CLONING AND EXPRESSION OF *chi*A FROM NATIVE *Serratia marcescens* AUDS227

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1. INTRODUCTION

The increasing world population will require an estimated additional agricultural production of $2.4x10^9$ tons/year. However, this additional production will not be based on an increase in the arable surface taken from temperate or rain forest, but on the improvement of crop productivity. This can be achieved in part by suitable control of losses due to biotic agents such as fungal pathogens, pests and weeds (Montesinos, 2003).

Disease management is a necessary condition for effective cultivation and preservation of crops. Methods using chemicals to support the increase in agricultural production currently prevail but are a serious threat to environment, secure food production and deteriorate the planet's health because of non-target effects (Montesinos, 2003). The development of resistance of pathogens to most commonly used synthetic fungicides have spurred the search for alternative disease management strategies that would reduce reliance on synthetic fungicides (Yaman, 2003; Rausell *et al*., 2004).

New methods of crop protection are based on historical observations in agriculture and forestry of the benefits obtained from naturally occurring microbial communities which exert a biological control of diseases. Biological control is sustained by beneficial interactions resulting from competition, antagonism and hyperparasitism of certain microorganisms against plant pathogens (Montesinos, 2003). 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).

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; Zhang and Yuen, 2001). Cell walls of most pathogenic microorganisms contain a considerable amount of chitin. Hence, the use of chitinases, that catalyze decomposition of chitin molecules, is a promising approach to control the pathogen.

Advances in molecular biology have laid the foundation for mining of valuable genes and their transfer to target plants through transgenic approach. Chitinase encoding genes are being used to improve plant defence against fungal pathogens. Bacteria *Serratia marcescens* are the most efficient in producing chitinases and excellent model for studying the degradation and utilization of chitin (Ferrer *et al*., 1996).

Several chitinases from bacteria have been cloned and expressed in *E. coli* (Sitrit *et al*., 1995). Transgenic tobacco plants expressing high levels of *S. marcescens chi*A exhibited increased tolerance to *Rhizoctonia solani* compared to untransformed control plants (Howie *et al*., 1994).

In the light of above background, this research programme is focused on:

1. Molecular characterization of *chi*A, *chi*B and *chi*C genes in native isolates of *S. marcescens.*

2. Cloning of novel *chi*A from *S. marcescens* and expression studies of cloned *chi*A in *E. coli.*

2. REVIEW OF LITERATURE

2.1 Fungal diseases and their impact

Fungal pathogens cause significant crop losses accounting to several billion dollars per annum. Control of diseases in agricultural and horticultural crops is vital with food shortages experienced in many regions as well as demand for improved efficiency in food production coupled with environmental protection (Johnson, 1992). Loss of fertility in soils due to improper management and erosion threatens to limit production of food crops in many areas of the world (Logemann and Schell, 1993). In addition some plant pathogens, including many causing root diseases, such as *Pythium* sp. or *Rhizoctonia* sp. infest soil to a point where it is not economical to grow a principal crop unless the soil is fumigated or crop rotations are established. Debilitating diseases weaken crops, resulting in depressed yields and/or poor quality. Plant diseases caused by fungal pathogens may also wipe out a crop completely (Lucas *et al*., 1992).

Disease management expenses constitute one of the major costs associated with crop production (Bridge *et al*. 2004). Several general approaches are under taken to control fungal diseases (a) management of agricultural land, (b) use of fungicides, and (c) breeding of resistant crop varieties.

Management includes chemical fallow, or soil cultivation that are expensive to implement and may cause undesirable side effects such as soil erosion (Leong, 2004). Furthermore, despite the great advances in chemical management of fungal diseases, some of the important plant pathogens causing vascular wilt, anthracnose and other root infections remain uncontrolled by the fungicides (Knight *et al*., 1997). In addition fungicides may become less effective due to the evolution of resistance among the pathogens (Faize *et al*., 2003).

Breeding for disease resistance is one method of protecting crops. Only a few resistance-genes have been shown to provide pathogen control for an extended period of time such as the cases of wheat stem rust and rice blast caused by *Puccinia graminis* and *Magnaporthe grisea* respectively (Rommens and Kishore, 2000). Traditional approaches to control epidemic spread of diseases are no longer efficient. Hence, the development of pathogen resistant plant genotypes has become an important target in plant biotechnology.

2.1.1 Disease resistance through genetic engineering

Pathogenic fungi are continually developing resistance to existing fungicides. Therefore, other methods of disease control are highly desirable (Rommens and Kishore, 2000). One such alternative is the identification of biological agents and identification of genes conferring resistance through the use of molecular biology tools for the control of plant diseases. Microorganisms with inhibitory activity against pathogens are a potential source of genes to confer disease resistance in plants (Herrera-Estrella and Chet, 1999).

Molecular techniques have facilitated the introduction of beneficial traits into model organisms to produce potential biocontrol agents (Herrera-Estrella and Chet, 1999). Also, the introduction of genetically determined traits by transformation eliminates the species boundaries that have traditionally limited germplasm sources. Research with pathogen avirulence genes has suggested that functionally similar disease resistance genes are shared between taxonomically diverse plants (Kobayashi *et al*., 1989).

Therefore cloned disease resistance genes as well as defence response genes such as those encoding for chitinases and glucanases, have important uses against pathogens that attack plant species. These genes have been cloned from a variety of sources such as bacteria, fungi and plants to engineer resistance. A number of these genes have been tested for their ability to control fungal pathogens in transgenic plants grown in the laboratory and to some extent in the field (Leong, 2004).

A number of studies have successfully demonstrated that chimeric genes can protect plants against infection by fungal pathogens (Broglie *et al*., 1991; Jach *et al*. 1995). Greater level of resistance can be achieved using combinations of genes (Jach *et al*. 1995; Zhu *et al*., 1994). The discovery of new and more efficient enzymes particularly from sources such as bacteria has become paramount to the success of engineered resistance against plant pathogens.

Thus, genetic engineering of plants for resistance has become an attractive alternative, as it represents a powerful tool for the improvement of existing cultivars and for the introduction and expression of genes outside the scope of conventional breeding (Pappinen *et al*., 2002). The most attractive candidates for manipulation of the single gene defence mechanism approach are the genes encoding chitinases because they hydrolyse chitin which are structural component of cell walls of fungi.

2.2 Fungal cell wall

Chitin and β-glucan are the main components of fungal cell walls of filamentous fungi. Chitin forms the backbone and β-1, 3-glucan (laminarin) is the filling material (Cohen-Kupiec and Chet, 1998). Fungal cell walls contain more than 605 laminarin which is hydrolysed mainly by β-1, 3-glucanases (Cohen-Kupiec and Chet, 1998).

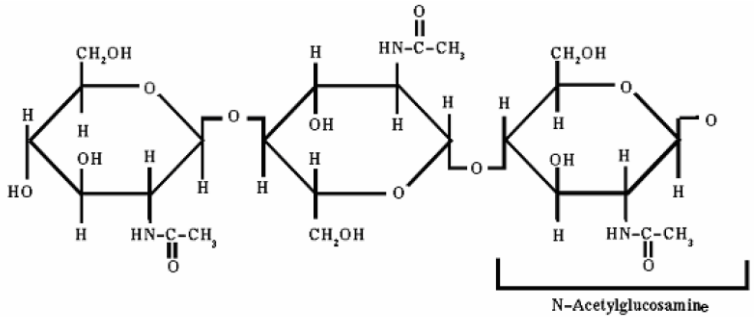


Fig. 1.1 Molecular structure of chitin. The diagram shows the *N*-acetyl –glucosamine monomers with β (1-4) linkages.

2.3 Chitin

Chitin, (1, 4)-β-linked homopolysaccharide of N-acetylglucosamine (Vaaje-Kolstad *et al*., 2004; Hoell *et al*., 2005; Sugnita *et al*., 2005) is the second most abundant biopolymer in nature next to cellulose (Vaaje and Kolstad *et al*., 2005). Chitin is present in diatoms, yeasts, fungi, protozoa, arachnids, insects, crustaceans, nematodes, other invertebrates and tunicates (Trudel and Asselin, 1989).

In insects, it is a major component of the cuticle and of the peritrophic membrane, and also functions as sleeve lining the gut of many insects (Hoell *et al*., 2005). Chitin application leads to increased populations of chitinolytic bacteria, especially actinomycetes, and fungi. These increases are correlated with reductions in pathogenic fungi and nematodes (Brown *et al*., 1982). Chitin has a remarkable compatability with living tissue, and has been looked for its ability to increase the healing of wounds. Chitosan is a polymer produced through enzymatic deacetylation of chitin (Amorim *et al*., 2001) and used in application from health care to agriculture. Hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extracellularly by the action of chitinases (Sitrit *et al*., 1995).

2.4 Chitinolytic bacteria

Some chitinolytic bacteria have been shown to be potential agents for biological control of plant diseases caused by various phytopathogenic fungi and insect species. The most notable chitinase producers among the gram-negative bacteria are *Aeromonas, Chromobacterium, Photobacterium, Pseudomonas* and *Serratia marcescens* (Thamthiankul *et al*., 2001). Among the gram-positive bacteria, chitinase producers are widespread among the *Actinomycetes*, e.g., the species of *Arthobacter* and *Streptomyces* and in spore forming genera *Bacillus* and *Clostridium* (Thamthiankul *et al*., 2001).

Alteromonas sp. strain 0-7 is a gram-negative, flagellated, motile and aerobic rod shaped bacterium of marine origin having chitinolytic 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 extra cellular environment (Watanabe *et al*., 1994). Inbar and Chet (1991) isolated a strain of *Aeromonas caviae* from soil which showed a high level of chitinolytic activity. The bacterial strain was found to secrete chitinase into the growth medium when grown on chitin as the sole carbon source. Nawani and Kapadnis (2003) reported the presence of chitin degrading potential in genera *Streptomyces, Kitasatosporia, Nocardioides, Nocardiopsis* and *Serratia*.

2.4.1 Screening microbes for chitinolytic activity

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

2.4.1.1 Substrate hydrolysis in media

Soil and aquatic systems harbor chitin degraders. Most fungi and bacteria produce chitinases only when 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, collodial chitin etc. Monreal and Reese (1969) found that *S. marcescens* (QMb1466) and a related bacterium, *Enterobacter liquefaciens* produced many times more enzymes on collodial chitin than on swollen chitin or native (wiley milled) chitin.

The bacterium *S. marcescens* strain (SR1) was found to cause a clear zone around its growth on chitin agar medium containing collodial chitin as substrate. As the bacterium starts to digest collodial chitin supplemented in agar medium, ultimately the production of red pigment was more (Parani and Saha, 2009). *S. marcescens* was found to be the most active over 100 organisms tested for the production of chitinase. 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 zone of hydrolysis.

2.4.1.2 Biochemical estimation

Chitinolytic activity was assayed by measuring the release of reducing sugars from collodial 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 p-nitrophenol (PnP) from p-nitrophenyl-*N*-acetyl-D-glucosaminide.

2.5 Antifungal molecules

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 cell wall degrading enzymes like chitinases (Grover and Pental, 2003; Ordentlich *et al*., 1988).

2.5.1 Cell wall degrading enzymes

Chitinases, glucanases and other hydrolytic enzymes have many roles in a wide range of different biological systems. These enzymes are usually extracellular, of low molecular weight and highly stable. In addition, they may be produced in multiple forms or isozymes that differ in charge, size, regulation, stability and ability to degrade cell walls. Chitinases catalyses the conversion of chitin and it is produced by wide range of bacteria (Koga *et al*., 1999).

2.5.1.1 Chitinases

Chitinases (EC 3.2.1.14) are the enzymes which hydrolyse the β-1, 4 linkages in the insoluble chitin microfibril, that are extensively distributed among plants, most fungi, yeasts and some prokaryotes (Koga *et al*., 1999). All organisms that contain chitin also contain chitinases which are required for morphogenesis of cell wall and exoskeletons. Other organisms that do not contain chitin may produce chitinases to degrade the polymer for food (Roberts and Selitrennikoff, 1988). In general chitinases, are classified into two major categories –

1. Endochitinases- that cleave chitin chain polymer randomly at internal sites.

2. Exochitinases that catalyze the progressive release of diacetylchitobiose starting at the non-reducing ends of chitin chains.

Based on amino acid sequence similarity of chitinases from various organisms, five classes of chitinases have been proposed. These classes can be grouped into two families of glycosyl hydrolases, family 18 and 19. Chitinases from classes I, II and IV are of plant origin and make up the family 19 glycosyl hydrolases. Class III chitinases are mainly plant and fungal in origin. Together with class V chitinases, they make up the family 18 glycosyl hydrolases which are structurally unrelated to family 19. Class III includes the bifunctional lysozyme/chitinase enzyme of *Havea brasiliensis*. Class V is mainly comprised of bacterial chitinases.

The wast majority of bacterial chitinases fall within family 18, which is subdivided into three groups, ChiA, ChiB, and ChiC, based on the differences in the amino acid sequences of their catalytic domains (Suzuki *et al*., 1999). ChiA and ChiB are processive chitinases that degrade chitin chains in opposite directions, while ChiC is a nonprocessive endochitinase (Horn *et al*., 2006). Due to their prevalence in nature, ChiA have been used for studying the diversity and distribution of chitinolytic bacteria in terrestrial systems as well as aquatic environment (Cottrell *et al*., 1999; Kirchman and white, 1999; Krsek and Wellington, 2001; Metcalfe *et al*., 2002; Ramaiah *et al*., 2000; Tsujibo *et al*., 2002; Williamson *et a*l., 2000) including the marine environment (Ramaiah *et al*., 2000).

2.5.1.2 Importance of chitinases

In higher plants, chitinases are used as defence against plant pathogens (Koga *et al*., 1999). These enzymes are found at low levels in healthy plants. However, their expression is increased during pathogen attack. The production of chitinases elicits other plant responses including the synthesis of antifungal phytoalexins (Gooday, 1999). The antifungal activity of chitinases cause rapid loss of fungal hyphal tips and germinating spores. These enzymes are an effective tool for the complete degradation of mycelial or conidial walls of phytopathogenic fungi (De la Cruz *et al*., 1995).

Plants produce chitinases as a defence against fungal pathogens and they are accompanied by β-1, 3-glucanases (Wen *et al*., 2002). Chitinases from bacteria and fungi are extremely important for maintaining a balance between the large amount of carbon and

nitrogen trapped in the biomass as insoluble chitin in nature (Cohen- Kupiec and Chet, 1998). In chitin containing organisms, chitinases play an important role in normal life cycle functions such as morphogenesis and cell division (Brurberg *et al*., 2000). Bacteria produce chitinases to meet their nutritional needs. They usually produce several chitinases, probably to hydrolyze the diversity of chitins found in nature (Cohen-Kupiec and Chet, 1998). Chitinases are reported to dissolve cell walls of various fungi, a property that has been used for the generation of fungal protoplasts. Chitinase-producing organisms are effectively used in the bioconversion process to treat shellfish waste and also to obtain value-added products from such wastes (Felse and Panda, 1999).

2.6 *Serratia marcescens* and its chitinases

Serratia sp. are gram negative bacteria, classified in the large family of Enterobacteriaceae. *Serratia* sp can be distinguished from other genera by its production of three special enzymes DNAase, lipase and gelatinase (Giri *et al*., 2004). It is one of the most effective bacteria for degradation of chitin (Brurberg *et al*., 1994; 1995; 1996; 2000; Watanabe *et al*., 1997; Nawani and Kapadnis, 2001; Suzuki *et al*., 2001; Uchiyama *et al*., 2003). 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; Suzuki *et al*., 1998). *S. marcescens* is selected as the source of chitinase for the following reasons:

(i) crude preparations of chitinases from *S. marcescens* are commercially available,(ii) an effective affinity chromatographic purification procedure for the *S. marcescens* chitinases has been reported, (iii) the gene(s) encoding chitinases and their associated regulatory signals are likely to be recognized and expressed directly in *E. coli* (Fuchs *et al*., 1986), (iv) the predominant *S. marcescens* chitinase is an endolytic enzyme that solubilizes chitin more rapidly than the exolytic enzymes and (v) *S. marcescens* chitinases hydrolyze “crystalline” chitin (Monreal and Reese, 1969).

2.7 Chitinolytic machinery of *Serratia marcescens*

Studies by number of groups clearly showed that *S. marcescens* produces at least three chitinases (ChiA, ChiB and ChiC), a chitobiase and a putative chitin-binding protein (CBP21). It represents the chitinolytic machineries of the bacterium (Perrakis *et al*., 1994). The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries known to date (Perrakis *et al*., 1994). Chitinolytic bacteria are typically detected by either the production of clearing zones on agar containing chitin or hydrolysis of fluorogenic substrate analogue of chitin. The colloidal chitin assay for clearing zones suggests that 10% of culturable bacteria degrade chitin (Okutani, 1975).

ChiB and ChiC are found in the periplasm or culture medium of *S. marcescens* but these two proteins do not contain typical *N*-terminal signal peptides (Melchers *et al*., 2000; Suzuki *et al*., 1999). There are no indications of any proteolytic processing of ChiB (Brurberg *et al*., 1995), whereas proteolytic processing of ChiC does not seem to be linked to export but rather to some kind of unspecific process. In *E. coli,* both enzymes undergo no *N*-terminal proteolytic processing and they are mainly found in the cytoplasmic fraction (Brurberg *et al*., 1995). Thus, it seems that *S. marcescens* contains a type of export machinery that is absent in *E. coli.* Upon induction with chitin, *S. marcescens* produces at least one more protein which has no enzymatic activity but which binds to chitin (Brurberg *et al*., 2000). The list of chitinases and chitin binding proteins, are tabled below.

