CLONING OF CHITINASE A GENE (*CHIA*) FROM *SERRATIA MARCESCENS* BN10 AND ITS EXPRESSION IN COLEOPTERA-SPECIFIC *BACILLUS THURINGIENSIS*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

SEZER OKAY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

SEPTEMBER 2005

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

CLONING OF CHITINASE A GENE (*CHIA*) FROM *SERRATIA MARCESCENS* BN10 AND ITS EXPRESSION IN COLEOPTERA-SPECIFIC *BACILLUS THURINGIENSIS*

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September 2005, 102 pages

Chitinases have been shown to be potential agents for biological control of the plant diseases caused by various phytopathogenic fungi and insect pests, because fungal cell walls and insect exoskeletons contain chitin as a major structural component. Chitinase has also been found to increase the efficacy and potency of *Bacillus thuringiensis* crystal (Cry) proteins toxic to larvae of insect pests. The reason of this synergy is the presence of chitin in the structure of the outer membrane of larval midgut.

In this study, the gene encoding chitinase A (*chiA*) from *Serratia marcescens* Bn10, a local isolate of Trabzon province was amplified by PCR and cloned into the *E.coli/Bacillus* shuttle vectors, pNW33N and pHT315. For the expression in *B.*

thuringiensis, the promoter region of *cry3*Aa11 gene of *B. thuringiensis* Mm2 was placed at the upstream of *chiA*. The vectors carrying both *chiA* and promoter site of *cry3*Aa11 was first introduced into *E. coli* and then into *Bacillus subtilis* 168 which were used as intermediate hosts in this study. pHT315PC carying *chiA* was then introduced into Coleoptera-specific *B. thuringiensis* cells (strain 3023) and the specific chitinase activity of the recombinant *B. thuringiensis* was measured as 5056 U/min/mg which was 6.3 fold higher than that of the parental strain. The specific activity corresponded to about one third of that produced by *S. marcescens* Bn10. The *chiA* gene was next sequenced and characterized. The sequence was submitted to GeneBank (Accession No. DQ165083). Chitinase A of *S. marcescens* Bn10 was found to be a 563 residue protein with a calculated molecular mass of 60.9 kDa. The mean G+C content of the gene is 58.75%. The deduced amino acid sequence was 99.3–91.5% identical to those of known chitinases from *S. marcescens*, *Burkholderia cepacia* and *Enterobacter* sp. It was found that the chitinase of *S. marcescens* Bn10 has six amino acids difference from the consensus sequence of aligned chitinases.

The production of chitinase by the local isolate *S. marcescens* Bn10 in different cultural conditions was also investigated. Optimum temperature and pH for chitinase production was found to be 30 ^{o}C and 7.5, respectively. Varying the concentration of colloidal chitin and the inclusion of NAG into the medium had no effect on chitinase production. The effect of different parameters such as temperature, pH, substrate concentration and certain inhibitory elements on enzyme activity were next assayed. The highest activity was obtained at 45 ^{o}C and in a pH range of 4.0 to 9.0. Activity of chitinase increased with increasing substrate concentration up to 35 μg/mL. Ca^{2+}, Co^{2+}, Cu^{2+}, EDTA, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were tested for their effects on the activity of enzyme. The enzyme was inhibited by only 4% in the presence of 10 mM EDTA, whereas 10 mM Co^{2+} included in the assay mixture increased the activity by 20%.

Keywords: *Serratia marcescens*; *Bacillus thuringiensis*; gene cloning; *chiA* gene; chitinase production

ÖZ

KİTİNAZ A GENİNİN (*CHIA*) *SERRATIA MARCESCENS* BN10'DAN KLONLANMASI VE COLEOPTERA-SPESİFİK *BACILLUS THURINGIENSIS*'TE İFADE EDİLMESİ

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Eylül 2005, 102 sayfa

Fungal hücre duvarı ve böcek dış iskeleti temel yapı bileşeni olarak kitin içerdiğinden kitinaz enzimleri hem çeşitli bitki hastalıklarına neden olan fitopatojen funguslara hem de zararlı böceklere karşı biyolojik kontrol ajanı olarak kullanılma potansiyeline sahiptirler. Bunun yanısıra kitinaz enziminin böcek larvalarına karşı ölümcül etki gösteren *Bacillus thuringiensis* kristal (Cry) proteinlerinin toksik etkisini artırdığı da gösterilmiştir. Bu sinerjinin nedeni, larva bağırsak epitelinin dış zarının kitin içermesidir.

