the environment. Plant diseases have affected the existence, adequate growth and productivity of all kinds of crop plants their domestication and began to practice agriculture more than 6000 years ago (Bruehl, 1991). In developed societies losses from diseases in food and feed produce result primarily in financial losses and higher prices.

Pesticides are widely used to control plant diseases. However, most pesticides are hazardous to the health of humans and animals, in addition to increasing the cost of production. They also have deleterious effects on ecological systems. Host plant resistant is the most economical environment friendly option. Genes for resistance to diseases are available in primery, secondary and tertiary germplasm for many diseases. Recent development in recombinant DNA technology has let the entire biological resources be considered as one gene pool, permitting transfer of genes across organisms. To identify genes that could be expressed in the plants to improve their resistance to fungal pathogens, a lot of basic work has been done in the area of host–pathogen recognition (Staskawicz *et al*., 1995). During the last decade, many genes, whose products are capable of interacting with invading pathogens have been identified and cloned (Takken *et al*., 2000).

Chitin, a β-1,4-linked polymer of *N*-acetyl glucosamine (GlcNAc), is an important structural component of insects, fungi, and nematodes. Several organisms, including higher plants, fungi, and bacteria, produce chitinases (EC 3.2.1.14) that cleave the glycosidic bonds in the chitin by either an endolytic or exolytic mechanism (Monreal and Reese, 1969). The first report on developing fungus-resistant transgenics came in 1991. Broglie *et al*., (1991) constitutively expressed bean chitinase gene in tobacco and *Brassica napus* and the plants showed enhanced resistance to *Rhizoctonia solani*. *Serratia marcescens* 2170, an active producer of chitinase and an excellent model for studying the degradation and utilization of chitin (Palomar *et al*., 1990) is also a good source of chitinase.

Six strains of *S. marcescens* were collected from the Department of Agricultural Microbiology, college of Agriculture Dharwad and were screened for their chitinolytic activity by growing on colloidal chitin plate in which chitin was the sole carbon source. The isolate *S. marcescens* 141 was found to be very effective in producing the chitinase enzyme. A gene encoding a chitinase from *S. marcescenns* BJL200 has been cloned and expressed in *E. coli* (May *et al*., 1995). Screening *S. maltophilia* 34S1 genomic library for chitinase activity in *B. cepacia* M53 resulted in the identification of two cosmid clones that caused bacterial colonies to clear colloidal chitin in agar within 5 days (Donald *et al*., 2002). The database at http://www.ncbi.nlm.nih.gov also houses sequences of chitinase encoding genes from different sources, Based on available information, specific primers were designed for *chiA, chiB* and *chiC.* About 1700bp, 1500bp and 1450bp amplicons picked up for *chiA, chiB* and *chiC* were cloned into pTZ57R/T and transformants having recombinants were isolated through blue-white assay. The white colonies were confirmed as recombinants by gene specific PCR and through restriction analysis. The inserts in clones pNKK0901 (*chiA*), pNKK1202 (*chiC*) were fully sequenced which yielded 1700 bp and 1450 bp, respectively. pNKK2502 (*chiB*) was partially sequenced (600 bp).

The genes encoding chitinase have been cloned extensively. Jones *et al*., (1986) cloned chiA from *S. marcescens*. It has 1699 bp and codes for 565 amino acids. The endochitinase gene *chiA74* from *Bacillus thuringiensis* serovar kenyae strain LBIT-82 was cloned in *Escherichia coli* DH5 and sequence of 676 amino acids was deduced (Barboza *et al*., 2003).

The comparative analysis showed that the nucleotide sequence of cloned *chiA* gene has 99 (1685/1692) per cent homology with *Serratia marcescens* (AB01599) gene for chitinase A precursor, 98 (1673/1692) percent homology with endo-chitinase(*chiA*) gene of *S. marcescens* (AF454462), 96 (1640/1692) per cent with chitinase (*chiA*) gene of *Enterobacter sp* (DQ013365) NRG-4 and 87 (1483/1692) percent homology with *chit60* of *S. plymuthica* (SPL488913).

In silico translated amino acid sequence (563 amino acids) of cloned *chiA* in this study has 99 (560/563) per cent homology with chitinase A precursor of *S. marcescens* (BAA31567], 95 (536/561) percent with endochitinase A of *S. liquefaciens* [AAK07482] and 92 (523/563) percent with chitinase of *S. plymuthica* (CAD32933].