Gene (protein)	SDS-PAGE (kDa)	Localization in *S. marcescens*	N-terminal signal peptide
*chi*A (ChiA)	57-58	Extracellular	Yes

*chi*B (ChiB)	52-54	Periplasm/extracelluar	No
*chi*C (ChiC1)	48-52	Extracellular	No
*chi*C (ChiC1)	35-36	Extracellular	No
chb	95	Periplasm	Yes
cbp (CBP21)	21-22	Extracellular	Yes

The best known of these is chitinase A (ChiA) which is an endochitinase. ChiA is produced as a 563-residue precursor, which is secreted from the cells with concomitant cleavage of an *N*-terminal signal peptide by a periplasmic signal peptidase when the exported protein reaches the periplasm. The resulting enzyme has 540 residues and a calculated molecular mass of ~58.5 kDa (Brurberg *et al*., 2000).

Gene encoding ChiA has been isolated from various strains of *S. marcescens* and their nucleotide sequences have been determined (Jones *et al*., 1986). The organization of chitinase genes on the *S. marcescens* chromosome is not precisely known. Hybridisation studies have shown that the genes encoding ChiA and ChiB are not closely linked. The genes encoding ChiB and CPB21 are linked, but the DNA sequence suggests that transcription of the two genes is not coupled. It is interesting to note that eight chitinase genes in *Streptomyces coelicolor* are scattered on the chromosome (Brurberg *et al*., 2000). CBP21 is a protein which binds to chitin but does not have hydrolyzing activity. This protein is produced only under the conditions which allow the production of chitinases. Therefore, coordinate regulation of the expression of CBP21 with those of chitinases was suggested (Suzuki *et al*., 2001). To identify the genes involved in chitinase production, transposon mutagenesis of strain 2170 was carried out and various mutants defective in chitinase production were isolated (Watanabe *et al*., 1997; Suzuki *et al*., 1998, 1999, 2001; Uchiyama *et al*., 2003).

2.8 Prodigiosin

A red pigment produced by the many strains of the bacterium like *S. marcescens* and some other unrelated microbial strains, such as *Vibrio psychroerythrus*, *Streptomycin griseoviridis* and *Hahella chejuensis* was found to exhibit antibacterial, antimycotic, immunomodulating, anti-tumor and anti-malarial properties (Frustner, 2003).

2.8.1 Prodigiosin structure:

The prodigiosin group of natural products is a family of tripyrrole red pigments that contains a common 4-methoxy, 2-2 bipyrrole ring system. The biosynthesis of the pigment is a bifurcated process in which mono and bipyrrole precursors are synthesized separately and then assembled to form prodigiosin. Prodigiosin have been shown to be associated in extracellular vesicles.

2.8.2 Potential of prodigiosin

Prodigiosin is a reddish antibiotic pigment that plays an important role in the biocontrol of plant diseases by the bacterium *Serratia marcescens*. (Bennett and Bentley, 2000) examined the effect of epiphytic microbes on the stability of prodigiosin used for biological control processes. Indigenous epiphytic microorganisms interfere with the interaction between plant pathogens and biocontrol agents by degrading the antibiotics produced by the agents. The pigment has a role in respiration and has some antibiotic properties. It is presumed that pigment biosynthesis acts as a protective mechanism in unfavourable conditions when the growth of cells is delayed.

2.9 *Serratia marcescens* as a biological control agent

Rhizosphere bacteria are excellent agents to control soil-borne plant pathogens. Bacterial species like *Serratia, Bacillus, Pseudomonas* and *Arthobacter* have been proved in controlling plant diseases. Microorganisms capable of lysing chitin, which is a major constituent of the fungal cell wall, play an important role in biological control of fungal pathogens.

S. marcescens, a gram negative bacterium, classified in the large family of *Enterobacteriaceae*, soil inhabitant, is very efficient in degradation of chitin because of its ability to produce different chitinolytic enzymes (Ulhoa and Peberdy, 1991). It can be distinguished from other genera by its production of three special enzymes DNAase, lipase and gelatinase (Giri *et al*., 2004).

Out of 50 bacterial isolates two potent isolates with higher nematocidal activity were recognized as *S. marcescens* and *P. fluorescens* using morphological and biochemical diagnosis tests. The identification and molecular characterization confirmed by RAPD screening and PCR sequence analysis. The effect of bacterial treatment as bio-control agent on the development of *Meloidogyne incognita* infecting faba bean was evaluated under greenhouse conditions. Both *S. marcescens* and *P. fluorescens* were effective as bio-control agent and significantly reduced the incidence of root-knot disease in soil artificially infested with *Meloidogyne incognita* (Zeinat *et al*., 2009).

The *chi*A and *chi*B genes from *S. marcescens* have been transformed into other bacterial species like *Pseudomonas fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens or to create new biocontrol agents (Brurberg *et al*., 2000). Furthermore, chitinase ChiA partially purified after cloning into *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 concentrations of the *B. thuringiensis* delta-endotoxin, a synergistic toxic effect was seen on insect larvae (Regev *et al*., 1996; Sampson *et al*., 1998). In the study of Inglis *et al*. (2000) the effects of *S. marcescens* was investigated on the F1 generation of laboratory-reared *Heliothis virescens* (F.). They showed that the number of eggs laid and the prevalence of eclosion of eggs from *Serratia* treatment adults were reduced relative to control treatment adults.

The gene coding for the major chitinase of *S. marcescens*, *chi*A, was cloned under the control of the *tac* promoter into the broad-host-range plasmid pKT240 and the integration vector pJFF350. *P. fluorescens* carrying *tacchi*A either on the plasmid or integrated into the chromosome is an effective biocontrol agent of the phytopathogenic fungus *Rhizoctonia solani* on bean seedlings under plant growth chamber conditions (Downing and Thomson, 2000). Chitinase ChiA partially purified after cloning into *E. coli* was found to reduce disease caused of *S. rolfsii* in beans and *R. solani* in cotton. Transgenic tobacco plants expressing high levels of *S. marcescens* ChiA exhibited increased tolerance to *R. solani* as compared to untransformed control plants (Brurberg *et al*., 2000).

The chitinase of *S. marcescens* plays important role in the virulence of the bacterium together with protease and lecithinase (Uchiyama *et al*., 2003). Chitinase produced by *Serratia plymuthica* C48 inhited spore germination and germ tube elongation of *Botrytis cinerea* (Frankowski *et al*., 2001). A synergistic inhibitory activity of prodigiosin and chitinolytic enzymes was observed against spore germination of *Botrytis cinerea* (Nobutaka *et al*., 2001). In chitin supplemented agar medium, the growth and production of red pigment by *S. marcescens* (SR1) was highly antagonist to the fungal pathogens viz; *Alternaria alternate*, *Aspergillus niger*, *Fusarium oxysporum*, *Helminthosporium* sp, *Cuvularia* sp, which suggests that degradation of hyphal cell wall/ cell death due to the production of chitinase enzymes (Minerdi *et al*., 2008).

The *S. marcescens* produces chitinolytic enzymes, which causes degradation of fungal cell walls and induction of plant defence reaction in addition release of antifungal low weight molecule, which suggest that the bacterium is an effective and persistant biocontrol agent for both air borne and soil borne plant pathogens (Parani and Saha, 2009). *S.*

marcescens produces a clear zone on chitin agar (CA) medium compared with Potato Dextrose Agar (PDA) medium. This bacterium was antagonistic to fungal pathogens viz. *Alternaria alternate*, *Aspergillus niger*, *Fusarium oxysporum*, *Helminthosporium* sp, *Cuvularia* sp, on CA and PDA media (Parani and Saha, 2009). Reports have shown that co-application of *B. thuringiensis* delta-endotoxins and bacterial chitinases significantly increased the insecticidal effect of the *B. thuringiensis* against insect larvae (Regev *et al*., 1996; Barboza-Corona *et al., 2003*; Downing *et al*., 2000; Arora *et al*., 2003; Lin and Xiong, 2004; Lertcanawanichakul *et al*., 2004).

2.10 PCR-RFLP technique for identification of novel gene

Whenever the information on the entire or conserved domains of the target gene is known, PCR based techniques are used to detect and pull out the desired sequences from genomic DNA or from clones. PCR primers were designed based on conserved nucleotide sequences of chitinase genes in cultured bacteria and used to amplify the chitinase genes in cultured and uncultured marine bacteria (Cottrell *et al*., 2000). The PCR based methodologies have been proposed to identify different cry genes in *B. thuringiensis* strains (Ben-Dov *et al*., 1997, Bourque *et al*., 1993, Ceron *et al*., 1994, Gleave *et al*., 1993 and Juarez-Perez *et al*., 1997). The PCR based RFLP method used to recognize the different *cry*-type genes in Bt11 strain (Liu *et al*., 2009). To detect the *cry2A*-type genes harbored in the JF19-2 genome, PCR analysis was performed with PCR-RFLP primer pairs. The 1.5 kb amplicon was obtained using the primer pair II(+) and II(-) and that amplicon was cloned into pGEM-T and transformed into *E. coli* DH5α. Among that ten positive clones were randomly selected, and these PCR products were digested with *Dde*I (Zheng *et al*., 2009).

18 different chitinase genes were detected by a set of degenerate PCR primer specific for *chi*A of family 18 chitinases. Most of them belonged to the *Serratia* chitinases. Eight genes had different amino acid sequences in the conserved motif, encompassing the catalytic site among the ChiA of family 18 glycosyl hydrolase's and clustered in an independent clade on the phylogenetic tree (Lian *et al*., 2007). Hassani *et al*. (2006) found a novel *w-gliadin* Gene in *Aegilops tauschii.* Through RFLP, twelve restriction enzymes were initially tested on genomic DNA of the two parents of which four restriction enzymes revealed polymorphism. Out of these four, only *Dra*I was associated with the novel *w-gliadin*.

Meyer *et al*. (1992) studied a PCR-RPLP fingerprinting technique to analyse the nine species aggregates of *Trichoderma*. Kuo and Chak (1996) designated PCR-RFLP typing system is a facile method to detect both known and novel *cry* genes existing in a *B. thuringiensis* strain. PCR-RFLP method helps to detect the organization and differential expression of cry genes. They have identified the novel *cry* genes existing in *B*. *thuringiensis* strains. The restriction fragment length polymorphism (RFLP) patterns of the PCR-amplified fragments revealed 14 distinct *cry*-types from 20 *B*. *thuringiensis* strains. Those *cry*-type genes included *cryIA(a)*, *cryIA(a)*+, *cryIA(b)*, *cryIA(b)*+, *cryIA(c)*, *cryIB+*, *cryIC*, *cryIC*+, *cryIC(b)*, *cryID*, *cryIE*, *cryIF*, *cryIF*+, and *cryIII*+. Among them, six *cry*-type genes were found to have different sequences from the corresponding published *cry* gene sequences. Kabadjova *et al*. (2002) established a rapid PCR-RFLP-based identification scheme for four closely related *Carnobacterium* species (*C. divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile)* that are of interest to the food industry. Heilig *et al*. (2002) developed a *Lactobacillus* group-specific PCR primer, which selectively amplify 16S ribosomal DNA (rDNA) from *lactobacilli* and related lactic acid bacteria, including members of the genera *Leuconostoc*, *Pediococcus*, and *Weissella*.

2.11 Cloning and expression

The *chi*A from *Serratia marcescens* has been cloned through PCR based testing. The complete gene was constructed into a pRSET vector and expressed in *E. coli*. The recombinant enzyme was purified to >90% homogeneity by hydrophobic interaction chromatography followed by ion-exchange separation. The recombinant *chi*A was characterized and exhibited an exo-type catalytic activity toward colloidal chitin and releasing both *N*-acetylglucosamine and *N*, *N*-diacetyl chitobiose as products (Wu *et al*., 2009). *N*, *N*-diacetyl chitobiose is an effective inducer of bacterial chitinase (Wu *et al*., 2009).

The *chiA* gene from *Aeromonas caviae* encodes an extracellular chitinase, 865 amino acids long, that shows a high degree of similarity to chitinase A of *S. marcescens*. Expression in *E. coli* yielded an enzymatically active protein from which a leader sequence was removed, presumably during transport of the enzyme across the cell membrane (Sitrit *et al*., 1995). *S. marcescens chi*A was PCR cloned, sequenced and heterologously expressed in an anti-coleopteran *B. thuringiensis* (strain 3023-SCHI). The specific *chi*A activity of recombinant *B. thuringiensis* reached its level even higher than that produced by the source organism (Okay *et al*., 2008).

By cointroduction of *cry1Ac7* gene of *B. thuringiensis* strain 234 and *chiA* gene of *S. marcescens* into strains of *P. fluorescens*, an increased biocontrol of sugarcane borer *E. saccharina* could be achieved Downing *et al*. (2000). A chitinase gene from the gram-negative bacterium *S. marcescens* BJL200 was cloned in *Lactococcus lactis subsp.lactis* MG 1363 and in the silage inoculum strain *Lactobacillus plantarum* E19b using the expression vectors Pmg36e and pGKV259 with lactococcal promoter fragments p32 and p59. The expression in *Lactococcus lactis* was found to have increased nine and twenty-seven fold (Brurberg *et al*., 1994). Jang *et al*. (2005) reported cloning, over expression, biochemical characterization of the novel chitinase A (*chi*A) in *E. coli*.

Sitrit *et al*. (1993) expressed *chi*A from *S. marcescens* in *R. meliloti* and demonstrated that the nodule extracts from *chi*A expressing alfalfa plants caused lysis of *Rhizoctonia solani* hypal tips. The cosmid library of *S. marcescens* DNA made in pLAFR1 and screened in *E. coli* for clones capable of degrading chitin (Fuchs *et al*, 1986). 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).

2.12 Transgenic plants

Broglie *et al*. (1991) were the first to show enhanced fungal resistance in transgenic plants brought about by the expression of single chitinase gene. Transgenic tobacco plants expressing high levels of *S. marcescens* ChiA exhibited increased tolerance to *R. solani* compared to untransformed plants (Howie *et al*., 1994). Liu and Xiong (2004) transformed rice plant with a gene encoding endochitinase (*ech*42) from the biocontrol fungus *T. atroviride*. The transformed plants showed increased resistance to sheath blight caused by *R. solani* and rice blast caused by *Magnaporthe grisea*. Chandrakanth *et al*. (2003) transferred the 42 kDa endochitinase from *T. virens* to cotton and these plants showed resistance to the pathogens i.e. *A. alternate* and *R. solani*. Shah *et al*. (2005) transformed tobacco plants with *ech*42 cloned from *T. virens* and confirmed the integration of endochitinase into tobacco genome by PCR amplification using specific primers.

3. MATERIAL AND METHODS

The present study was conducted to clone and express a novel *chi*A gene from *S. marcescens* in *E. coli*. The material and methods employed for achieving the objectives are detailed below.

3.1 Bacteria and vectors used in this study

During this study, from the culture collection of the Department of Biotechnology, UAS Dharwad, *S. marcescens* isolated from the soils of the Western Ghats of India in the Dandeli region was used (Table 1) and *Serratia marcescens* 141 was used as a reference strain. Figure 1 indicates the Dandeli region of Western Ghats of India.

Defined vectors have been used for cloning the *chi*A gene and analyse its expression. They have been mentioned below.

Sl. No.	Vector	Source
1	pTZ57R/T	MBI, Fermentas, USA
2	pET32C+	Novagen, Germany

3.2 Screening the isolates for chitinolytic activity

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

3.2.1 Preparation of colloidal chitin

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

Five gram of chitin powder (Himedia Chemicals Co. Mumbai) was added slowly into 60 ml of concentrated HCL (Sd. Fine Chemicals, Mumbai) 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 (28℃). The precipitate was collected by centrifugation at 5000xg at 20 minutes at 4℃ and was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Collodial chitin solution (5%) was prepared and stored at 4℃ until further use.

3.3 Molecular characterisation of *chi*A, *chi*B and *chi*C

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.

Ten ml of overnight grown culture in Luria broth grown at 28℃ was centrifuged at 10,000 rpm at 4℃ for 10 minutes. The pellet was re-suspended 10 mM Tris, 100 mM sodium chloride solution and centrifuged at 10, 000 rpm for 10 min at 4℃. After discarding the supernatant, the pellet was re-suspended in 2.5 ml of $T_{50}E_{20}$ buffer containing 500 µl of lysozyme (50 mg/ml) and incubated at 37℃ for 20 min in that solution 25 µl of RNase (10 mg/ml) was added and incubated at room temperature for 10 min. After adding 2.5 ml of 2 % SDS in $T_{50}E_{20}$, it was incubated at 50℃ for 45 min. 50 µl of proteinaseK (20 mg/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

Fig 1. Dandeli region of wetern Ghats of India

Table 1. Isolates of *Serratia marcescens* from Dandeli region of Western Ghats of India used in this study

Sl. No.	Isolates	Sl. No.	Isolates
1	AUDS190	23	AUDS263
2	AUDS191	24	AUDS264
3	AUDS192	25	AUDS265
4	AUDS220	26	AUDS266
5	AUDS221	27	AUDS272
6	AUDS224	28	AUDS273
7	AUDS226	29	AUDS274
8	AUDS227	30	AUDS275
9	AUDS228	31	AUDS276
10	AUDS229	32	AUDS278
11	AUDS230	33	AUDS285
12	AUDS231	34	AUDS286
13	AUDS232	35	AUDS287
14	AUDS241	36	AUDS288
15	AUDS242	37	AUDS294
16	AUDS243	38	AUDS307
17	AUDS256	39	AUDS308
18	AUDS257	40	AUDS309
19	AUDS258	41	AUDS311
20	AUDS259	42	AUDS312
21	AUDS260	43	AUDS365
22	AUDS261	44	AUDS366
45	AUDS287	53	AUDS531
46	AUDS288	54	AUDS532
47	AUDS294	55	AUDS533
48	AUDS307	56	AUDS534
49	AUDS308	57	AUDS643
50	AUDS309	58	AUDS644
51	AUDS311	59	AUDS645
52	AUDS312	60	AUDS646

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 isopropanol was added and centrifuged. The supernatant was discarded and pellet was washed with 70 percent alcohol, dried and dissolved in 100 µl of $T_{10}E_1$. The components of reagents are listed in Appendix I.