Bu çalışmada, kitinaz A enzimini kodlayan gen (*chiA*) Trabzon ili çevresinden izole edilmiş olan *Serratia marcescens* Bn10'dan PCR yoluyla çoğaltılmış ve klonlanarak *E. coli*/*Bacillus* vektörlerine (pNW33N ve pHT315) yerleştirilmiştir. *B.*

thuringiensis'te gen ifadesinin sağlanması için *B. thuringiensis* Mm2'ye ait *cry3*Aa11 geninin promotor bölgesi *chiA* geninin 5' ucuna yerleştirilmiştir. Cry3Aa11 promotoru altında *chiA* genini taşıyan vektörler ara konakçılar olarak önce *E. coli*'ye, sonra *B. subtilis* 168'e aktarılmıştır. *chiA*'yı taşıyan pHT315PC vektörü daha sonra Kınkanatlılar'a karşı toksik etkiye sahip olan *B. thuringiensis* hücrelerine aktarılmıştır. Rekombinant *B. thuringiensis*'in spesifik kitinaz aktivitesi 5056 Ünite/dakika/mg olarak ölçülmüştür. Bu değer ana suş *B. thuringiensis* 3023'ün kitinaz aktivitesinin 6.3 katı, *S. marcescens* kitinaz aktivitesinin ise yaklaşık üçte biri kadardır. Klonlanan *chiA* geninin nükleotid dizisi analiz edilmiştir. Dizi GenBankası'na sunulmuş ve DQ165083 numarasını almıştır. *S. marcescens* Bn10'a ait *chiA* geni 60.9 kDa moleküler ağırlığa sahip 563 amino asitlik bir protein kodlamaktadır. Genin ortalama G+C içeriği % 58.75'dir. Amino asit dizisinin *S. marcescens*, *Burkholderia cepacia* ve *Enterobacter* cinsine ait türlerin bilinen kitinaz dizileriyle karşılaştırılması sonucunda bu kitinazlara %99.3-91.5 oranında benzerlik gösterdiği ve korunmuş diziler içerisinde altı amino asitinin farklı olduğu tesbit edilmiştir.

Yerli izolat *S. marcescens* Bn10 tarafından farklı kültür koşullarında kitinaz üretimi incelenmiştir. Kitinaz üretimi için optimum sıcaklık ve pH sırasıyla 30 ^{o}C ve 7.5 olarak bulunmuştur. Kolloidal kitinin farklı konsantrasyonlarının ve besiyerine eklenen NAG'nin kitinaz üretimine belirgin bir etkisi saptanmamıştır. Daha sonra sıcaklık, pH, substrat konsantrasyonu ve elementlerin inhibisyonu gibi farklı parametrelerin enzim aktivitesi üzerine etkisi araştırılmıştır. *S. marcescens* Bn10'a ait kitinaz pH 4.0–9.0 aralığında 45 ^{o}C'de en yüksek aktivite vermiştir. Kitinaz aktivitesi 35 μg/mL'ye kadar artan substrat konsantrasyonlarına bağlı olarak artmıştır. Daha sonra, Ca^{+2}, Co^{+2}, Cu^{+2}, EDTA, Fe^{+2}, Mg^{+2}, Mn^{+2} ve Zn^{+2}'nin enzim aktivitesi üzerine etkileri araştırılmıştır. 10 mM EDTA nın enzimi %4 oranında inhibe ettiği, 10 mM $Co^{+2'}$ın ise aktiviteyi %20 oranında artırdığı görülmüştür.

Anahtar kelimeler: *Serratia marcescens*; *Bacillus thuringiensis*; gen klonlanması; *chiA* geni; kitinaz üretimi

ACKNOWLEDMENTS

I would like to express my deepest gratitude and sincerest appreciation to my supervisor Prof. Dr. Gülay Özcengiz for her guidance, continuous advice, invaluable help and insight throughout the research. I am grateful to my co-supervisor Assist. Prof. Dr. Melek Özkan for her invaluable help, continuous encouragement and constructive criticism.

I would also like to thank to Assist. Prof. Dr. Ebru İnce Yılmaz for her help and criticism throughout the study. I would like to thank my lab mates for their friendship and cooperation.

My special thanks also go to Aslıhan Kurt for her understanding, endless help, encouragement and great friendship that made easier for me to overcome diffuculties in all hard times.