Similarly the comparative analysis showed that the nucleotide sequence of cloned *chiC* gene has 99(1449/1461) percent homology with *S. marcescens chiC* gene (AB019238), 90 (30/33) percent homology with chitinase (chit37) gene of *Trichoderma harzianum* (AF525753), 96 percent with endochitinase *ChiA* precursor of *Vibrio cholerae* (AF097314) and 91 per cent with chitinase D precursor of *Bacillus circulans* (BACCHIDA).

The translated amino acid sequences (480 amino acids) of cloned *chiC* has 98 (475/480) percent homology with chitinase C of *Serratia marcescens* [BAA76623]. Similarly, partial nucleotide sequence of *chiB* has 99 percent homology with reported *chiB* nucleiotide and amino acid sequences.

The *chiA* amino acid sequence has conserved domain that is similar to Glyco_domain and glycosyl hydrolase's domain. The sequence of *chiA* amplicon of 1721 bp had an ORF of 1691 nucleotides.

The *chiC* amino acid sequence had conserved domain similar to glyco_domain, carbohydrate binding domain, chitinase [carbohydrate transport and metabolism], fibronectin type III domain and glycosyl hydrolase domain. The sequence of *chi*C amplicon of 1461bp had an ORF of 1442bp.

As pTZ57R is more of a cloning vector, these chitin genes were further subcloned into a prokaryotic expression vector pET28a(+), to study their expression in *E. coli* BL21 (plysS). The recombinant clones were analysed by PCR amplification and restriction. The confirmed *E. coli* containing pNKK2603, pNKK0207 and pNKK1005 were subjected to SDS-PAGE. The protein bands corresponding to 58.5 kDa was observed for *chiA,* indicating expression of the cloned gene.

Clear zone was produced around the colonies of recombinant clones of *chiA* on colloidal chitin plate, but no clear zone around the control colonies and in case of *chiB* and *chiC* clones. It is possible that they may not be produced in sufficient quantity, for detection by SDS-PAGE. It is necessary to purify the protein. Similar results were observed by Suzuki *et al.*, (2002). The individual role of chitinases from *Serratia marcescens* 2170, chitinse A, B and C1 were produced in *Escherichia coli* and their enzymatic effect on chitin degradation were studied by Suzuki *et al.*, (2002) and found that *chiA* was the most active enzyme in hydrolyzing insoluble chitin among the three chitinases.

Further, the genes *chiA*, *chiB* and *chiC* isolated during the study were directionally cloned into a plant transformation vector pHS100, under CaMV35S tandem promoter at *Xba* I and *Bam* HI sites. The ligated product was transferred into *E. coli* DH5α. As *chiB* sequence has an internal *Bam* HI site, the amplicon was completely restricted with *Xba* I enzyme first and then partially restricted with *Bam* HI to secure a *Xba* I and *Bam* HI DNA fragment of about 1.5 kbp was eluted and cloned in pHS100. The recombinant pHS100 clones obtained were confirmed for the insert by size through PCR amplification and restriction digestion with *Xba* I and *Bam* HI.

The confirmed clones were transferred to *Agrobacterium tumefaciens* LBA4404 by triparental mating. The *Agrobacterium,* with the construct of *chiA* pNKK0205 was used to transform tobacco by following protocol from Hooykaas and Suchilperoort (1992). The plants which were rooted on medium with kanamycin (200µg/ml) were checked for the presence of inserts and more than 50 per cent plants were found to be PCR positive. The transgene expression was checked by one step RT-PCR. RT-PCR amplicon of 1700 bp was observed in transgenics and no band was seen in control plants. This implies expression of transgene at transcription level. The intensity of amplicon is a direct indication of transcription of the transgene. However, there is a need to do a detailed analysis of gene expression in these plants by southern confirmation, SDS-PAGE and bioassy by challenge infection by pathogenic isolates of fungal diseases of tobacco.

The utility of *S. marcescens* (Howie *et al*., 1994) and other chitinases have been demonstrated using transgenic tobacco. while *S. marcescens chiA* increased tolerance to *R. solani* (Howie *et al*., 1994), *Saccharomyces cerviciae CTCI* gene showed decreased in susceptibility to ranging from 50-70 percent to *Botrytis cinerea* in transgenic tobacco.

The usefulness of the cloned *chiA* construct developed during the study needs to be explored in specific crops and checked against *R. solani* and other phytopathogenic fungi. Microbes are a good source of genes for chitinases. It is necessary to clone a large number of such genes from different sources, study their effectiveness against different pathogens and biosafety and then deploy them in crop plants.

VI. SUMMARY

The most significant development in the area of varietal development for disease resistance is the use of the techniques of gene isolation and genetic transformation to develop transgenics resistant to fungal diseases. Improvements in genetic transformation technology have allowed the genetic modification of almost all important food crops. Antifungal genes from microbe origin are useful to develop transgenic crops. Chitinase genes have proved to be important alone or in combination with other insecticidal genes like glucanase against phytopathogenic fungi. In the present study, an attempt was made to clone chitiase genes from *Serratia marcescens* and express them in *E. coli* and in tobacco. The results obtained are summarized below.