3.3.2 Quantification of genomic DNA

Genomic DNA was quantified using the spectrophotometer as described in Sambrook and Russell (2001). The extracted DNA was subjected to analysis in the spectrophotometer using $T_{10}E_1$ buffer as blank, absorbance at 260 and 280 nm was recorded.

3.3.3 PCR primers employed for amplification of *chi*A, *chi*B and *chi*C

Gene	Primer			Expected amplicon size (bp)	Reference
	Name	Sequence	Tm(°C)		
*chi*A	F	5'gcccatggaaggaatcagttatgcgcaaat3'	55.0	1692	This study
	R	5'gcggatcccaacgcactgcaaccgattat 3'			
*chi*B	F	5'atccactcaggagaaatgcca 3'	64.2	1500	This study
	R	5'tgaaaaccccgcagcatt3'			
*chi*C	F	5'gcccatggaggccaccatgagcacaaataac3'	60.0	1450	This study
	R	5'gcggatcccgattagcgattaggcgatgag 3'			

3.3.3.1 PCR components

Template DNA : The genomic DNA obtained from *S. marcescens* isolates was diluted to 100 ng and used as template DNA for further studies.

Taq DNA polymerase : (3 u/µl) 10X assay buffer, $MgCl_2$ and dNTPs were obtained from M/S Bangalore Genei Pvt. Ltd., Bangalore.

Primers : Three sets of the specific primers at (5pM) concentration were found optimum and used in all further studies.

Thermal cycler : Eppendorf Master cycler (5331) was used to run the PCR programme.

3.3.3.2 Standardisation of primer concentration for designed primers

Specific primers of 5 pM concentration were standardized by keeping a gradient PCR for each set of primer along with the template DNA of the reference strain *S. marcescens* 141 and the temperature at which amplicons of good intensity of expected size were obtained was used for further studies.

3.3.3.3 Contents of PCR reaction mixture

Reagents	Volume (µl)
Taq assay buffer(10x)	2.0
dNTPs (1 mM)	2.0
$MgCl_2$ (25 mM)	1.0

Forward primer (5 pM)	0.5
Reverse primer (5 pM)	0.5
Taq DNA Polymerase (3U/ µl)	0.5
Template (DNA 100 ng/µl)	1
Sterile distilled water	11.5
Total	20

3.3.4 PCR programmes for amplification of chitinase genes

Steps	*chiA*		*chiB*		*chiC*	
	Temp	Time	Temp	Time	Temp	Time
Initial denaturation	95.0℃	7.0 min	95.0℃	5.0 min	95.0℃	8.0 min
Denaturation	95.0℃	1.0 min	95.0℃	1.0 min	95.0℃	1.0 min
Annealing	55℃	1.0 min	64.2℃	1.0 min	60℃	1.0 min
Extension	72.0℃	1.0 min	72.0℃	2.0 min	72.0℃	3.0 min
Repeat	35 cycles					
Final extension	72.0℃	10 min	72.0℃	10.0 min	72.0℃	8.0 min
Hold	4.0℃					

3.3.5 Separation of amplified products

After the set programme was completed, the contents of the tube were electrophoresed to separate amplified products. About 20 µl of the amplified products from tube along with 3 µl of loading dye (Appendix III) was loaded onto 1 percent agarose gel (Appendix III) along with λ *Hind*III /*Eco*RI double digest as DNA molecular weight marker. Electrophoresis was done at 70V for 1 hour and the buffer used was 1X TAE at pH 8.0 (Appendix III). After separation, the bands in gels were visualized under UV light and documented using gel documented system, Syngene, Germany.

3.3.6 Chitinase profile analysis

The *chi*A, *chi*B and *chi*C profile analysis of native isolates of *S. marcescens* was done on the basis of reference *S. marcescens* 141.

3.4 Cloning of novel *chi*A

3.4.1 *In silico* restriction analysis of reference *Sm*141*chi*A

The restriction sites present in *chi*A previously cloned from reference strain *S. marcescens* 141 Accession number DQ990373.1 deposited in the NCBI database earlier (Ningaraju, 2005) were analyzed by using BTI Gene tool software. Based on these analysis different restriction enzymes *Pst*I, *Xma*I, *Sma*I and *Sal*I (Bangalore Genie Pvt. Ltd, Bangalore) were selected further for restriction fragment length polymorphism of c*hi*A.

3.4.2 Elution of PCR product

A large-scale amplification was done and the expected amplicon of 1692 bp were separated out through electrophoresis. The gel portion containing the *chi*A amplicons of *S. marcescens* AUDS227, AUDS274, AUDS365 and *S. marcescens* 141 were excised using a sharp sterile scalpel by keeping the gel at low intensity UV transilluminator. The gel pieces were collected in sterile pre-weighed 2.0 ml micro centrifuge tubes. The Qiagen Min Elute kit (Qiagen, Germany) was used to elute the DNA from agarose gel as described in user's manual.

3.4.3 RFLP analysis of *chi*A positive isolates

The bands corresponding to 1692bp of *chi*A from reference strain *S. marcescens* 141 and Dandeli isolates AUDS227, AUDS274 and AUDS365 were excised from preparative gels and restricted with *Pst*I enzyme. The DNA (eluted product) with concentration of 25 ng/µl and volume (4 µl) with *Pst*I restriction enzyme with concentration of 10 u/µl of 0.5 µl and buffer D of (2 µl) was added in 0.5 ml micro centrifuge tube and mixed well by gentle pipetting and kept at 37^0C for 1.30 hr in water bath for restriction. The resulting restriction pattern of the *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A were compared with the reference *Sm*141chiA. The restricted products were separated on freshly prepared 1.2 per cent low melting agarose gel along with 2 µl of loading dye with λ *Hind*III */Eco*RI double digest as DNA molecular weight marker and 100 bp ladder (Bangalore Genie Pvt. Ltd, Bangalore). The gel was observed under a mid range UV trans-illuminator and gel image was documented using gel documentation system, Syngene, Germany.

3.4.4 Elution of *Sm*AUDS227*chi*A

Based on the chitinolytic activity and difference in RFLP profile of the *Sm*AUDS227*chi*A with the reference *Sm*141*chi*A, the *Sm*AUDS227*chi*A was selected further for cloning and expression studies. A large scale amplification of *Sm*AUDS227*chi*A was done and the expected amplicon of 1692 bp was separated out through electrophoresis as described in section 3.4.2.

3.4.5 Ligation and transformation of *E. coli* DH5α

Excised PCR amplicon of *Sm*AUDS227*chi*A was ligated to pTZ57R/T cloning vector (2886bp), as described in InsT/A clone TM 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 a 0.5 ml microcentrifuge tubes and incubated at 16°C for 16 hrs for ligation.

3.4.6 Preparation of competent cells

The competent 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* DH5α plate was inoculated in 5 ml Luria broth with appropriate selection pressure and incubated at 37°C overnight at 200 rpm. Next day, the culture was diluted to 1:100 using Luria broth *i.e.*, 0.5 ml of culture was added to 50 ml of Luria broth. It was incubated for 2 to 3 hr till an OD of 0.3 to 0.4 at 600 nm was attained. The culture was chilled on ice for 30 min and 25 ml of culture was dispensed into two sterile centrifuge tubes. The cells were pelleted at 6000 rpm for 5 min at 4°C, the supernatant discarded and the pellet was suspended in 12.5 ml ice-cold 0.1 M $CaCl_2$. The centrifuge tubes were again kept in ice for 30 min and later centrifuged at 5000 rpm for 5 min and the pellet was dispensed in 1 ml of ice-cold 0.1 M $CaCl_2$. About 100 µl of the competent cells were distributed to pre-chilled 1.5 ml micro centrifuge tubes and used immediately for transformation.

3.4.7 Transformation of *E. coli* DH5α

About 100 µl of freshly prepared competent cells were taken in a chilled micro centrifuge tube and 10 µl of ligation mixture was added, mixed gently and chilled on ice for 30 min. Heat shock was given by shifting the chilled mixture to 42°C water bath for exactly 2 min, and immediately chilled on ice for 5 min. To this 900 µl of Luria broth was added and incubated at 37°C at 200 rpm for 45 min to allow bacteria to recover and express the antibiotic marker encoded by the construct. The cells were pelleted at 13,000 rpm for 1 min, 900 µl of supernatant discarded and the pellet was dissolved in the remaining 100 µl of supernatant and spread on Luria agar plates having ampicillin @100 µg/ml, X –gal, IPTG and incubated overnight at 37°C.

3.4.8 Confirmation of the clones.

The transformed cells were picked up and streaked on Luria agar containing ampicillin (100 µg/ml), X-gal and isopropyl B-D thiogalactoside (IPTG). The clones containing recombinant molecules were selected based on blue-white assay. Plasmids were isolated from clones containing of *chi*A (as described in the section 3.5.1) and the clones were confirmed through PCR amplification by using specific primers and further, confirmation was also done by restriction analysis using *EcoR*I and *Hind*III, which has released 1692bp insert. Further, these recombinants were restricted with *Sma*I for insert orientation confirmation.

3.5 Subcloning of the cloned *chi*A into prokaryotic expression vector

3.5.1 Vector and clone isolation

The alkaline lysis protocol of Sambrook *et al*. (2001), with certain modifications was used for isolation of plasmids.

Colonies were inoculated to 10 ml LB with ampicillin (100 µg/ml) and incubated over night at 37°C with 175 rpm. Overnight grown culture was centrifuged at 5000 rpm for 2 min in 2.0 ml micro centrifuge tubes. The supernatant was removed and pellet was washed with STE buffer (0.25 volume of original culture) and centrifuged at 5000 rpm for 2 min. The pellet was resuspended in 200 µl of ice-cold alkaline lysis solution I by vigorous vortexing. Later, 400 µl of freshly prepared alkaline lysis solution II was added to each tube and the contents were mixed by inverting the tubes for 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 4-5 times. The tubes were stored on ice for 5 minutes and centrifuged at 13,000 rpm for 8 min at 4°C. The supernatant was transferred to fresh tubes and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to precipitate proteins and mixed well. It was 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 was added. The contents were mixed and allowed to stand for 2 min. at room temperature. The solution was later centrifuged at 13,000 rpm for 5 min and supernatant discarded. The pellet was washed with 70 per cent ethanol and centrifuged for 1 min at 13,000 rpm to recover the plasmid. Further, supernatant discarded and the pellet dried completely and dispensed into 30 µl of $T_{10}E_1$ (pH 8.0) containing 1 µl of RNase A (10 mg/ml). The solution was kept at 50°C for 15 min and then stored at -20°C (Appendix I). After completion, the samples were stored at 4°C in refrigerator for further use.

3.5.2 Electrophoresis

About 2 µl of the isolated DNA from pSBK101A (pTZ57R/T plus *chi*A) along with 1 µl of loading dye was electrophoresed in 0.7 per cent agarose gel along with λ - DNA *Hind*III /*EcoR*I double digest as DNA molecular weight marker. Electrophoresis was done at 70 V for 1 hr. and the buffer used was 1X TAE.

3.5.3 Linearising the vector and clone

For analysing the expression of cloned *chi*A, prokaryotic expression vector pET32C$^+$ (Novagen, Germany) was used. Recombinant pTZ57R/T containing *chi*A was restricted with *Eco*RI and *Hind*III restriction enzymes. Accordingly, expression vector pET32C$^+$ was digested with the same enzymes to have compatible cohesive ends and the complete restriction was confirmed by electrophoresis.

3.5.4 Ligation and transformation of *E. coli* BL21 (DE3)

The single sharp bands corresponding to prokaryotic expression vector pET32C$^+$ of 5901 bp and *chi*A insert from pSBK101A (pTZ57R/T plus *chi*A) of 1692 bp were eluted from gels, as described previously (section 3.4.2).

Equimolar concentration, approximately 0.54 pmoles of free ends of insert and vector backbone was used for ligation under 1X cohesive end ligation buffer with 1 U of T4 DNA ligase, incubated over night at 16^{o}C for 16 hrs.

Around 15 µl of ligation mixture was used for transforming 100 µl of competent *E. coli* BL21 (DE3) (Novagen, Germany) cells. Preparation of competent cells and transformation was done as per the protocol described earlier (3.4.6 and 3.4.7). The pellet containing transformed cells was dispensed in about 100 µl of supernatant and plated on Luria agar containing ampicillin @100 µg/ml and incubated at 37°C for 8-12 hrs. The colonies were further streaked on Luria agar with ampicillin @100 µg/ml and incubated overnight at 37°C.

3.5.5 Confirmation of transformed clones

The transformants grown on Luria-Burtani agar containing 100 µg/ml ampicilin were randomly picked up and screened for the presence of recombinant plasmids by PCR using *chi*A gene specific primers and by restriction through *Eco*RI and *Hind*III and labelled as pSBK201A, pSBK201B, pSBK201C and pSBK201D.

3.6 Induction and expression of *chi*A

3.6.1 Confirmation of expression by SDS-PAGE analysis

Induction and isolation of the ChiA was done as per the procedure outlined in pET system manual, 11th edition provided by Novagen with minor modifications. A single isolated colony of *E. coli* BL21(DE3) containing pSBK201A (pET32C$^+$ plus *chi*A), pSBK201B, pSBK201C and pSBK201D was inoculated separately in 1 ml of LB + 100 µg/ml Amp and incubated overnight at 37^{o}C with shaking at 200 rpm. The overnight grown culture was diluted in 10 ml of fresh LB in 1:100 ratio and incubated till the OD_{600} reached 0.5-1.0 (approximately 3 hours). The log phase cells were induced by adding 1 mM IPTG and incubated for additional 5 hours. *E. coli* BL21 (DE3) containing plain pET32C$^+$ was inoculated and induced. A batch of uninduced culture was maintained in each case.

Following the induction, cells were pelleted at 13,000 rpm for 10 min. and the pellet resuspended in 100 µl of 100 mM Tris (pH 8.0). 100 µl of 1X SDS loading dye was added to 100 µl of sample heated at 95^{o}C for 10 min. and the supernatant was collected after spinning at 10,000 rpm for 5 min. To confirm the expression of *chiA* and to fractionate the expressed protein from crude extract, SDS-PAGE was done as per the protocol given in Sambrook and Russell (2001).

3.6.2 Polyacrylamide gel electrophoresis

3.6.2.1 Preparation of the polyacrylamide gel

The glass plates were assembled as per the manufacturer's instruction. Seventeen ml of 12 per cent (Appendix VII) polyacrylamide gel was poured into the gap between the glass plates leaving sufficient space for the stacking gel. The 0.1 per cent SDS was poured on the top of the polyacrylamide gel in order to make surface smooth and level. The gel was left for 45 minutes or until it polymerized. After polymerisation, the SDS layer was removed by washing with the water for three times. About 5 ml of 5% stacking gel was poured on the resolving gel after inserting the comb. After the polymerization, the comb was carefully removed and wells were washed with the deionized water for three times to remove the unpolymerised acrylamide. While pouring the resolving gel and stacking gel, enough care was taken to avoid air bubble formation in the gel.

3.6.2.2 Preparation of sample for the SDS-PAGE

The sample for the SDS PAGE was prepared as described in the Section 3.7.1. To this supernatant, equal volume of loading dye (Appendix VII) was added and heated up to 95°C for 10 minutes. To each well about ~100 µg of the sample and equal volume of dye mixture was loaded.

3.6.2.3 Running of the polyacrylamide gel electrophoresis

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

3.6.2.4 Staining and destaining of polyacrylamide gel

After running the polyacrylamide gel electrophoresis, the gel was carefully removed and placed in the staining solution (Appendix VII). The gel was stained for 3 hours in shaking condition. After staining with coomassie brilliant blue, the excess dye was removed by de-staining solution (Appendix VII) under shaking condition. The de-staining solution was replaced for three times until the clear blue colour bands appeared and the gel was documented in UV Doc and sealed in polyethylene bag, and stored at 4℃.

3.7 Expression of target protein for bioassay

The transformed *E. coli* colony was inoculated in 5 ml of Luria-Bertani agar containing 100 µg/ml ampicillin and incubated overnight at 37^{o}C. Next day, the culture was diluted in 1:100 ratio without selection pressure and again incubated at 37^{o}C with shaking condition until it reaches the log phase of growth. Later the induction of expression of target protein was done by adding optimal concentration of IPTG (1mM) and incubated for 5 hrs at 37^{o}C. After that the cells were harvested by centrifugation at 5000g for 15 min at 4^{o}C and resuspended in 4ml of binding buffer pH 7.8. Lysozyme of final concentration of 1 mg/ml was added and the cell suspension was incubated in ice for 30 minutes. TritonX-100, DNase, and RNase were added to the final concentration of 1 per cent, 5 µg/ml and 5 µg/ml respectively and incubated at 4^{o}C on rocking platform for 10 min. Finally, the suspension was centrifuged at 3000g for 30 min at 4^{o}C and the supernatant was collected as raw active protein in fresh tube.

3.7.1 Anti-fungal bioassay

For anti-fungal bioassay the fungal pathogens *Sclerotium rolfsii* and *Rhizoctonia solani* were selected. 5 mm agar discs of fungal pathogens were placed on the center of the PDA plates and incubated at 28±2 for two days separately. After two days of incubation / growth, the target protein of different concentration 50, 100 and 200 µg/µl extracted from clones viz. pSBK201A, pSBK201B and pSBK201D were spotted against the fungal pathogens.