I am grateful to Serdar Atılgan for his help in spectral measurements and being a precious friend. I would also like to thank to the graduate students of Prof. Dr. Hüseyin Avni Öktem for making their laboratory facility available.

I would like to express my heartful gratitude to my mother Hatice, my father Memet, my brothers Serkan, Sertaç and Samet for their endless love, support, patience and understanding.

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LIST OF ABBREVIATIONS

BGSC : Bacillus Genetic Stock Center

bp(s) : Base pair(s)

CBP : Chitin Binding Protein

EDTA : Ethylenediaminetetraacetic acid

$(GlcNAc)_n$: Chito-oligosaccharides

ICP : Insecticidal Crystal Protein

kb : Kilobase

kDa : Kilodalton

NAG, GlcNAc : *N*-acetylglucosamine

NCBI : National Center for Biotechnology Information

SD : Shine-Dalgarno

SDS-PAGE : Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

U : Unit

CHAPTER 1

INTRODUCTION

1.1. Biological Pest Control and Chitin as a Target

Increase of the world population will require an estimated additional agricultural production of 2.4×10^9 tons/year. However, this additional production should 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 (pests, diseases, weeds) (Montesinos, 2003). The therapeutic approach of killing pest organisms with toxic chemicals has been the prevailing pest control strategy for over 50 years (Lewis *et al*., 1997). However, an increase in the use of chemical pesticides to support the increase in agricultural activity needed to sustain the expected population growth can severely deteriorate the planet's health because of non-target effects (Montesinos, 2003). Cassidy *et al*. (2005) showed that the insecticides heptachlor epoxide and estradiol contribute to initiation, promotion and progression of breast cancer. In the study of Schreinemachers (2003), it was found that the widespread use of chlorophenoxy herbicides causes birth malformations and infant death from congenital anomalies. Chemical pesticides utilized to control the pests have hazardous effects on the environment. Increasing problems with resistance of these pests to most commonly used synthetic insecticides have spurred the search for alternative pest management strategies that would reduce reliance on synthetic insecticides (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 pests and diseases. Biological control is sustained by beneficial interactions resulting from competition, antagonism and hyperparasitism of certain microorganisms against plant pathogens, insects and weeds. Currently, several microorganisms involved in such processes are the active ingredients of a new generation of microbial pesticides or are the basis for many natural products of microbial origin (e.g. elicitors) or after chemical modification (e.g. phenylpyrrole fungicides) (Montesinos, 2003).

Some chitinolytic bacteria have been shown to be potential agents for biological control of the plant diseases caused by various phytopathogenic fungi and insect pests, because fungal cell walls and insect exoskeletons both contain chitin as a major structural component. The most notable chitinase producers among the gram-negative bacteria are *Aeromonas*, *Chromobacterium*, *Photobacterium*, *Pseudomonas*, *Serratia*, *Vibrio*, and the gliding bacteria *Chitinophaga*, *Cytophaga*, and *Lasobacter*. Among the gram-positive bacteria, chitinase producers are widespread among the actinomycetes, e.g., the species of *Arthrobacter*, *Nocardia*, and *Streptomyces*, and in the spore-forming genera *Bacillus* and *Clostridium* (Thamthiankul *et al.*, 2001).

1.2. Chitin Degradation

1.2.1. Chitin

Chitin, (1,4)-β-linked homopolysaccharide of *N*-acetylglucosamine (Sampson and Gooday, 1998; Vaaje-Kolstad *et al*., 2004; Hoell *et al*., 2005; Suginta *et al*., 2005) is the second most abundant biopolymer in nature next to cellulose (Lonhienne *et al*., 2001; Vaaje-Kolstad *et al*., 2005). It is sometimes considered to be a spin-off of cellulose, because the two are molecularly very similar except that cellulose contains a hydroxy group while chitin contains acetamide. The structure of chitin is shown in Figure 1.1.