- 1691bp *chiA*, 1499bp *chiB* and 1442bp chi*C* DNA fragment of *Serratia marcescens* were amplified using specific primers designed from the reported sequences.
- The amplicons were cloned in pTZ57R/T containing T overhangs at *Eco* 321 site and *E. coli* DH5α was transformed with recombinant plasmids pNKK0901 pNKK2502and pNKK1202, respectively with *chiA*, *chiB* and *chiC.*
- The presence of inserts was confirmed by sequence analysis and nucleotide-nucleotide BLASTn search. Recombinant clones pNKK0901, pNKK2502 and pNKK1202 had 1691 bp, 1499 and 1442 bp inserts, respectively. The sequence search indicated 99 per cent homology with reported nucleotide and amino acid sequences.
- The *chiA*, *chiB* and *chiC* were cloned into prokaryotic expression vector pET28a(+). SDS-PAGE analysis of the recombinant clones containg *chiA* pNKK2503 showed the expression of a 58.5 kDa protein and there was no expression from pNKK0204 and pNKK1004.
- The *chiA*, *chiB* and *chiC* inserts were further subcloned into a plant transformation vector pHS100 to facilitate transformation of crop plants.
- The recombinant clones of pHS100; pNKK0205, pNKK1005 and pNKK2505 were mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating.
- The *A. tumefaciens* with recombinant clone having *chiA* pNKK0205 was used to transform tobacco. In two PCR positive plants, expression of a 1700 bp transcript was confirmed by one step RT-PCR.
- The transgenic plants need to be checked for tolerance to fungal pathogens.

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APPENDIX I

Extraction buffer

0.35M	-	Sorbitol
0.1M	-	Tris-HCL (pH 7.5)
5.0mM	-	EDTA
20.0mM	-	SDS

Lysis buffer

0.1M	-	Tris-HCL (pH 7.5)
20.0mM	-	EDTA
2.0M	-	NaCl
55.0mM	-	CTAB

For extraction buffer (10ml)

Polyviny Pyrrolidone(PVP)	-	100mg
Dextrose anhydrous AR	-	900mg
Sodium hydrogen sulphite EP	-	40gm

APPENDIX II

a. Loading dye composition

Loading dye (6x)	:	0.25% bromophenol blue 40% (w/v) sucrose 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

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

APPENDIX IV

a. Ligation reaction recipe

Plasmid vector pTZ57R/T DNA (0.165 µg, 0.18 pmol ends)	30 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

APPENDIX IX

Composition of yeast extract mannitol agar (YEMA)

For 100ml:

D-Mannitol	-	1g
KH_2PO_4	-	20mg
K_2HPO_4	-	20mg
Yeast Extract	-	100mg
$MgSo_4.7H_2O$ (1M)	-	80µl
$CaCl_2$ (1M)	-	40 µl
Agar	**-**	**1.8g**

APPENDIX V

a. Components of luria agar (LA)

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

Luria agar Amp_{50} : To 100 ml Luria agar 50 µl of Amp_{100} (antibiotic) was added at 50°C.

Luria agar Amp_{100} : To 100 ml Luria agar 100 µl of Amp_{100} (antibiotic) was added at 50°C.

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 0^0C. 40ul/plate was used.

b. Recipe for 0.7 per cent agarose gel (40 ml)

Agarose - 280 mg

1x TAE - 40 ml

EtBr (10 mg/ml) - 2 µl

APPENDIX VI

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)	: 10 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 VII

Components of restriction of PCR amplicon and pHS-100 vector

PCR amplicon DNA	:	6 µl	
Enzyme *Xba* I (5U)	:	2 µl	
10x buffer		:	2 µl
1x BSA	:	2µl	
Sterile water	:	8µl	
Total		:	20 µl

APPENDIX VIII

Components of ligation of *chiA* insert and pHS-100

chiA insert	3 µl
Plasmid DNA (pHS-100)	1.5 µl
T_4 DNA ligase enzyme (10 U)	1 µl
Buffer (10 x)	1 µl
Sterile H_2O	3.5 µl
Total	10 µl

Components of ligation of *chiB* inserts and pHS-100

chiB insert	4 µl
Plasmid DNA (pHS-100)	2 µl
T_4 DNA ligase enzyme (10 U)	1 µl
Buffer (10 x)	1 µl
Sterile H_2O	2 µl
Total	10 µl