3.8 Sequencing of clones

For sequencing, the clones (pTZ57R/T plus *chi*A) were selected on the basis of efficacy of expression in pET32C^{+}. The corresponding pTZ57R/T plus *chi*A clones were sequenced using M13 forward 5'gtaaaacgacggccagt 3' and reverse 5' caggaaacagctatgac 3' primers and further as primer walking by designing internal primers, at Ocimum Biosolutions, Hyderabad. After sequencing, the complete nucleotide sequences of *Sm*AUDS227*chi*A and the reference *Sm*141*chi*A were assembled in BIO EDIT software and subjected to *in silico* analysis using CLUSTALW and BTI Gene Tool software for ORF finding, restriction site and *in silico* translation. BLAST analysis was done with the DNA sequences obtained by available online nucleic acids data base at http://www.ncbi.nlm.nih.gov.

4. EXPERIMENTAL RESULTS

The results of experiments conducted during the present investigation in order to understand chitinase profile in native *S. marcescens* isolates and to clone and express novel *chi*A from an efficient native isolates of *S. marcescens* are presented herein.

4.1 Screening the isolates for chitinolytic activity

The *S. marcescens* isolates AUDS227, AUDS232, AUDS242, AUDS243, AUDS263, AUDS264, AUDS265, AUDS274, AUDS288 and AUDS365 isolated from the soils of the Dandeli region of the Western Ghats of India were found to be very effective in releasing chitinase enzyme when different strains of *S. marcescens* were grown on colloidal chitin plate in which chitin was added as sole carbon source with a range of zone of hydrolysis of 5-7 mm (Plate1).

4.2 Diversity of chitinase genes in the native *S. marcescens* isolates

Chitinase specific PCR was carried out using specific primers designed from reported *S. marcescens chi*A, *chi*B and *chi*C nucleotide sequences available in the NCBI database. The amplicons so obtained was separated on 0.7% agarose gel are presented in plate 2, plate 3 and 4 respectively. From the gels, it is clear that an amplicon of around ~1692 bp, 1500 bp and 1450 bp was obtained from *chi*A, *chi*B and *chi*C respectively. Based on the chitin resolution, the highly potent *chi*A positive isolates of *S. marcescens* AUDS227, AUDS274 and AUDS365 were selected further for restriction fragment length polymorphism analysis.

4.2.1 *S. marcescens chi*A, *chi*B and *chi*C positive isolates

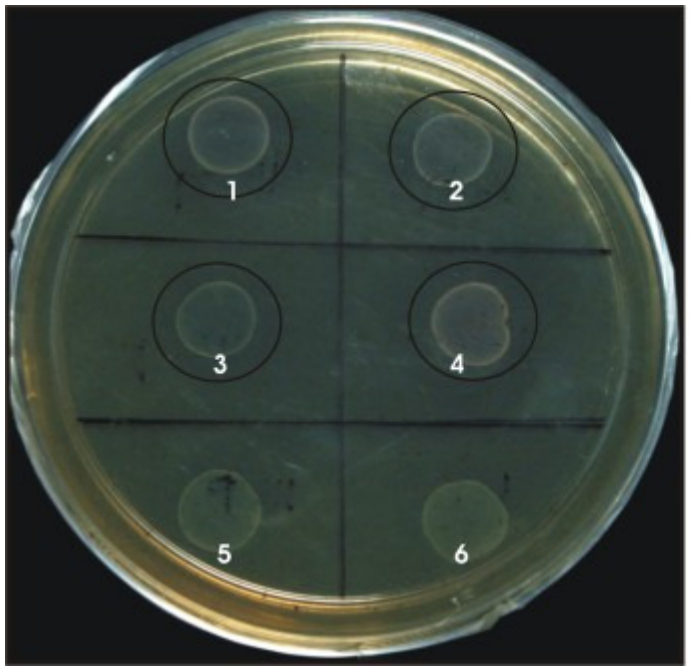
All the isolates tested, amplified both *chi*A and *chi*C. However, only nine of the isolates viz., AUDS221, AUDS263, AUDS264, AUDS265, AUDS266, AUDS273, AUDS274, AUDS276 and AUDS278 were found to be positive for *chi*B. The results are presented in Table 2.

4.3 *In silico* analysis of *Sm*141*chi*A (reference) for PCR-RFLP

The restriction sites present in reference *Sm*141*chi*A nucleotide sequence obtained from NCBI database were analyzed by using BTI Gene tool software. Based on this analysis *Pst*I, *Xma*I, *Sma*I and *Sal*I restriction enzymes were selected for restriction fragment length polymorphism of c*hi*A. The complete nucleotide sequence and restriction site map of the *Sm*141*chi*A are shown in Fig. 2 and 3. The sequence information of *Sm*141*chi*A revealed the presence of *Xma*I at 948th, *Sma*I at 950th, *Sal*I at 526th and *Pst*I site at 905th position (Fig. 4, 5, 6 and 7).

4.3.1. RFLP analysis of *chi*A positive isolates

The restriction of *chi*A (1692bp) eluted product from *S. marcescens* isolates AUDS227, AUDS274 and AUDS365 were done by *Pst*I, *Xma*I, *Sma*I and *Sal*I enzymes (Bangalore Genei Private Ltd., Bangalore) separately. The resulting RFLP pattern of respective *chi*A positive isolates was compared with the reference *Sm*141*chi*A. The RFLP pattern of *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A generated by *Sal*I revealed the 526 bp and 1166 bp fragments, by *Xma*I 948 bp and 744 bp fragments and by *Sma*I 950 bp and 742 bp fragments which gave the similar banding pattern compared to the reference *Sm*141*chi*A. Through *Pst*I, the *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A revealed 787 bp, 361 bp, 283 bp and 261 bp fragments, which were found to be different compared to the reference *Sm*141*chi*A. The reference *Sm*141*chi*A gave 905 bp and 787 bp fragments (Plate 5). The restriction position and the fragments generated after



1. *Sm*AUDS227
2. *Sm*AUDS274
3. *Sm*AUDS365
4. *Sm*141 (Reference strain)
5. *Sm*AUDS515
6. *Sm*AUDS643

Plate 1. Chitinoltic activity of Dandeli isolates od S. *marcescens* on colloidal chitin media

restriction of *Sm*141*chi*A, *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A by *Xma*I, *Sma*I, *Sal*I and *Pst*I are presented in table 3.

Based on the chitinolytic activity and difference in RFLP profile of the *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A with the reference *Sm*141*chi*A, the *Sm*AUDS227*chi*A was selected further for cloning and expression studies.

4.4 Ligation and transformation of *chi*A

The 1692bp *chi*A amplicon from *S. marcescens* isolate AUDS227 was eluted from preparative gel and ligated to pTZ57R/T cloning vector. The recombinant product pTZ57R/T containing *chi*A was transformed into *E. coli* DH5α using 10 µl ligation mixture and transformants were randomly picked up, confirmed and labelled as pSBK101A, pSBK101B, pSBK101C and pSBK101D. Super coiled plasmid DNA of pTZ57R/T was used as positive control and transformation efficacy was noted on the basis of positive control. The vector map of pTZ57R/T and pSBK101A is presented in Fig. 8 and 9.

4.4.1 Confirmation of clones

The confirmation of pSBK101A was done by PCR and restriction analysis. The template DNA of reference strain *Sm*141 was used as a positive control and the plain pTZ57R/T used as a negative control for PCR amplification of pSBK101A. The PCR positive clones were confirmed by restriction analysis using *Eco*RI and *Hind*III (Plate 6 and 7).

4.5 Ligation of *chi*A into prokaryotic expression vector

The pTZ57R/T cloning vector was restricted with *EcoR*I and *Hind*III to release the 1692 bp insert and the eluted insert further ligated to pET32C^{+} expression vector. The recombinant products of vector pET32C^{+} plus *chi*A were transformed into *E. coli* BL21 (DE3) using 15 µl of ligation mixture and the obtained transformants were labelled as pSBK201A, pSBK201B, pSBK201C and pSBK201D and super coiled plasmid DNA of pET32C^{+} was used as positive control. The transformation efficacy was found to be 0.26×10^{4} CFU/µg of the recombinant.

Plate 2. PCR amplification of *chi*A from the genomic DNA of native *S. marcescens*

M. Lambda DNA/ *Eco*RI +*Hind*III marker

1. *Sm*141*chi*Aspecific primer (55℃)
2. *Sm*AUDS227*chi*A specific primer (55℃)
3. *Sm*AUDS365*chi*A specific primer (55℃)
4. *Sm*AUDS274*chi*A specific primer (55℃)
5. *Sm*AUDS288*chi*A specific primer (55℃)

Plate 3. PCR amplification of *chi*B from the genomic DNA of native *S. marcescens*

M. Lambda DNA/ *Eco*RI +*Hind*III marker

1. *Sm*141*chi*B specific primer (64.2℃)
2. *Sm*AUDS274*chi*B specific primer (64.2℃)
3. *Sm*AUDS263*chi*B specific primer (64.2℃)
4. *Sm*AUDS264*chi*B specific primer (64.2℃)
5. *Sm*AUDS265*chi*B specific primer (64.2℃)

Plate 4. PCR amplification of *chi*C from the genomic DNA of native *S. marcescens*

M. Lambda DNA/ *Eco*RI +*Hind*III marker

1. *Sm*141*chi*C specific primer (60℃)
2. *Sm*AUDS227*chi*C specific primer (60℃)
3. *Sm*AUDS365*chi*C specific primer (60℃)
4. *Sm*AUDS274*chi*C specific primer (60℃)
5. *Sm*AUDS288*chi*C specific primer (60℃)

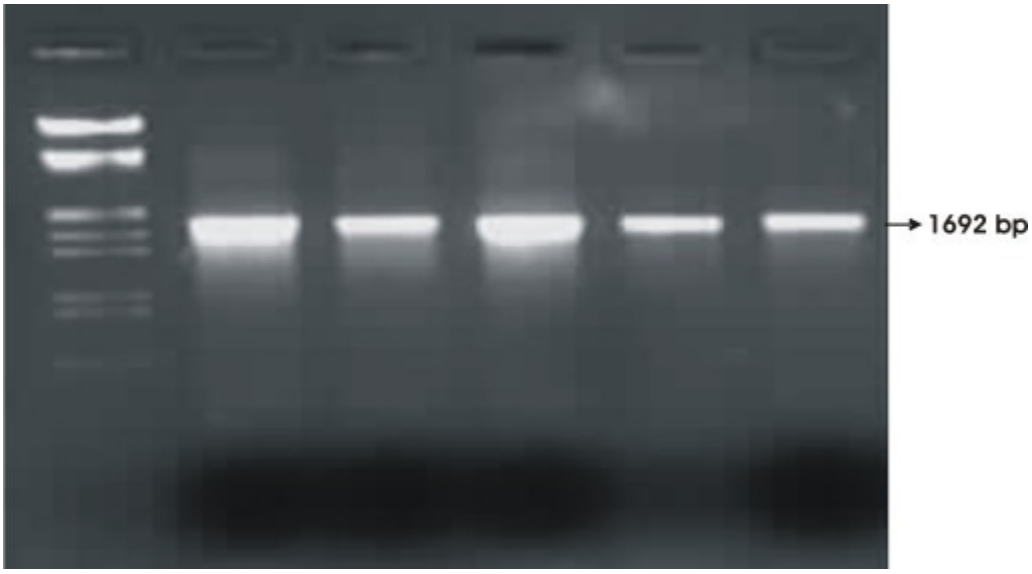


Plate 2. PCR amplification of chiA from genomic DNA

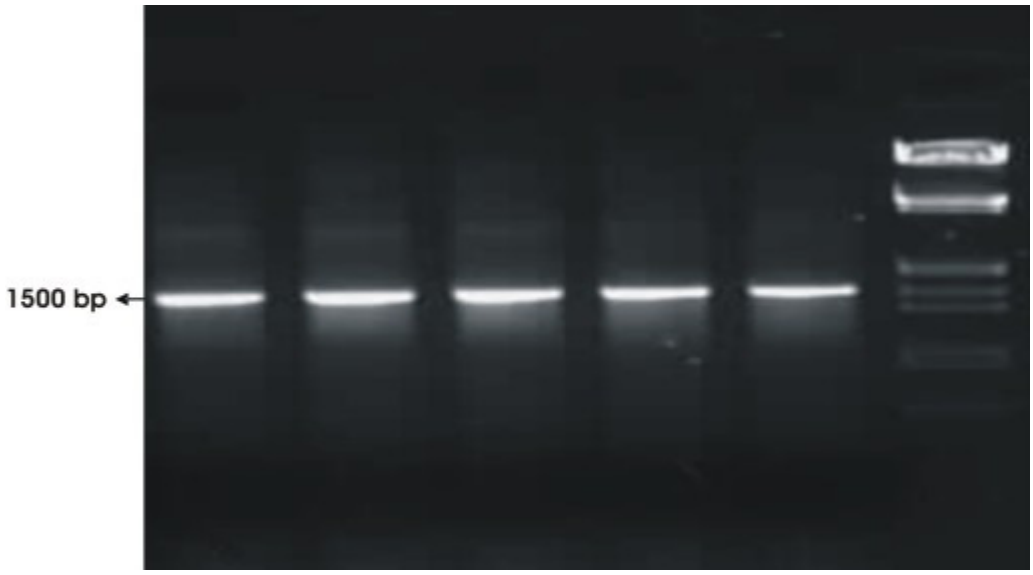


Plate 3. PCR amplification of chiB from genmic DNA

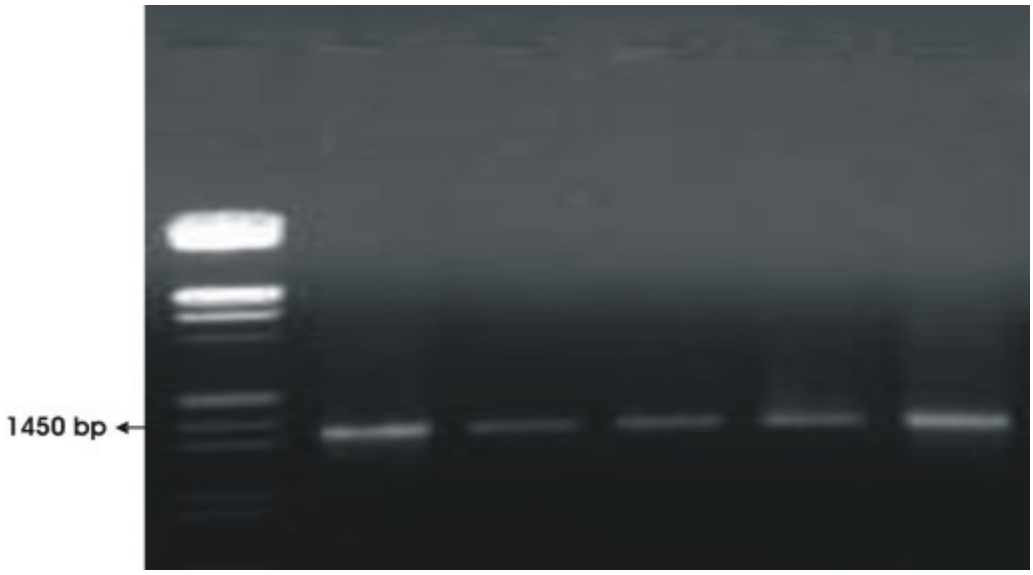


Plate 4. PCR amplification of chic from genomic DNA

Table 2: Screening of *S. marcescens* (Dandeli) isolates with *chi*A, *chi*B and *chi*C specific primers and their chitinolytic activity are tabled below

Sl. No.	Isolate	*chi*A	*chi*B	*chi*C	Zone of hydrolysis (mm)
1	AUDS190	Positive	Negative	Positive	2
2	AUDS191	Positive	Negative	Positive	2
3	AUDS192	Positive	Negative	Positive	2
4	AUDS220	Positive	Negative	Positive	4
5	AUDS221	Positive	Positive	Positive	2
6	AUDS224	Positive	Negative	Positive	2
7	AUDS226	Positive	Negative	Positive	4
8	AUDS227	Positive	Negative	Positive	5
9	AUDS228	Positive	Negative	Positive	4
10	AUDS229	Positive	Negative	Positive	3
11	AUDS230	Positive	Negative	Positive	2
12	AUDS231	Positive	Negative	Positive	2
13	AUDS232	Positive	Negative	Positive	7
14	AUDS241	Positive	Negative	Positive	3
15	AUDS242	Positive	Negative	Positive	5
16	AUDS243	Positive	Negative	Positive	5
17	AUDS256	Positive	Negative	Positive	4
18	AUDS257	Positive	Negative	Positive	2
19	AUDS258	Positive	Negative	Positive	2
20	AUDS259	Positive	Negative	Positive	4
21	AUDS260	Positive	Negative	Positive	2
22	AUDS261	Positive	Negative	Positive	3
23	AUDS263	Positive	Positive	Positive	5
24	AUDS264	Positive	Positive	Positive	5
25	AUDS265	Positive	Positive	Positive	5
26	AUDS266	Positive	Positive	Positive	4
27	AUDS267	Positive	Negative	Positive	2
28	AUDS268	Positive	Negative	Positive	2
29	AUDS272	Positive	Negative	Positive	2
30	AUDS273	Positive	Positive	Positive	3
31	AUDS274	Positive	Positive	Positive	5

Contd…

32	AUDS275	Positive	Negative	Positive	2
33	AUDS276	Positive	Positive	Positive	2
34	AUDS278	Positive	Positive	Positive	2
35	AUDS285	Positive	Negative	Positive	4
36	AUDS286	Positive	Negative	Positive	3
37	AUDS287	Positive	Negative	Positive	3
38	AUDS288	Positive	Negative	Positive	5
39	AUDS294	Positive	Negative	Positive	2
40	AUDS307	Positive	Negative	Positive	2
41	AUDS308	Positive	Negative	Positive	2
42	AUDS309	Positive	Negative	Positive	2
43	AUDS311	Positive	Negative	Positive	2
44	AUDS312	Positive	Negative	Positive	2
45	AUDS365	Positive	Negative	Positive	5
46	AUDS366	Positive	Negative	Positive	3
47	AUDS367	Positive	Negative	Positive	2
48	AUDS368	Positive	Negative	Positive	3
49	AUDS370	Positive	Negative	Positive	3
50	AUDS409	Positive	Negative	Positive	2
51	AUDS515	Positive	Negative	Positive	2
52	AUDS518	Positive	Negative	Positive	2
53	AUDS531	Positive	Negative	Positive	4
54	AUDS532	Positive	Negative	Positive	2
55	AUDS533	Positive	Negative	Positive	2
56	AUDS534	Positive	Negative	Positive	4
57	AUDS643	Positive	Negative	Positive	2
58	AUDS644	Positive	Negative	Positive	4
59	AUDS645	Positive	Negative	Positive	2
60	AUDS646	Positive	Negative	Positive	2

4.5.1 Confirmation of clones by restriction digestion

The PCR positive clones were further confirmed by restriction analysis. The release of 1692 bp fragment from pSBK201A, pSBK201B, pSBK201C and pSBK201D and the absence of such fragment in pET32C$^+$ plain vector when restricted with *EcoR*I and *Hind*III confirmed that the clones were recombinant (Plate 6 and 7). The vector map of pET32C+ and pSBK201A is presented in Fig. 10 and 11.