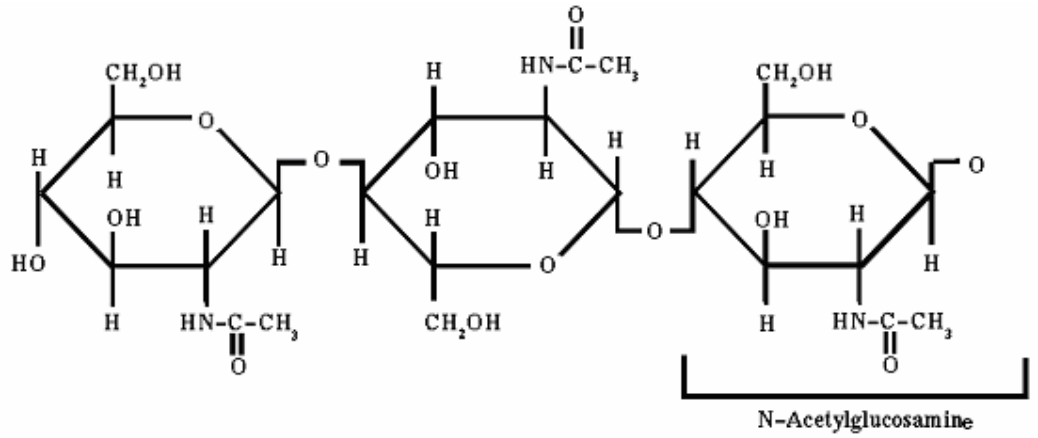


Figure 1.1. Molecular structure of chitin (http://www.faunistik.net/DETINVERT/MORPHOLOGY/GEWEBE/chitin_01.html).

Chitin is present in diatoms, yeasts, fungi, protozoa, arachnids, insects, crustaceans, nematodes, other invertebrates and tunicates (Trudel and Asselin, 1989). Table 1.1 shows the percentages of chitin found in different organisms. 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. Pathogens that infect through the gut must penetrate this chitin-rich barrier (Shen and Jacobs-Lorena, 1997; Sampson and Gooday, 1998; Hoell *et al*., 2005). Hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extracellularly by the action of chitinases (Sitrit *et al*., 1995).

The combination of chitin's remarkable properties makes it extremely versatile. Chitin is a non-toxic, non-allergenic, anti-microbial and biodegradable polymer. It has a strong positive charge which allows it to bind with negatively charged surfaces or materials including metals, skin and macromolecules such as proteins. In addition to being a naturally-occurring molecule, chitin is also notable for the process in which it is broken down because many sea creatures shed shells of this material every year. There are bacteria in the ocean that can sense small chains of sugars sent from a discarded chitin shell. Once these bacteria (*Vibrio furnisii*) have determined that they have found a shell, they begin to synthesize enzymes that can break down the shell. In

a series of nine steps, these bacteria reduce chitin to chains of simple sugars and ammonia (http://wywy.essortment.com/whatischitin_rkkh.htm).

Table 1.1. Percentages of chitin found in some organisms (http://www.psrc.usm.edu/macrog/sea/chitin.htm).

Organism	Percentage
Fungi	5–20
Worms	20–38
Squids/Octopus	3–20
Scorpions	30
Spiders	38
Cockroaches	35
Water Beetle	37
Silk Worm	44
Hermit Crab	69
Edible Crab	70

The past decade has yielded a large increase in knowledge of the enzyme and mechanisms involved in degradation of insoluble abundant polymers such as cellulose and chitin. This new knowledge brings us closer to a comprehensive understanding of how nature accomplishes the turnover of enormous amounts of polymers (Brurberg *et al.*, 2000). At least 10 gigatons of chitin are synthesized and degraded each year in the biosphere. One of the more important things that chitin and its products could be used for is in treatment of burn patients. Chitin has a remarkable compatibility with living tissue, and has been looked at for its ability to increase the healing of wounds. Chitosan is a polymer produced through enzymatic deacetylation of chitin (da Silva Amorim *et al.*, 2001) and is used in applications from health care to agriculture to dyes for fabrics. There are even medical applications and companies who use products made with chitosan as part of weight loss programs. There is also evidence that chitosan can reduce serum cholesterol levels. More research has also indicated that chitosan can increase crop yields and clean and clear up pools. It promotes healing of

ulcers and lesions, is an antibacterial and acts as an antacid, inhibits the formation of plaque/tooth decay, helps to control blood pressure, helps to prevent constipation, acts as a calcium enhancer/bone strengthener, reduces blood levels of uric acid and has an anti-tumor action (http://dalwoo.com/chitosan/whatischitosan.html).