Components of ligation of *chiC* insert and pHS-100

chiC insert	3 µl
Plasmid DNA (pHS-100)	1.5 µl
T_4 DNA ligase enzyme (10 U)	1 µl
Buffer (10 x)	1 µl
Sterile H_2O	3.5 µl
Total	10 µl

APPENDIX X

Components of Murashige and Skoog (1962) medium (modified)

	Component	mg/l concentration
Macronutrients	NH_4NO_3	1650.00
	KNO_3	1900.00
	$MgSO_4 . 7H_2O$	370.00
	KH_2PO_4	170.00
	$CaCl_2 . 2H_2O$	440.00
Micronutrients	$FeSO_4 . 7H_2O$	27.80
	Na_2 EDTA	60.00
	$MnSO_4 . 4H_2O$	22.30
	$ZnSO_4 . 7H_2O$	8.60
	H_3BO_3	6.30
	KI	0.83
	$Na_2MoO_4 . 2H_2O$	0.25
	$CuSO_4 . 5H_2O$	0.025
	$CoCl_2 . 6H_2O$	0.025
Organics	Thiamine HCl	10.00
	Pyridoxine HCl	1.00
	Nicotinic acid	1.00
	Glycine	10.00
	Myoi*nos*itol	100.00
	Biotin	0.50

APPENDIX XI

Extraction buffer for DNA isolation (RAPID method)

Tris-HCl (pH 7.5)	-	200mM
NaCl	-	250ml
EDTA (pH 8.00)	-	25mM
SDS	-	0.5%

ABBREVIATIONS

ABA	Abscisic acid
BARC	Baba Atomic Research Centre
BCA's	Biological control agent
bp	Base pair
Bt	*Bacillus thuringiensis*
cDNA	Complementary DNA
CDS	Coding DNA sequences
ChBD	chitin binding domain
CTAB	N-Cetyl N, N, N, - trimethyl ammonium bromide
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
Gly	Glycine
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria broth
M	Molar
mM	Milimoles
MS	Murashige and Skoog
µl	Micro liter
nM	Nanomoles
OD	Optical density
ORF	Open reading fragment

PCR	Polymerase chain
PGIP's	Polygalactouridinase inhibitor proteins
P^H	Hydrogen ion concentration
pM	Picomoles
	Polymerase Chain Reaction
PR	Pathogenesis related proteins
PVP	Polyvinyl pyrrolidone
rmp	Rotation per minute
RT-PCR	Reverse transcription
SA	Salicyclic acid
SDS	Sodium deodecyl sulphate
T_{10}	Tris 10ppm
TAE	Tris acetic acid
UV	Ultraviolet
V	Volts
YEM	Yeast extract mannitol
YEMA	Yeast extract mannitol agar

Amino acids

A	Alanina
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	leusine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

CLONING AND CHARACTERIZATION OF CHITINASE GENES FROM NATIVE ISOLATES OF *Serratia marcescens*

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Abstract

Serratia marcescens, a chitinolytic bacterium, produces chitinases capable of degrading fugal cell wall. Chitinases are known to be involved in resistance to fungal diseases in plants. In the present study, an attempt was made to isolate the genes for these enzmes from the bacterium.

Six strains of S. *marcescens* obtained from the culture collection of the Department of Agricultural Microbiology, UAS, Dharwad were screened to check their chitinolytic ability on colloidal chitin media, *S. marcescens* isolate 141 was found to be very effective. The total DNA isolated from *S. marcescens* 141 was used to clone *chiA, chiB and chiC* genes in pTZ57R/T by designing gene-specific primers to the reported sequences of *chiA, chiB* and *chiC*. Amplicons of 1.7kb, 1.5kb and 1.45kb size were obtained and confirmed through PCR and restriction analysis. These constructs were named as pNKK0901, pNKK2502 and pNKK1202. In order to express the cloned gene/s, they were sub-cloned into a prokaryotic expression vector pET28a (+) and the constructs were named as pNKK2603, pNKK0207 and pNKK1004, which contained *chiA, chiB* and *chiC* respectively. SDS-PAGE analyses showed that *chiA* expressed as a 58.0 kDa protein. The *chiA, chiB and chiC* genes were further sub-cloned into plant transformation vector pHS100 and the constructs were named as pNKK0205, pNKK1006 and pNKK2505, respectively. They were transferred to *A. tumefaciens* LBA4404 by tri-parental mating using *E. coli* (pRK2013) as helper strain. The *A. tumefaciens* with recombinant clone having *chiA* pNKK0205 was used to transform tobacco and two plants were found to be PCR positive. These plants were further confirmed by one step RT-PCR.