4.6 Expression studies in *E. coli* BL21 (DE3)

To analyse the expression of the cloned *chi*A from *S. marcescens* AUDS227, the total protein from IPTG induced *E. coli* BL21 (DE3) pSBK201A, *E. coli* BL21 (DE3) pSBK201B, *E. coli* BL21 (DE3) pSBK201C and *E. coli* BL21 (DE3) pSBK201D recombinants and plain pET32C$^+$ (as a control) were subjected to SDS-PAGE. The protein band corresponding to approximately 79.86 kDa (Plate 8 and 9) in pSBK201A, pSBK201B and pSBK201D indicated

ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCGGCGCA

GGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATTGTTGAAGTT

GACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCCGATGTTTCCGTCTC

CTGGAATTTATGGAATGGCGACACCGGCACGACGGCAAAAGTTTTATTAAATGGCAAAGAGGCGT

GGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTTAAAGTGAATAAAGGCGGCCGTTAT

CAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGCACCGCCAGTGACGCCACCGAAATTGTGG

TGGCCGACACCGACGGCAGCCATTTGGCGCCGTTGAAAGAGCCGCTGCTGGAAAAGAATAAACC

GTATAAACAGAACTCCGGCAAAGTGGTCGGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCA

ATTTCACCGTCGACAAGATCCCGGCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATC

TGCGGCGGCAATGGCATCAACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTGCAGC

GCTCCTGCCAGGGCCGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAA

AGCGCAGAAGGGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCG

CTGAAGCAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACC

CGTTCTTCTTCATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTCCTG

CAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAAGGCGCCA

ACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGCTGCTGATGAAGGAGCTGCGGAC

GATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACCTCCGCCATCAGCGCC

GGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAACTCGATGGATCACATCTTCCT

GATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAACCTGGGGCATCAGACCGCGCTGAATG

CGCCGGCCTGGAAACCGGACACCGCCTACACCACGGTGAACGGCGTCAATGCGCTGCTGGCGCA

GGGCGTCAAGCCGGGCAAAATCGTGGTCGGCACCGCCATGTATGGCCGCGGCTGGACCGGGGTG

AACGGCTACCAGAACAATATTCCGTTCACCGGCACCGCCACCGGGCCGGTTAAAGGCACCTGGG

AGAACGGTATCGTGGACTACCGCCAAATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTATAC

CTACGACGCCACGGCGGAAGCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCG

ACGATGCCCGCTCGGTGCAGGCCAAAGGCAAGTACGTGTTGGATAAGCAGCTGGGCGGCCTGTT

CTCCTGGGAGATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAAC

AGCGCCGGCGTTCAATAA

Fig 2. Complete nucleotide sequence of *Sm*141*chi*A (Reference)

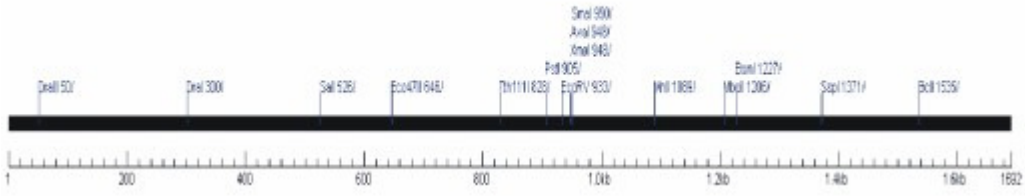


Fig 3. Restriction site map of SM141chiA with common enzymes

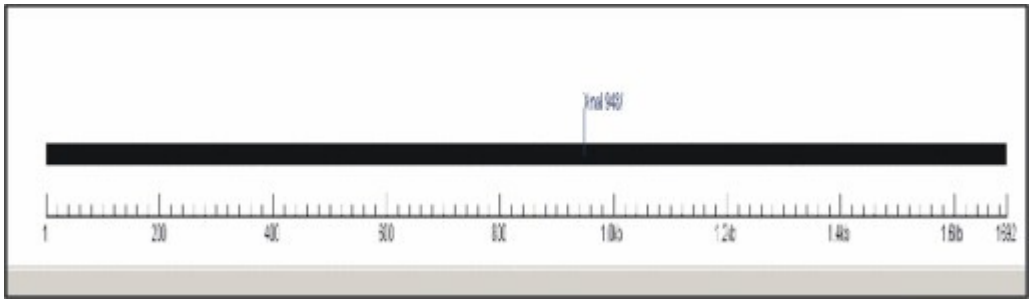


Fig 4. Restriction site map of Sm141chiA (Reference) with Xma1

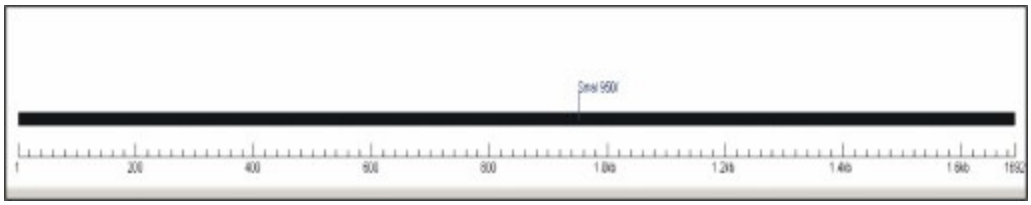


Fig 5. Restriction site map of Sm141chiA with Smal

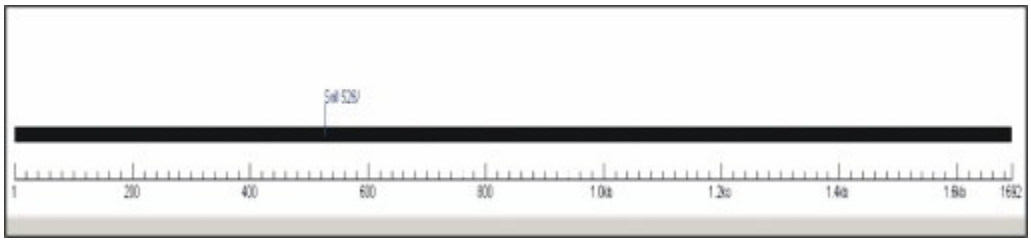


Fig 6. Restriction site map of Sm141chiA Sall

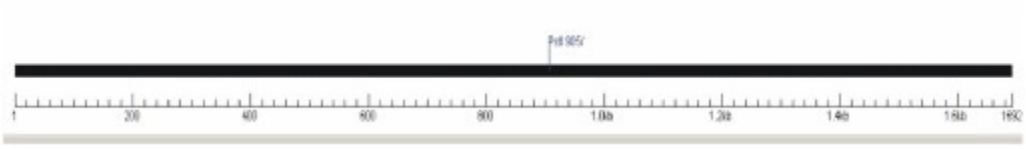


Fig 7. Restriction site map of Sm141chiA with PstI

Table 3: PCR-RFLP profile of *chi*A positive isolates with *Pst*I, *Xma*I, *Sma*I and *Sal*I

Restriction fragment sizes in (bp)					
Sl. No.	**Enzymes**	***Sm*141*chi*A (Reference)**	***Sm*AUDS227*chi*A**	***Sm*AUDS274*chi*A**	***Sm*AUDS365*chi*A**
1	*Xma*I	948, 744	948, 744	948, 744	948, 744
2	*Sma*I	950, 742	950, 742	950, 742	950, 742
3	*Sal*I	1100, 526	1100, 526	1100, 526	1100, 526
4	*Pst*I	905, 787	787, 361, 283, 261	787, 361, 283, 261	787, 361, 283, 261

the expression of *chi*A in *E. coli* BL21 (DE3) and absence of such band in control and pSBK201C when loaded at equal amount of crude protein (≈100 µg). For further functional analysis of the clones, antifungal bioassay was employed.

4.7 Antifungal bioassay

The pSBK201A, pSBK201B and pSBK201D construct carrying *chi*A in *E. coli* BL21 (DE3) were induced with IPTG and the active protein was isolated. The isolated protein of different concentration viz. 50, 100 and 200 µg/µl from respective confirmed clones were subjected for bioassay against the mycelial growth of fungal pathogens viz. *S. rolfsii* and *R. solani* and after 12 hrs of incubation at 28 ± 2℃, the crescent shaped zone of growth inhibition of S*. rolfsii* and *R. solani* was observed compared to the control (Plate 10 and 11).

4.8 Sequencing and *In silico* analysis of the clones

The nucleotide sequences of clones pSBK101A, pSBK101B, pSBK101C and pSBK101D were analysed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. *Sm*AUDS227*chi*A had 99 per-cent homology with S. *marcescens* Bn10 endochitinase (DQ165083), 98 per-cent homology with *chi*60 of S. sp. TV09 (AY040610), 98 per-cent homology with *S. marcescens* BJL200 *chi*A (Z36294), 97 per-cent homology with *S. marcescens* strain 141 *chi*A (EV753246). The results are presented in Table 4.

The BLASTp results of amino acid sequence of cloned *chi*A showed 99 per -cent homology with reported *chi*A of *S. marcescens* (ABS70983.1), 99 per-cent homology with chitinase of *Sanguibacter* sp. C4 (ABB91448.1), 99 per-cent homology with *S. marcescens chi*A (ABI79317.1), 99 per-cent homology with *chi*A of *S. marcescens* (ACE78180.1). The results are presented in Table 4.

Sequence analysis using BTI gene tool software revealed the length of amplicon is 1692 bp for *Sm*AUDS227*chi*A. It starts with ATG codon and terminates at position 1692 with a TAA stop codon (Fig. 12) having 1691 bp ORF. The *Sm*AUDS227*chi*A has *N*-terminal signal peptide of 23 residues which is rich in leucine (Fig. 13) and the gene product is a 563 residue protein with the estimated size of ~79.86 kDa with *N*-terminal tag.

The pair-wise alignment of amino acid sequences *Sm*AUDS227*chi*A and *Sm*141*chi*A (reference) was done using BTI Gene tool software and it has revealed the variation in the

Plate 5. RFLP profile of *chi*A positive isolates

1. *Sm*141*chi*A uncut
2. *Sm*AUDS227*chi*A with *Pst*I
3. *Sm*AUDS274*chi*A with *Pst*I
4. *4. Sm*AUDS365*chi*A with *Pst*I
5. Lambda DNA/ *Eco*RI +*Hind*III marker
6. 100 bp ladder.
7. *Sm*141*chi*A with *Pst*I

Plate 6. PCR confirmation of pSBK101A and pSBK201A

M. Lambda DNA/ *Eco*RI +*Hind*III marker

1. *Sm*141*chi*A (positive control)
2. plain pTZ57R/T (2886bp)
3. pTZ57R/T with insert (1692 bp)
4. plain pET32C$^+$ (5901bp)
5. pET32C$^+$ with insert (1692 bp)

Plate 7. Restriction confirmation of pSBK101A and pSBK201A

M1. 1 kb ladder

M2. lambda DNA/*Hind*III digest

1 Plain pTZ57R/T (2886 bp)

2 pSBK101A (pTZ57RT+*chi*A)

3 Plain pET32C$^+$ (5901 bp)

4 pSBK201A (pET32C$^+$ + *chi*A)

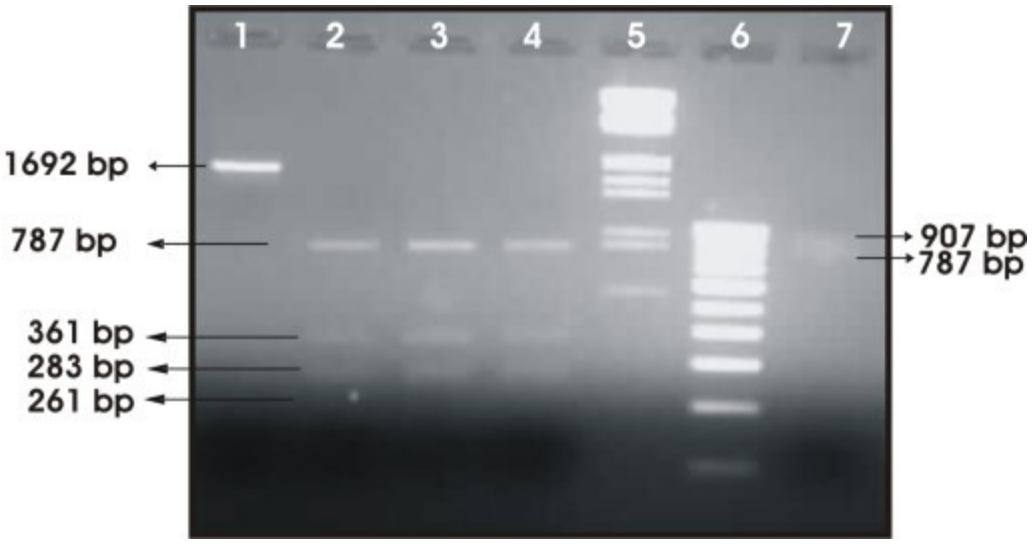


Plate 5. RFLP pattern of chiA positive S. marcescens isolates

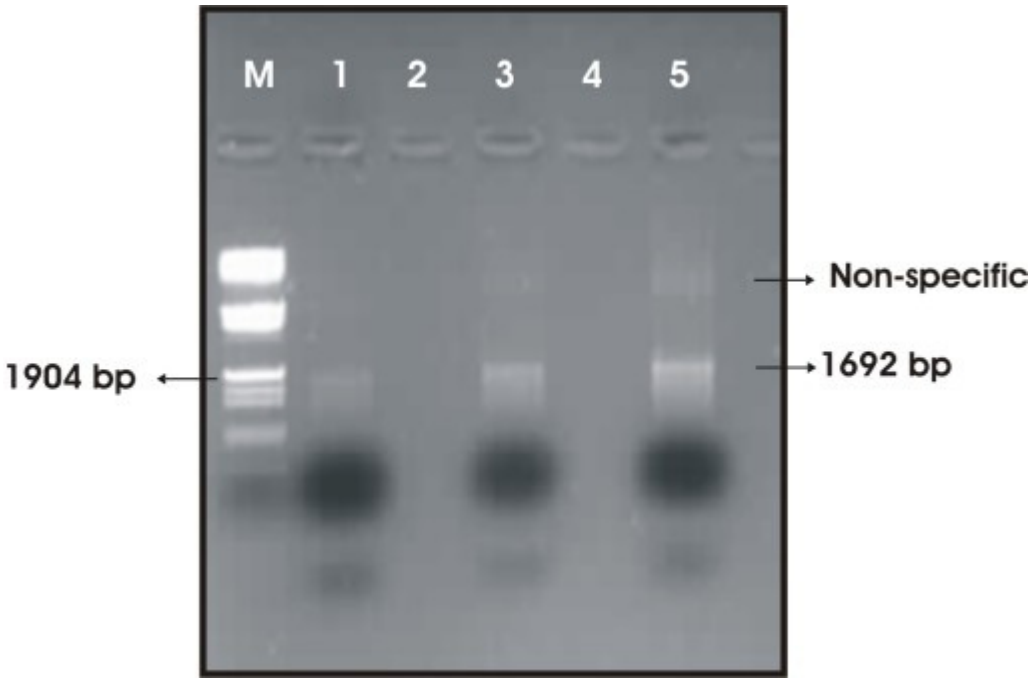


Plate 6. PCR confirmation of pSBK101A and pSBK201A

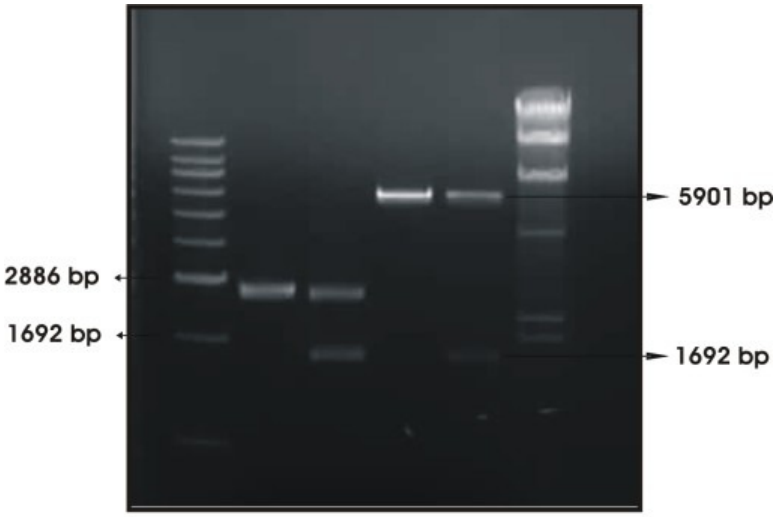


Plate 7. Restriction confirmation of pSBK101A and pSBK201A

Plate 8. SDS-PAGE analysis for expression of *chi*A

1. Uninduced *E. coli* BL21 (DE3)
2. Induced *E. coli* BL21 (DE3)
3. Page ruler^{TM} plus prestained protein ladder # SM1811
4. Uninduced pET32C^{+}
5. Induced pET32C^{+}
6. Uninduced pSBK201A
7. Induced pSBK201A
8. Uninduced pSBK201B
9. Induced pSBK201B