1.2.2. Chitinases

Chitin hydrolysis is performed by two types of enzymes: The first, chitinases [poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolases, EC 3.2.1.14] are the major enzymes; endochitinase which produces multimers of *N*-acetylglucosamine and exochitinase which catalyzes the progressive release of soluble low molecular mass dimers starting at the non-reducing end of the polymer. The second, chitobiases (*N*-acetyl glucosaminidase, EC 3.2.1.30) which hydrolyze chitobiose to monomers of *N*-acetylglucosamine (Roberts and Selitrennikoff, 1988; Botha *et al*., 1998; Souza *et al*., 2003; Ruiz-Sánchez *et al*., 2005; Suginta *et al*., 2005).

All organisms that contain chitin also contain chitinases which are presumably 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). Plants produce chitinases as a defense against fungal pathogens (Wen *et al*., 2002). Chitinases in plants are accompanied by β-1,3-glucanases, both enzymes seem to be necessary to cause a high degree of lysis of *Armillaria mellea* for example, since the hyphae contain significant quantities of β-1,3-glucan in addition to chitin. By this mechanism the enzymes are effective as deterrent to infection (Muzzarelli, 1977).

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. Bacteria produce chitinases to meet their nutritional needs. They usually produce several chitinases, probably to hydrolyze the diversity of chitins found in nature (Figure 1.2); however, two class V proteins with endochitinase activity resembling bacterial chitinases have been isolated from tobacco (Cohen-Kupiec and Chet, 1998).

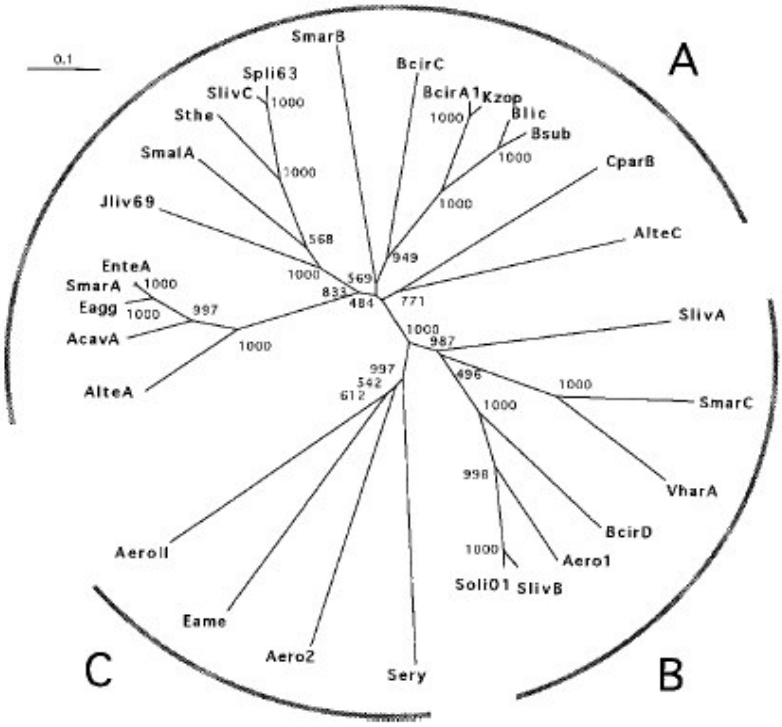


Figure 1.2. Relationships among family18 bacterial chitinases. The unrooted phylogenetic tree was calculated for 29 chitinases using the neighbour-joining method implemented in the program CLUSTAL W and drawn using the program TreeView. Numbers at branch points indicate bootstrap analysis values obtained using 1000 resampled data sets. The horizontal scale bar corresponds to 0.1 amino acid change per position. SmarA: *Serratia marcescens* 2170 chitinase A, EnteA: *Enterobacter* sp. G-1 chitinase A, Eagg: *Enterobacter agglomerans* chitinase (Chia-Entag), AcavA: *Aeromonas caviae* chitinase A, AlteA: *Alteromonas* sp. O-7 chitinase A (Suzuki *et al*., 1999).

Based on the published 3D-structure information, the active site of family 18 endochitinases has a long, deep substrate-binding cleft with an opening on both sides. On the other hand, the active site of exochitinases has a tunnel-like morphology with a closure of the roof at the end of the tunnel (Suginta *et al*, 2004). The catalytic domains of family 18 chitinases have a TIM-barrel ($(\beta\alpha)_8$ barrel) fold. The β-strand four of the TIM-barrel contains a characteristic DXDXE sequence motif including a glutamate residue that protonates the oxygen in the scissile glycosidic bond. In addition to the catalytic domain, this enzyme has a small chitin-binding domain that extends the substrate-binding cleft towards the reducing end of the polysaccharide chain. Catalysis in family 18 chitinases involves the *N*-acetyl group of the sugar bound in the -1 subsite of the enzyme. Protonation of the glycosidic bond and leaving group departure are accompanied by nucleophilic attack by the carbonyl oxygen of the *N*-acetyl group on the anomeric carbon, thus yielding an oxazolinium ion intermediate (Vaaje-Kolstad *et al*., 2004). Figure 1.3 shows the catalytic mechanism of family 18 chitinases.