Plate 9. SDS-PAGE analysis for expression of *chi*A

1. Page ruler^{TM} plus prestained protein ladder # SM1811
2. Uninduced BL21
3. Induced BL21
4. Uninduced pET32C^{+}
5. Induced pET32C^{+}
6. Uninduced pSBK201C
7. Induced pSBK201C
8. Uninduced pSBK201D
9. Induced pSBK201D

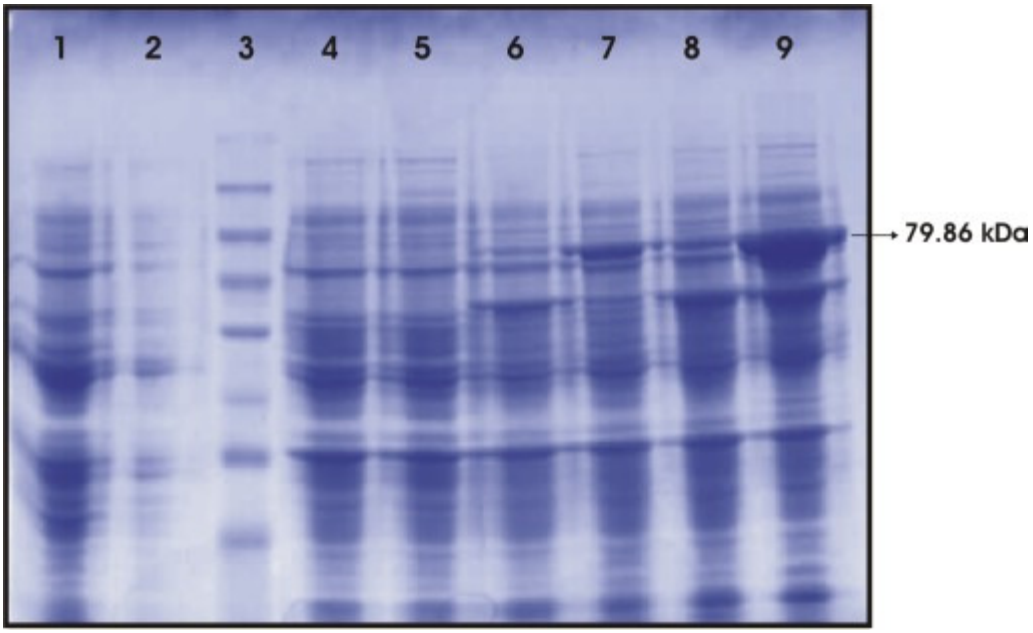


Plate 8. SDS-PAGE analysis for expression of chiA

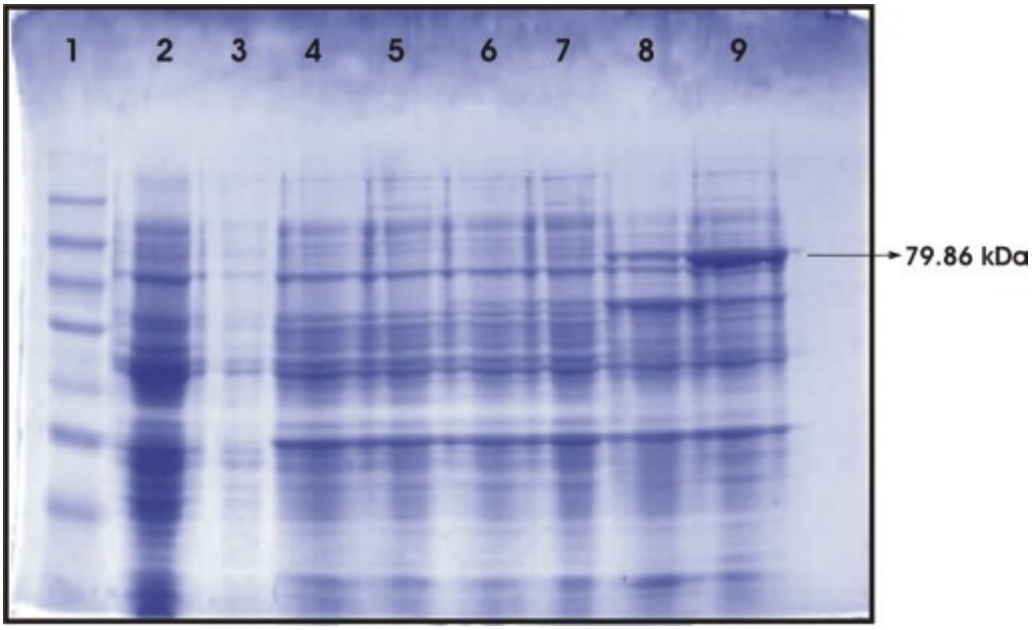


Plate 9. SDS-PAGE analysis for expression of chiA

catalytic site at 96th and 240th position in *Sm*AUDS227*chi*A (Fig. 19). The 1692 bp nucleotide sequence of *Sm*AUDS227*chi*A has shown the *Pst*I site at 361th, 644th and 905th position whereas, reference *Sm*141*chi*A nucleotide sequence has unique site for *Pst*I enzyme at 905thposition. The ORF of *Sm*AUDS141*chi*A and *Sm*AUDS227*chi*A are presented in Fig. 15 and 16. The common restriction enzymes and their sites in *Sm*AUDS227*chi*A are presented in Fig. 17 and 18.

4.9 Theoretical calculation of the deduced protein

*Sm*AUDS227*chi*A has an ORF of 1691 bp encoding 563 amino-acid residues with the first 163 amino acids represent as *N*-terminal tag. The SDS-PAGE analysis revealed the size of ChiA is ~79.86 kDa. The average molecular weight of amino acid is ~0.11 kDa. Therefore, estimated size of recombinant ChiA with 563 and 163 amino-acid residues is ~79.86 kDa.