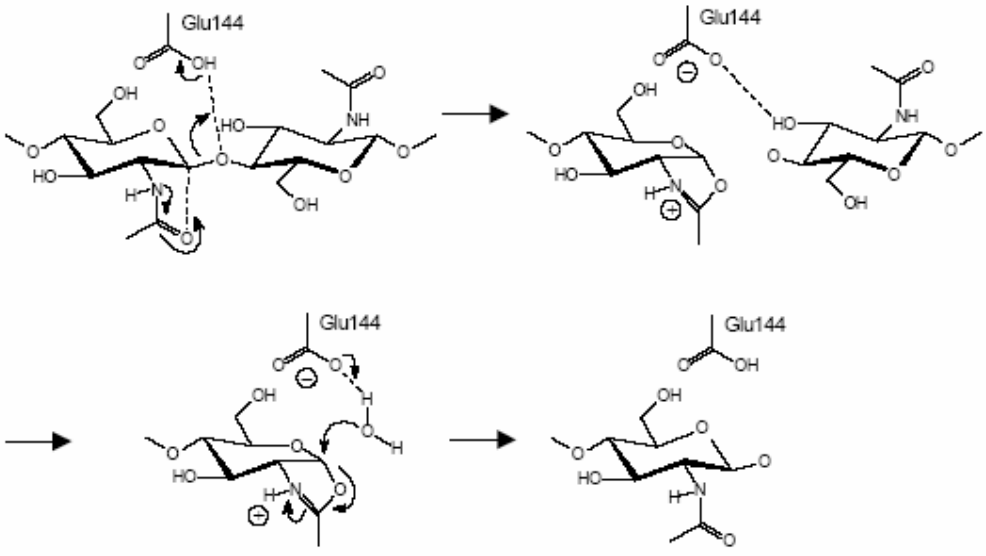


Figure 1.3. Catalytic mechanism of family 18 chitinases. Glu^{144} is the catalytic glutamate residue as found in ChiB (the corresponding residue in ChiA is Glu^{315}) (Brurberg *et al*., 2000).

These enzymes operate by a mechanism leading to overall retention of the anomeric configuration of the hydrolysed residue. It has been demonstrated that conserved glutamic acid and aspartic acid residues (four amino acids apart) of chitinase A1 of *Bacillus circulans* WL-12 were essential for the hydrolysis of chitin. These residues are also conserved in ChiA of *S. marcescens;* however, the determination of the crystal structure of ChiA demonstrated that the same glutamic acid residue but a more distant aspartic acid residue (76 amino acids distant) are the most likely amino acid residues to constitute the active site of the enzyme. The chitin-binding domain in bacterial chitinases can be located either in the amino-terminal or carboxy-terminal domains of the enzymes (Cohen-Kupiec and Chet, 1998).

The hydrolysis of chitin by chitinases is the most critical step in the degradation and utilization of chitin by bacteria. However, the study of chitinases is not sufficient to elucidate the process by which chitin is degraded and utilized by bacteria. The process involves a number of steps, including the recognition of chitin outside of the cell, the induction of chitinases, the maintenance of proper levels of chitinase production, and the incorporation and catabolism of degradation products.

For biotechnological interests, in addition to the potential applications of chitinase itself, the chito-oligosaccharides [$(GlcNAc)_n$] have been found to function as anti-bacterial agents, elicitors, lyzozyme inducers, immuno-enhancers (Wen *et al*., 2002) and chitinase inhibitors are used to inhibit growth of chitin-containing (plant-) pathogens and plague insects that need chitinases for normal development (Brurberg *et al*., 2000). 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).

The number of studies dealing with bacterial chitinases — their biochemical properties, the structure of the genes encoding them, the catalytic mechanism involved, and their tertiary structures — has been increasing rapidly (Watanabe *et al.*,

1997). Several chitinases from bacteria have been cloned and expressed in *Escherichia coli* (Sitrit *et al*., 1995; Lonhienne *et al*., 2001).

1.3. *Serratia marcescens* and Chitinases It Produces

Gram-negative, facultative anaerobe, motile and rod-shaped soil bacterium *Serratia* is 0.5-0.8 x 1.0-5.0 μ in size, produces characteristic red pigment, prodiginines and form highly mucoid colonies. It occurs naturally in soil and water as well as the intestines. Pathogenicity of this bacterium is important as a nocosomial infection; associated with urinary and respiratory tract infections, endocarditis, osteomyelitis, septicemia, wound infections, eye infections and meningitis. Its transmission occurs through direct contact or droplets and it has been found growing on catheters, in saline irrigation solutions and in other supposedly sterile solutions. Treatment includes cephalosporins, gentamicin, amikacin, but most strains are resistant to several antibiotics because of the presence or R-factors on plasmids (http://www.sunysccc.edu/academic/mst/microbes/23smarc.htm). The genus *Serratia,* of the family *Enterobacteriaceae,* in the tribe *Klebsielleae,* contains approximately 13 species. Among these species most widely studied is the species *S. marcescens*. *Serratia*, named after an Italian Physicist who invented the steamboat, has been widely considered a red-pigmented organism. For many years, it contained only one species, *S. marcescens* and was differentiated from other enteric bacteria due to its characteristic red pigmentation. However, many species of *Serratia* are non-pigmented or vary widely in pigmentation. Due to many similarities to the genera *Klebsiella* and *Enterobacter*, *Serratia* was often misidentified. Since 1972, DNA homologies and many intense biochemical comparison studies with other groups and cultures have made possible the proliferation of other species within the genus. Other well known species includes *S. odorifera, S. liquifaciens, S. rubidaea, S. ficaria, S. pymuthica* and *S. fonticola. S. marcescens* is the type species. Chitinase production by *S. marcescens* is much higher than the other species of *Serratia* genus (http://soils1.cses.vt.edu/ch/biol_4684/Microbes/Serratia.html).

S. marcescens is selected as the source of chitinase for the related studies 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 *Escherichia coli*, (iv) the predominant *S. marcescens* chitinase is an endolytic enzyme that solubilizes chitin more rapidly than the exolytic enzymes and should, therefore, be more efficacious, and (v) *S. marcescens* chitinases hydrolyze "crystalline" chitin (Fuchs *et al*., 1986).

S. marcescens is one of the most efficient 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; Ruiz-Sánchez *et al*., 2005; Vaaje-Kolstad *et al*., 2005). When this bacterium is cultivated in the presence of chitin, a variety of chitinolytic enzymes and chitin binding proteins can be detected. The studies by a number of groups clearly showed that *S. marcescens* produces at least three chitinases (ChiA, ChiB, ChiC), a chitobiase and a putative chitin-binding protein (CBP21). It is conceivable, but not certain, that these five proteins represent the complete chitinolytic machinery of the bacterium (Table 1.2) (Brurberg *et al*., 2000; Suzuki *et al*., 2001).

Table 1.2. Chitinases and CBP from *S. marcescens* (Brurberg *et al*., 2000).

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

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). Genes encoding ChiA have been isolated from various strains of *S. marcescens* and their nucleotide sequences have been determined. The three-dimensional structure of ChiA has been described (Figure 1.4) (Jones *et al*., 1986; Brurberg *et al*., 1994; 1996; Uchiyama *et al*., 2003). It consists of an all-β-strand amino-terminal domain and a catalytic $(\beta/\alpha)_8$-barrel domain with a small α+β domain inserted between the seventh and eighth β strands of the $(\beta/\alpha)_8$-barrel. In contrast to the rapidly accumulating data on the structure-function relationship of chitinases and related proteins, little is known about the mechanism of induction of chitinase production and of utilization of degradation products of chitin by *S. marcescens* cells (Uchiyama *et al*., 2003).