Plate 10. Antifungal activity od chitinase A against *Sclerotium rolsfii*

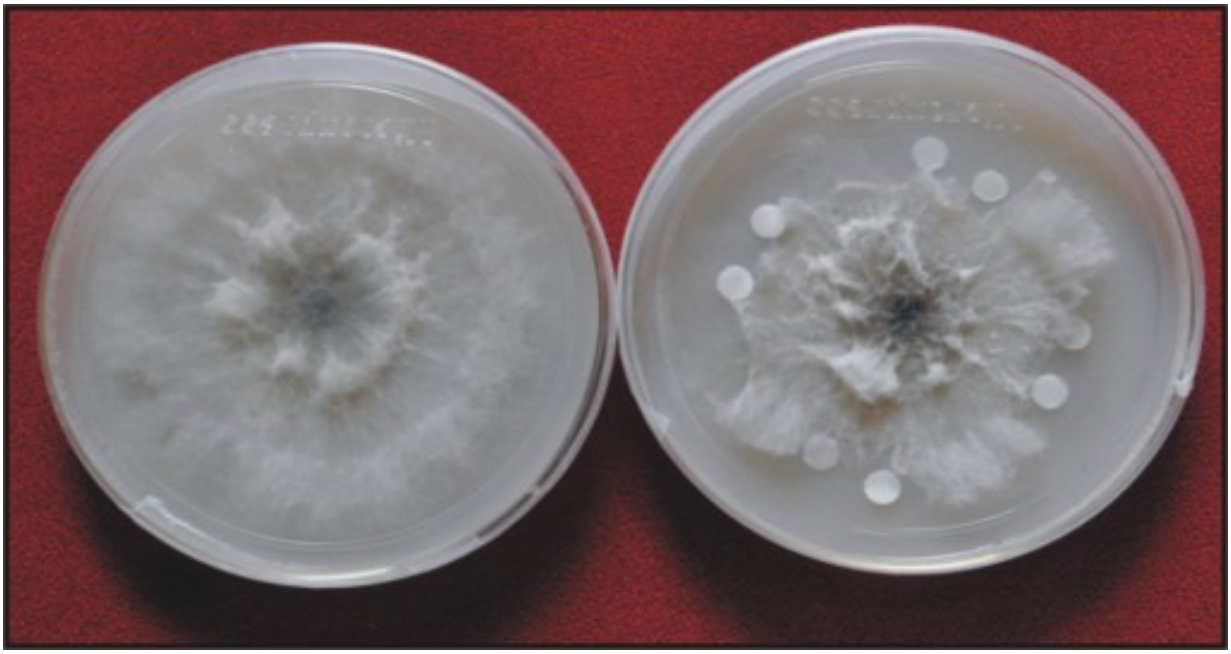


Plate 11. Antifungal activity of chitinase A against *Rhizoctonia solani*

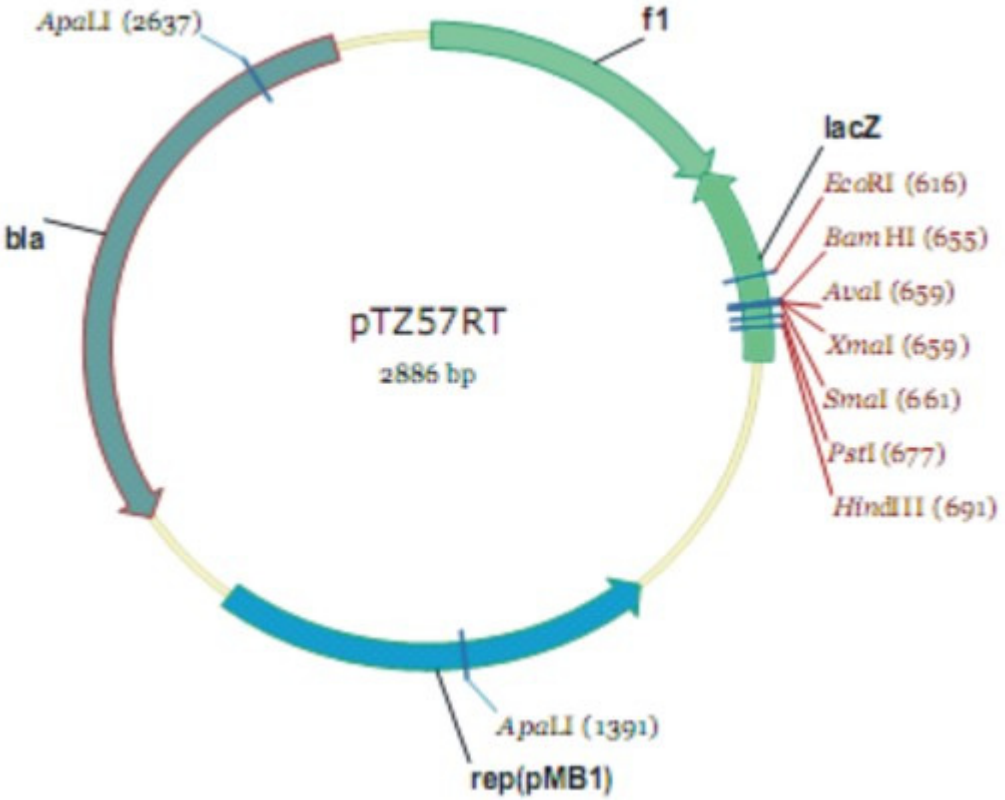


Fig 8. Vector map of pTZ57R/T

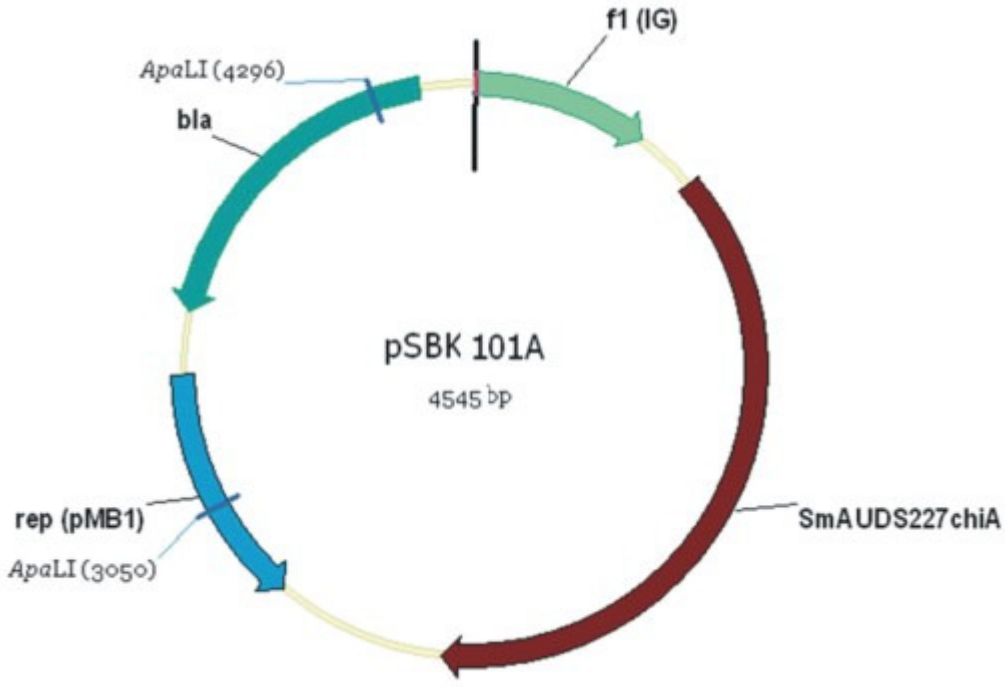


Fig 9. Vector map of pSBK101A

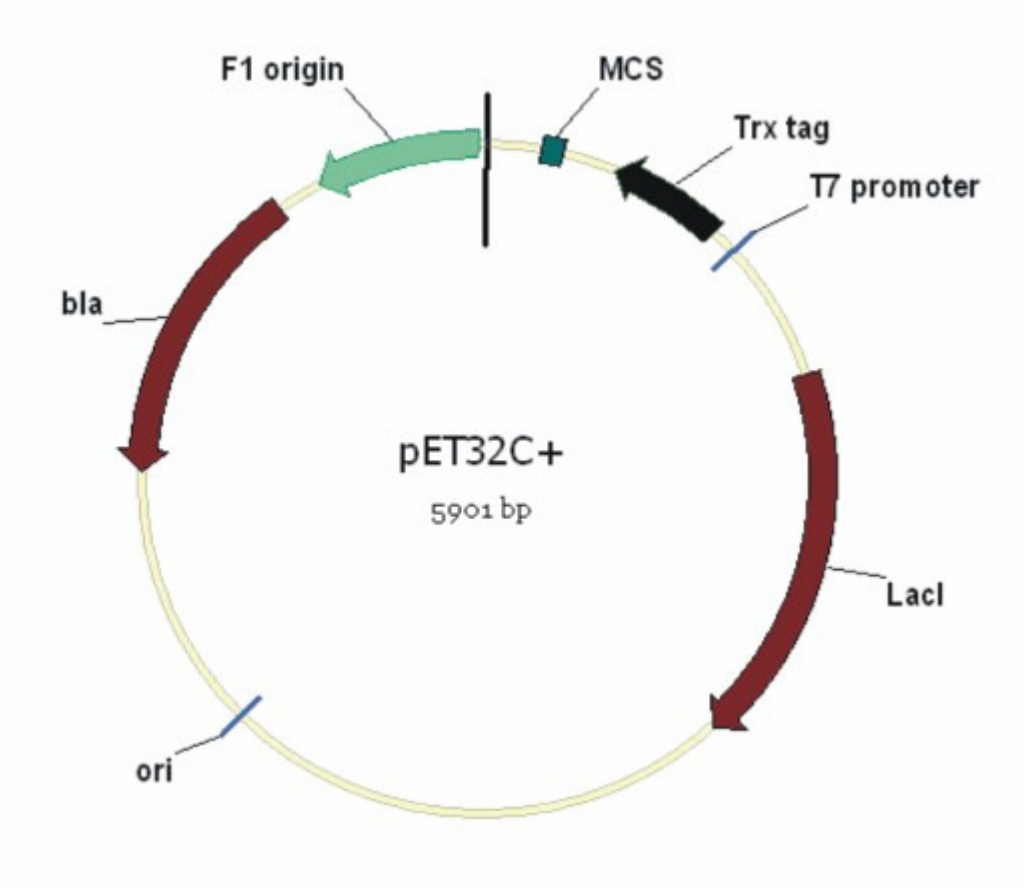


Fig 10. Vector map of pET32C+

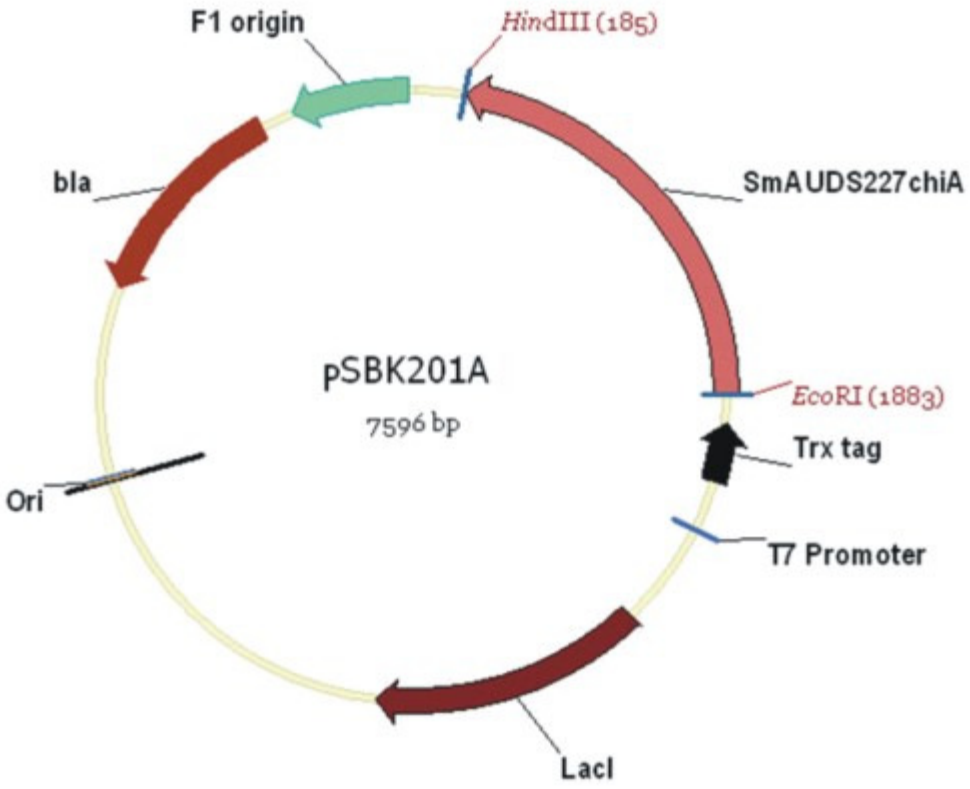


Fig 11. Vector map of pSBK201A

ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCG
CGGCGCAGGCCGCCGCGCCGGGCAAACCGACCATCGCCTGGGGCAACACCAAGTTCG
CCATCGTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAAT
GCCGCCGATGTTTCGGTCTCTTGGAATTTATGGAATGGCGACACCGGTACGACGGCAA
AAGTTTTATTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTAC
GGCGAATTTTAAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAAT
GCCGACGGCTGCAGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGC
AGCCATTTGGCGCCGTTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGA
ACTCCGGCAAAGTGGTGGGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTT
CACCGTCGACAAGATCCCGGCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCG
ATCTGCGGCGGCAACGGCATCAACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGG
CGCTGCAGCGCTCCTGCCAGGGCCGCGAGGACTTCAAAGTCTCGATCCACGATCCGTT
CGCCGCGCTGCAAAAAGCGCAGAAGGGCGTTACCGCCTGGGATGACCCCTACAAGGG
CAACTTCGGCCAGCTGATGGCGCTGAAACAGGCGCATCCTGACCTGAAAATTCTACCG
TCGATCGGCGGCTGGACGCTGTCCGACCCGTTCTTCTTCATGGGCGATAAGGTGAAGC
GCGATCGCTTCGTCGGTTCGGTGAAAGAGTTCCTGCAGACCTGGAAGTTCTTCGATGG
CGTGGATATCGACTGGGAGTTCCCGGGCGGCAAAGGCGCCAACCCGAACCTGGGCAG
CCCGCAGGACGGGGAAACCTATGTGCTGCTGATGAAGGAGCTGCGGGCGATGCTGGA
TCAGCTGTCGGCGGAAACCGGCCGCAAATATGAACTGACCTCCGCCATCAGCGCCGGC
AAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAACTCGATGGATCACATTT
TCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAACCTGGGGCATCAGAC
CGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACGGTGAACGGCGT
CAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAAATCGTGGTCGGCACCGCCAT
GTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTCACCGGT
ACCGCCACCGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGCCAA
ATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTACACCTACGACGCCACGGCAGAA
GCGCCATACGTGTTCAAGCCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCT
CGGTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCTTG
GGAGATCGACGCGGACAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAAC
AGCGCCGGCGTTCAATAA

Fig 12. Complete nucleteoide sequence of *Sm*AUDS227*chi*A

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNNLVKVKNAA

DVSVSWNLWNGDTGTTAKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNAD

GCXASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDK

IPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK

GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF

LQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRXMLDQLSAETGRKYEL

TSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKPDTAYT

TVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPVKGTWENGIVD

YRQIAGQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFS

WEIDADNGDILNSMNASLGNSAGVQ*

Legend:

Signal peptide :..... 1-23

N- terminal domain:.....24-140

Hinge region:.....141-158

Catalitic domain:.....159-443

Small α + β domain:.....444-516

Catalitic domain :.....517-563

Fig 13. Deduced amino acid sequence of *Sm*UDS227*chi*A

Fig 14. rps-BLAST result of *Sm*AUDS227*chi*A showing conserved domain

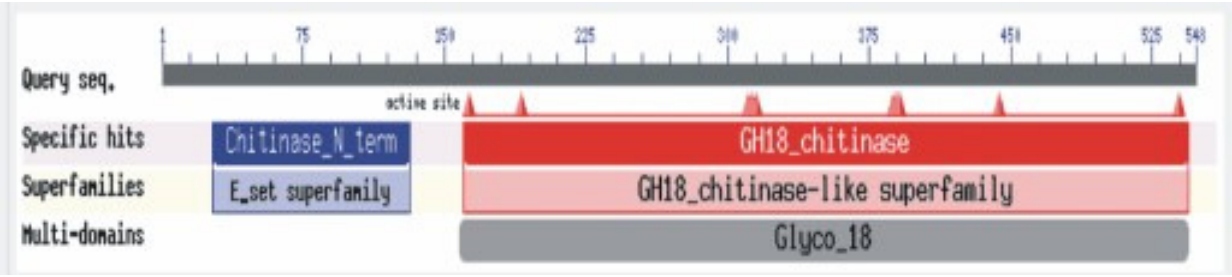


Table 4. BLASTn result of *Sm*AUDS227*chi*A

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
DQ165083.1	Serratia marcescens Bn10 endochitinase (chiA) gene, complete cds	3064	3064	100%	0.0	99%	
AY040610.2	Serratia sp. TU09 chitinase (Chi60) gene, complete cds	3031	3031	100%	0.0	98%	
Z36294.1	S.marcescens (BJL200) chiA gene for chitinase	3025	3025	100%	0.0	98%	
DQ282126.1	Sanguibacter sp. C4 chitinase (chit58) gene, complete cds	2926	2926	100%	0.0	97%	
EU753246.1	Serratia marcescens strain C8-8 chitinase A (chiA) gene, complete	2898	2898	100%	0.0	97%	
DQ990373.1	Serratia marcescens strain 141 chitinase A (chiA) gene, complete c	2892	2892	100%	0.0	97%	
EF451957.1	Serratia proteamaculans strain 18A1 endochitinase (chiA) gene, cc	2881	2881	100%	0.0	97%	
DQ285568.1	Serratia proteamaculans chitinase A gene, complete cds	2881	2881	100%	0.0	97%	

Table 5. BLASTp result of *Sm*AUDS227*chi*A

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
ABS70983.1	chitinase [Serratia marcescens]	1126	1126	99%	0.0	
ABB91448.1	chitinase [Sanguibacter sp. C4]	1125	1125	99%	0.0	
ABI79317.1	chitinase A [Serratia marcescens]	1124	1124	99%	0.0	
CAA85291.1	unnamed protein product [Serratia marcescens] >gb|AAK72610.2|	1124	1124	99%	0.0	
AAZ86539.1	endochitinase [Serratia marcescens]	1123	1123	99%	0.0	
AAL57854.1	endo-chitinase [Serratia marcescens] >gb|ABB86291.1| chitinase A	1123	1123	99%	0.0	
ACE78180.1	chitinase A [Serratia marcescens]	1122	1122	99%	0.0	
BAA31567.1	chitinase A precursor [Serratia marcescens]	1121	1121	99%	0.0	
ABF48722.1	chitinase A precursor [Serratia marcescens]	1118	1118	99%	0.0	

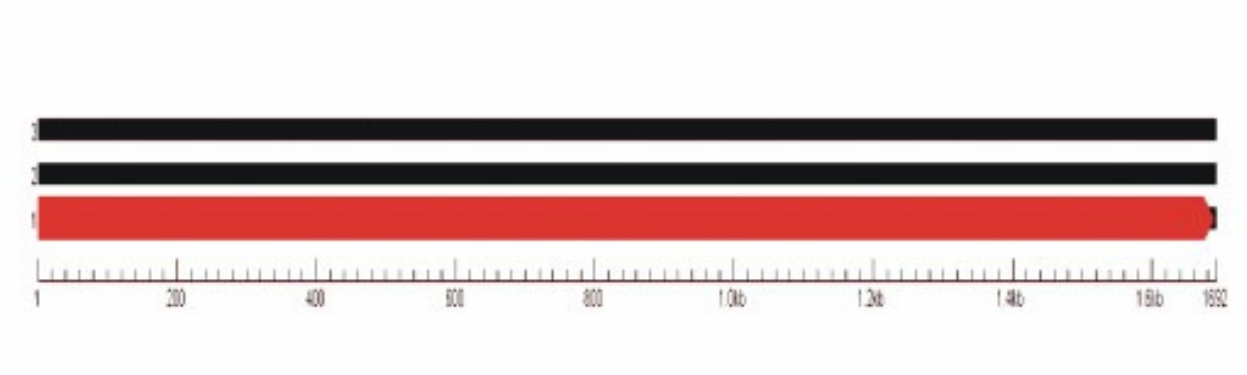


Fig 15. Open reading frame of (Reference) *Sm*141*chi*A

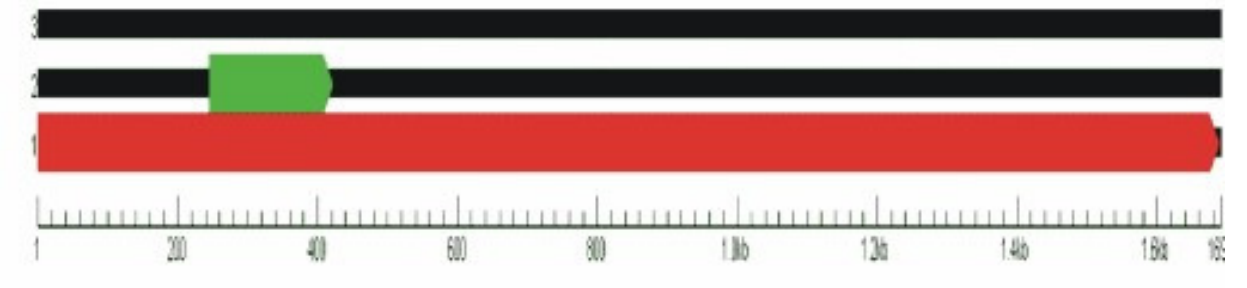


Fig 16. Open reading frame of *Sm*AUDS227*chi*A

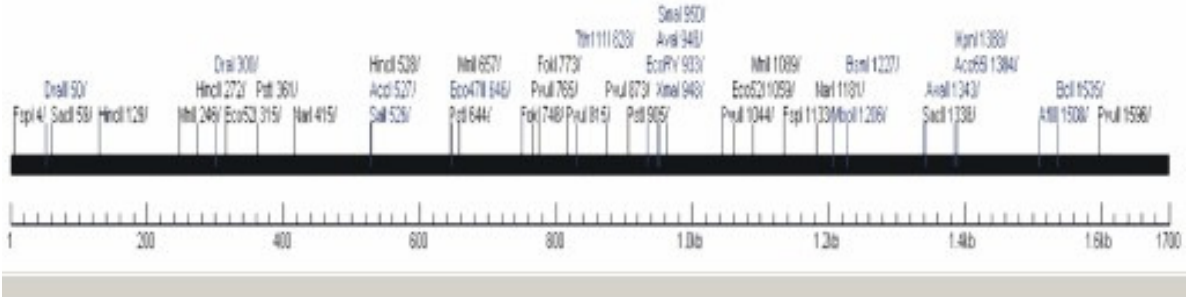


Fig 17. Common restriction sites found in *Sm*AUDS227*chi*

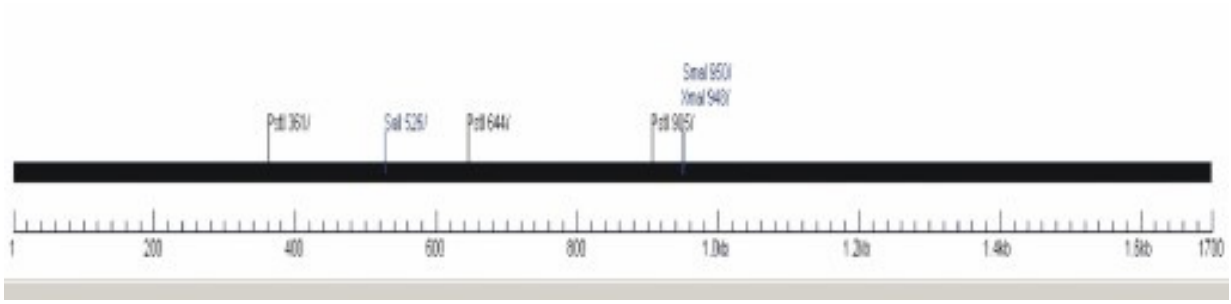


Fig 18. Restriction sites of *PstI, SalI, XmaI and SmaI* in *Sm*AUDS227*chiA*

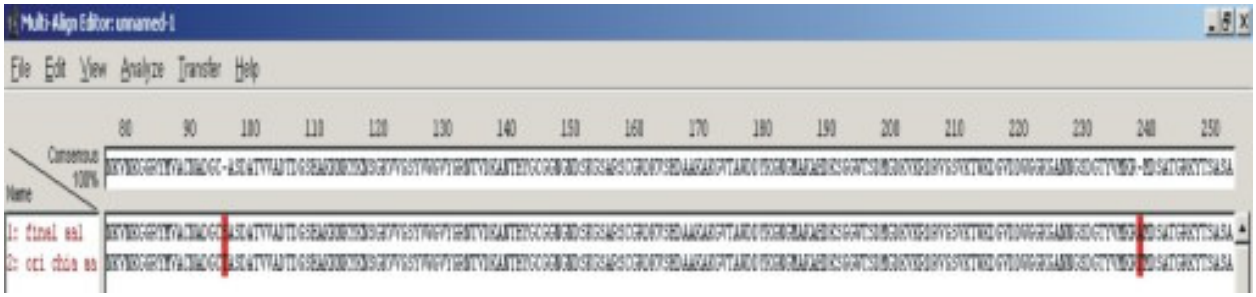


Fig 19. Pairwise alignment of amino acid sequences

5. DISCUSSION

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. Fungicides are widely used to control the plant diseases. However, most of the fungicides are hazardous to the health of humans and animals (Rommens and Kishore 2000). In order to tackle these problems, effective alternatives to reduce or eliminate the use of toxic chemicals are being investigated. Mining of useful genes from microbial sources and their validation in crop plants has been considered as a useful option. Therefore, cloned disease resistance genes as well as defence response genes such as those encoding for chitinases and glucanases, have important uses against pathogens that attack plant species (Leong, 2004).

Chitin is a β-1, 4-linked polymer of *N*-acetyl glucosamine (GlcNAc), is an important structural component of insects, fungi and nematodes. Hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extracellularly by the action of chitinases. These enzymes have been shown to play an important role in biological control of soil plant pathogens (Monreal and Reese, 1969).

In the present study, sixty Dandeli isolates of *S. marcescens* available in the culture collection of Department of Biotechnology, UAS, Dharwad were screened for their chitinolytic activity by growing on colloidal chitin plate in which chitin was the sole carbon source. The *S. marcescens* isolates AUDS227, AUDS232, AUDS242, AUDS243, AUDS263, AUDS264, AUDS265, AUDS274, AUDS288 and AUDS365 were found to show very high chitin resolution indicating that they could be very effective in producing chitinase enzyme in comparison with the reference strain *Sm*141. To detect the chitinase activity of *S. maitophila* 34S1, genomic library was constructed in *B. cepacia* M53 and the randomly selected cosmid clones resulted in clearance of colloidal chitin agar within 5 days (Donald *et al*., 2002). Hence, these isolates were employed for understanding the diversity of presence of chitinase genes as well as in identifying the presence, cloning and expression of *chi*A.

Based on the available information, primers were designed and used for amplification of *chi*A, *chi*B and *chi*C. About 1692 bp, 1500 bp and 1450 bp amplicons of *chi*A, *chi*B and *chi*C were amplified from the genomic DNA of *S. marcescens*. All the sixty isolates of *S. marcescens* were found to be positive for *chi*A and *chi*C and only nine isolates *viz*. AUDS221, AUDS263, AUDS264, AUDS265, AUDSA266, AUDS273, AUDS274, AUDS276 and AUDS278 were found to be positive for *chi*B.

On the basis of colloidal chitin degradation assay, the highly potent isolates of *S. marcescens* were selected further for PCR-RFLP analysis to identify the presence of novel variant of *chi*A using an approach similar to that of Kuo and Chak, 1996. Kuo and Chak (1996) designated PCR-RFLP typing system is a facile method to detect both known and novel *cry* genes existing in a *B. thuringiensis* strain. PCR-RFLP method helps to detect the organization and differential expression of cry genes. They have identified the novel *cry* genes existing in *B. thuringiensis* strains. The restriction fragment length polymorphism (RFLP) patterns of the PCR-amplified fragments revealed 14 distinct *cry*-types from 20 *B. thuringiensis* strains. Meyer *et al*. (1992) studied a PCR-RPLP fingerprinting technique to analyse the nine species aggregates of *Trichoderma*. The sequence diversity of *groESL* genes among *Streptococcus bovis* group isolates was analysed through PCR-RFLP including five reference strains and 36 clinical isolates (Chen *et al*., 2008).

The nucleotide sequence of *Sm*141*chi*A was analyzed using BTI Gene tool software. It revealed the presence of *Sma*I at 950th, *Xma*I at 948th, *Sal*I at 526th and *Pst*I site at 905th position. The RFLP pattern of *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A generated by *Sal*I revealed 526 bp and 1166 bp fragments, by *Xma*I 948 bp and 744 bp fragments and by *Sma*I 950 bp and 742 bp fragments which were similar to banding pattern obtained by *in vitro* restriction digestion of the reference *Sm*141*chi*A. Through *Pst*I, however, the *chi*A amplicon obtained from *S. marcescens* isolates AUDS227, AUDS274 and AUDS365 produced 787 bp, 361 bp, 283 bp and 261 bp sized fragments, which were found to be different compared to fragments produced in the reference *Sm*141*chi*A. The reference *Sm*141 *chi*A gave two fragments, 905 bp and 787 bp.

The novel *chi*A from *S. marcescens* AUDS227 identified through the RFLP analysis was cloned into pTZ57R/T vector and the transformants having recombinants were isolated through blue-white assay. The white colonies were confirmed as recombinants by *chi*A gene specific primer and through restriction analysis. The orientation of insert was confirmed with *Sma*I.

Jones *et al*. (1986) cloned *chi*A (1699 bp) from *S. marcescens* which encoded for 565 amino acids. The endochitinase gene *chi*A74 from *Bacillus thuringiensis* serovar kenyae LBIT-82 was cloned in *Escherichia coli* DH5α and sequence of 676 amino acids was deduced (Barboza *et al*., 2003). *S. marcescens chi*A was PCR cloned, sequenced and heterologously expressed in an anti-coleopteran *B. thuringiensis* (strain 3023-SCHI). The specific *chi*A activity of recombinant *B. thuringiensis* reached its level even higher than that produced by the source organism (Okay *et al*., 2008). By co-introduction of *cry1*Ac7 gene of *B. thuringiensis* strain 234 and *chi*A gene of *S. marcescens* into strains of *P. fluorescens*, an increased biocontrol of sugarcane borer *E. saccharina* could be achieved (Downing *et al*., 2000). Jang *et al*. (2005) reported cloning, over expression and biochemical characterization of the novel chitinase A (*chi*A) in *E. coli*.

As pTZ57R/T is more of a cloning vector, the *Sm*AUDS227*chi*A was further sub cloned into a prokaryotic expression vector pET32C^{+}, to study the expression of cloned *chi*A in *E. coli* BL21 (DE3) and the recombinants were analyzed by PCR amplification with *chi*A gene specific primers and restriction with *EcoR*I and *Hind*III. *E. coli* BL21 (DE3) containing pSBK201A, pSBK201B, pSBK201C and pSBK201D construct harboring *chi*A from *Sm*AUDS227 were subjected to SDS-PAGE. The protein bands corresponding to ~79.86 kDa was observed for *chi*A in pSBK201A, pSBK201B and pSBK201D indicating the expression of the cloned gene. There was no such expression of ChiA protein in case of pSBK201C.

For functional validation of the cloned *Sm*AUDS227*chi*A, the clones pSBK201A, pSBK201B and pSBK201D were induced with 1 mM IPTG and the extracted protein was subjected for bioassay against the fungal pathogens viz. *S. rolfsii* and *R. solani*. After 12 hrs of incubation at 28°C the zone of mycelial growth inhibiton of pathogens was observed. The inhibition was more in case of *R. solani* than *S. rolfsii*. The antifungal activity of chitinases cause rapid loss of fungal hypal tips and germinating spores. These enzymes are an effective tool for the complete degradation of mycelial or conidial walls of phytopathogenic fungi (De la Cruz *et al*., 1995).

*S. marcescens chi*A increased tolerance to *R. solani* (Howie *et al*., 1994). The *chi*A and *chi*B genes from *S. marcescens* have been transformed into other bacterial species like *Pseudomonas fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens or to create new biocontrol agents (Brurberg *et al*., 2000). Furthermore, chitinase ChiA partially purified after cloning into *E. coli* was found to reduce diseases caused by *Sclerotium rolfsii* in beans and *Rhizoctonia solani* in cotton (Shapira *et al*., 1989).

The nucleotide sequences of *chi*A in pSBK101A, pSBK101B, pSBK101C and pSBK101D were analysed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. Cloned *chi*A had 99 per cent homology with *S. marcescens* Bn10 endochitinase (DQ165083), 98 per cent homology with *chi*60 of *Sanguibacter* sp. TV09 (AY040610), 98 per cent homology with *S. marcescens* (BJL200) *chi*A (Z36294), 97 per cent homology with *chi*A of *S. marcescens* strain 141 (EV753246).

The BLASTp results of amino acid sequence of cloned *chi*A showed 99 per cent homology with reported *chi*A of *S. marcescens* (ABS70983.1), 99 per cent homology with chitinase of *Sanguibacter* sp. C4 (ABB91448.1), 99 per cent homology with *S. marcescens chi*A (ABI79317.1).

The length of amplicon is 1692 bp for *Sm*AUDS227*chi*A and sequence analysis using BTI gene tool software revealed 1691 bp ORF. It has the *N*-terminal signal peptide of 23 amino acid residues, which is rich in leucine and the gene product, is a 726 residue protein and estimated size is about 79.86 kDa. The *Sm*AUDS227*chi*A amino acid sequence has conserved domain that is similar to Glyco_domain and glycosyl hydrolase's domain.

The pair-wise alignment of amino acid sequences of *Sm*AUDS227*chi*A and reference *Sm*141*chi*A was done using BTI Gene Tool software and it has revealed the variation in the catalytic site at 96th and 240th position in *Sm*AUDS227*chi*A. The 1692 bp nucleotide sequence of *Sm*AUDS227*chi*A has the *Pst*I site at 361th, 644th and 905th position whereas, reference *Sm*141*chi*A nucleotide sequence has unique site for *Pst*I enzyme which, is at 905th position. The *Sm*AUDS227*chi*A has the same biochemical and functional properties as the *Sm*141*chi*A except the variation in the catalytic site.

The *N*-terminal chitin binding domain connected with a small hinge region which enables flexibility to the protein and α + β domain makes up one of the walls of substrate-binding groove in *chi*A (Brurberg *et al*., 2000). The *Sm*AUDS227*chi*A contains the catalytic domain which has a role in substrate binding with the characteristic sequence motifs SXGG (residues 271-274) and DXXDXDXE (residues 308-315) of a family 18 glycosyl hydrolase (Brurberg *et al*., 2000). The sequence also contains the catalytic residue amino acids Glu315, acting as a proton donor and Asp391 (Suginta *et al*., 2004).

The evaluated construct need to be explored in eukaryotic system and checked against different phytopathogenic fungi. Site specific mutagenesis will be useful to demonstrate the importance of amino acid residues in the catalytic properties of *chi*A.

As microbes are the major sources for chitinases, the current study encourages one to use PCR amplicon-RFLP as a tool to identify variants of *chi*A and explore the probability of discovery of novel chitinase genes. Thus, it is necessary to clone large number of such genes from different sources, study their effectiveness against different pathogens, identify variants of *chi*A, bio safety and deploy them in agricultural crop plants.

6. SUMMARY AND CONCLUSIONS

The present study was undertaken to characterize presence of *chi*A, *chi*B and *chi*C, to clone a novel *chi*A from *S. marcescens* isolated from the soils of the Western Ghats of India in the Dandeli region and assess the expression of cloned *chi*A in *E. coli* BL21 (DE3). The results obtained are summarized below.

- Chitinolytic activity of native isolates of *S. marcescens* on colloidal chitin agar was scored in comparison with reference strain *Sm*141. The chitinolytic bacteria were found to cause a clear zone around its growth on chitin agar medium containing colloidal chitin as substrate after incubation at 28±2℃ for 48 hrs. Based on the chitinolytic activity of native isolates of S. *marcescens*, the highly potent isolates were selected further for PCR-RFLP analysis.

- 1692bp *chi*A, 1500bp *chi*B and 1450bp *chi*C were amplified from native isolates of *S. marcescens* using specific primers designed from the reported nucleotide sequences obtained from the NCBI database.

- The chitinase profile analysis revealed that the *S. marcescens* AUDS221, AUDS263, AUDS264, AUDS265, AUDS266, AUDS273, AUDS274, AUDS276 and AUDS278 were positive for the presence of *chi*A, *chi*B and *chi*C.

- *In silico* analysis of reference *Sm*141*chi*A was done with Vector NTI and BTI Gene tool software. Based on the reference *Sm*141*chi*A nucleotide sequence *Xma*I, *Sma*I, *Sal*I and *Pst*I enzymes were selected for RFLP analysis of *chi*A.

- The RFLP analysis of *chi*A positive isolates were done along with the reference strain *Sm*141 to find out variability amongst *chi*A in the native *S. marcescens* isolates. The *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A gave different restriction fragments compared to the reference *Sm*141*chi*A. Based on the chitin resolution and difference in banding pattern of *Sm*AUDS227*chi*A with the reference *Sm*141chiA with *Pst*I, the *Sm*AUDS227*chi*A was selected further for cloning and expression studies.

- The novel *chi*A from *S. marcescens* AUDS227 was cloned in pTZ57R/T containing T overhangs at *Eco*321 site and transformed into *E. coli* DH5α to obtain the recombinant plasmid (pTZ57R/T plus *chi*A). The colonies of *E. coli* DH5α were randomly picked up and the presence of *chi*A insert was confirmed by PCR and restriction analysis and labelled as pSBK101A, pSBK101B, pSBK101C and pSBK101D.

- The novel *chi*A from pTZ57R/T was cloned into prokaryotic expression vector (pET32C+) and transferred to *E. coli* BL21 (DE3). The *E. coli* BL21 (DE3) colonies were randomly picked up, confirmed and labelled as pSBK201A, pSBK201B, pSBK201C and pSBK201D. SDS-PAGE analysis was done to analyse the expression of cloned *chi*A. *E. coli* BL21 (DE3) containing pSBK201A, pSBK201B and pSBK201D showed the expression of *chi*A. The estimated size of ChiA was about 79.86 kDa along with the *N*-terminal tag.

- The confirmed clones pSBK201A, pSBK201B and pSBK201D were induced with IPTG and the induced protein from different clones was subjected for bioassay against the fungal pathogens viz, *S. rolfsii and R. solani* and it showed crescent shaped growth inhibition of fungal pathogens compared to the control.

- For sequencing the pTZ57R/T plus *chi*A clones were selected with different efficacy of expression in pET32C+and the nucleotide sequences of the clones were subjected to BLAST algorithm.

- The nucleotide sequence analysis of the *Sm*AUDS227*chi*A revealed *Pst*I site at 361th, 644th and 905th position whereas, reference *Sm*141*chi*A nucleotide sequence has unique site for *Pst*I enzyme which, is at 905th position. The *Sm*AUDS227*chi*A has the

same biochemical and functional properties as the *Sm*141*chi*A except the variation at the catalytic site. The *Sm*AUDS227*chi*A has the *N*-terminal signal peptide of 23 amino acid residues, which is rich in leucine and the gene product, is a 726 residue protein with estimated size of about 79.86 kDa.

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Appendix I: Media preparation

A. Media for bacterial culture

a) Luria agar media		
Sl. No.	**Name of Component**	**Quantity of component for 1 liter of media (in gm)**
1	Tryptone	10
2	Yeast extract	5
3	Sodium chloride	5
4	Agar	18
pH 7.2 Autoclaved the media		

b) Luria broth		
Sl. No.	**Name of Component**	**Quantity of component for 1 liter of media (in gm)**
1	Tryptone	10
2	Yeast extract	5
3	Sodium chloride	5
pH 7.2 Autoclaved the media		

B. Media for fungal culture

a) Potato Dextrose Agar		
Sl. No.	**Name of Component**	**Quantity of component for 1 liter of media (in gm)**
1	Peeled potato	200g
2	Dextrose	20g
3	Yeast Extract	100mg
4	Agar	20g
5	Water	Make up the volume to 1000 ml
	Autolaved the media	

Appendix II: DNA isolation

a) Lysozyme preparation (10 ml)	
Lysozyme (10 mg/ml)	100 mg
10 mM Tris-Cl	10 ml

b) RNase solution (5ml)	
RNase (10 mg/ml)	50 mg
Distilled water	5 ml

c) ProteinaseK solution (5ml)	
ProteinaseK (20 mg/ml)	100 mg
50 mM Tris (pH 8)	30.28 mg
1.5mM calcium acetate	1.5 mg
Distilled water	4868 µl

d) Tris-Cl solution 1 M (100 ml)	
Tris-Base	12.11gm
HCl to make pH 8.0	
Distilled water to make up volume 100 ml	

Appendix III: Agarose gel electrophoresis

a) Loading dye composition(6X)	
1	0.25% Bromophenol blue (BPB)
2	40.0% Sucrose
3	Water to make up the volume

b) Ethidium bromide	
1	10 mg/ml in distilled water. Stored at 4℃ in dark bottle

c) Preparation of 1 per cent Agarose gel (100 ml)		
1	Agarose	1 gm
2	1X TAE	100 ml
3	EtBr (10 mg/ml)	4µl

d) 50x TAE Composition		
1	Tris base	242 g
2	Glacial acetic acid	57.1 ml
3	0.5 M EDTA (pH 8.0)	100 ml

Total volume 1000 ml with double distilled water.

Appendix IV: Selection Plate preparation

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

Appendix V: Plasmid isolation by alkali lysis method

a) Solution-I		
1	Glucose	50 mM
2	Tris-Cl (pH 8.0)	25 mM
3	EDTA (pH 8.0)	25 mM
Autoclaved and stored at 4ºC		

b) Solution-II		
1	NaOH	0.2 N
2	SDS	1% (w/v)
Prepared fresh and used at room temperature		

c) Solution III		
1	5 M potassium acetate	60 ml
2	Glacial acetic acid	11.5 ml
3	Double distilled water	28.5 ml
Autoclaved and stored at 4ºC		

STET Buffer		
1	Tris-Cl (pH 8.0)	10 mM
2	NaCl	0.1 M
3	EDTA (pH 8.0)	1 mM
4	Triton X-100	5 per cent (w/v)
5	Water	Make up the volume
	pH 8.0	

Appendix VI

a) Binding Buffer for Protein Isolation (In Non-Denaturing Form)		
1	Sodium phosphate	20 mM
2	NaCl	500 mM
	Autoclaved and stored at 4℃	

Appendix VII

Buffers and solutions for SDS PAGE

a. 1X SDS gel loading buffer		
1	Tris-Cl (pH 6.8)	50 mM
2	Dithiothreitol	100 mM
3	SDS	2 per cent (w/v)
4	Bromophenol blue	0.1 per cent
5	Glycerol	10 per cent

b. 1X Tris-Glycine Buffer	
Tris-Cl (pH 6.8)	25 mM
Glycine	250 mM
SDS	0.1 per cent (w/v)

c. 12 per cent Resolving Gel (SDS-PAGE) - 40ml		
1	Water	13.2 ml
2	30 per cent acrylamide mix	16.0 ml
3	1.5 M Tris (pH 8.8)	10.0 ml
4	10 per cent SDS	0.4 ml
5	10 per cent ammonium persulphate	0.4 ml
6	TEMED	0.016 ml

d. 5 per cent Stacking Gel (SDS-PAGE) – 10 ml		
1	Water	6.8 ml
2	30 per cent acrylamide mix	1.7 ml
3	1.0 M Tris (pH 6.8)	1.25 ml
4	10 per cent SDS	0.1 ml
5	10 per cent ammonium persulphate	0.1 ml
6	TEMED	0.011 ml

e. SDS Polyacrylamide Gel Staining Solution (1000 ml)		
1	Coomassie Brilliant Blue	0.25 g
2	Methanol	500 ml
3	Glacial acetic acid	100 ml
4	Water	400 ml

f. SDS Polyacrylamide Gel Destaining Solution (1000 ml)		
1	Methanol	500 ml
2	Glacial acetic acid	100 ml
3	Water	400 ml

CLONING AND EXPRESSION OF *chi*A FROM NATIVE *Serratia marcescens* AUDS227

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ABSTRACT

Serratia marcescens, a chitinolytic bacterium, produces chitinases capable of degrading fungal cell wall. Chitinases are the enzymes which hydrolyze the β-1, 4 linkages in the chitin micro fibril 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 enzymes from the bacterium.

Sixty native isolates of *S. marcescens* obtained from the culture collection of the Department of Biotechnology, UAS, Dharwad were screened to check their chitinolytic activity on collodial chitin media in comparison with the reference strain. The total DNA isolated from the native isolates of *S. marcescens* was used to identify *chi*A, *chi*B and *chi*C with diagnostic primers. In order to find out variability amongst *chi*A, RFLP analysis of *chi*A positive isolates of *S. marcescens* was done along with the reference strain *Sm*141. The *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A revealed different restriction fragments compared to the reference *Sm*141*chi*A. Based on the chitin resolution and difference in banding pattern of *Sm*AUDS227*chi*A with the reference *Sm*141*chi*A with *Pst*I, the *Sm*AUDS227*chi*A was selected further for cloning and expression studies.

The novel *chi*A from *S. marcescens* AUDS227 was cloned in pTZ57R/T transformed and maintained in *E. coli* DH5α and these constructs were named as pSBK101A, pSBK101B, pSBK101C and pSBK101D. In order to assess the expression of cloned *chi*A, it was sub cloned into prokaryotic expression vector pET32C+ and the constructs were named as pSBK201A, pSBK201B, pSBK201C and pSBK201D and the SDS-PAGE analysis showed that *chi*A has expressed 79.86 kDa protein. In order to find out the functionality, the confirmed clones were induced with IPTG and the induced protein from different clones was subjected for bioassay against fungal pathogens viz., *S. rolfsii* and *R. solani* and it showed crescent shaped growth inhibition of fungal pathogens compared to the control.