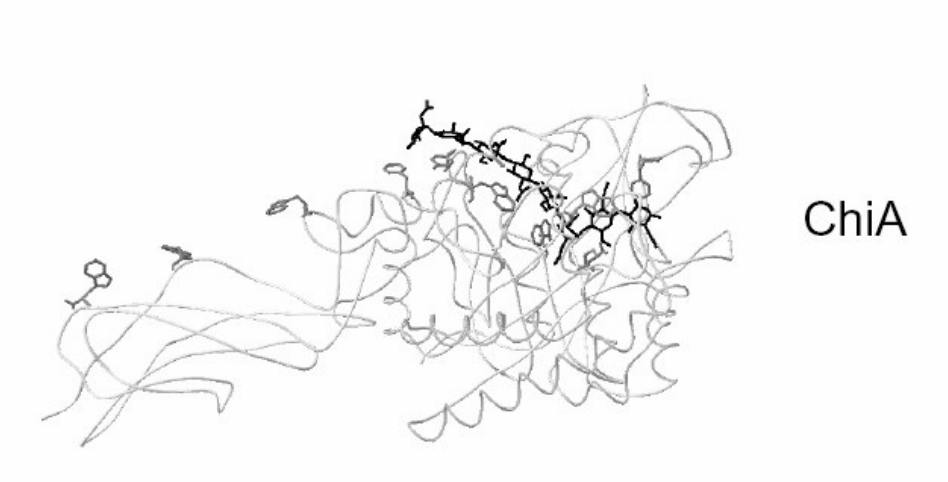


Figure 1.4. Crystallographic 3-D structures of ChiA showing the catalytic domain co-crystallized with an oligosaccharide and the position of the aromatic residues (Hult *et al*., 2005).

Substrate-assisted catalytic mechanism of family 18 chitinases seemed to involve protonation of the leaving group by the catalytic residue followed by substrate distortion into a 'boat' conformation at subsite -1 and the stabilization of an oxazolinium intermediate by the sugar acetamido moiety. The experimental data that

showed glycosidic bond cleavage by family 18 chitinases yielding retention of β-anomeric configuration in the products supported the mechanism obtained from the structural information. The X-ray structures of the hevamine chitinase/lysozyme complexed with allosamidin and of the *S. marcescens* ChiA mutants complexed with octa- and hexasaccharide substrates indicated that the catalytic sites of these two enzymes contained six substrate binding subsites, designated subsites -4, -3, -2, -1, +1, and +2. The scissile glycosidic bond is located between subsites -1 and +1 (Suginta *et al*., 2004).

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*., 2001; Uchiyama *et al*., 2003). Some of them were entirely defective in chitinase and CBP21 production. A detailed analysis of two such mutants, N1 and N22, resulted in identification of an essential gene for chitinase and CBP21 production. This gene, designated as *chiR*, encodes a LysR-type transcriptional activator that is essential for the expression of chitinases and CBP21 (Suzuki *et al*., 2001; Uchiyama *et al*., 2003). Production of chitinolytic enzymes in bacteria is normally induced by the presence of chitin in the culture medium. Since chitin is insoluble, the microorganisms are unable to utilize it unless it has been hydrolysed to soluble oligomers of *N*-acetyl-glucosamine. Several proposals have been made to explain how induction may occur in this situation. The most probable inducers of *S. marcescens* chitinolytic enzymes are soluble oligomers derived from chitin. Such soluble oligomers could be produced by the action of trace amounts of

chitinolytic enzymes that are present because of constitutive low level expression of the chitinase genes. Alternatively, a signal for induction of synthesis of chitinases could result from physical contact between the cell and the insoluble substrate. However, it has been shown that production of chitinolytic enzymes could indeed be induced by adding only $(GlcNAc)_2$ or $(GlcNAc)_{3–4}$ to the growth medium of *S. marcescens*. Whether all the chitinases in *S. marcescens* are induced with oligomers remains to be tested. In addition to being induced by chitin, the chitinases of *S. marcescens* are also induced by the SOS-response inducers mitomycin C and nalidixic acid. The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries known to date (Brurberg *et al*., 2000).

1.4. *S. marcescens* as a Biological Control Agent

One of the first biotechnological applications of chitinases concerned their use in biocontrol of plant pathogens. *S. marcescens* cultures, its chitinases and its chitinase genes have shown potential as biocontrol agents in a variety of experimental set-ups (Brurberg *et al*., 2000). This bacterium is a potential insect pathogen and chitinase plays important role in the virulence of this bacterium together with protease and lecithinase (Uchiyama *et al*., 2003). Someya *et al*. (2005) showed that *S. marcescens* strain B2 inhibited mycelial growth of the rice sheath blight pathogen *Rhizoctonia solani* AG–1 IA. Rice plants were treated with bacterial suspension and then challenge inoculated with the pathogen. Application of *S. marcescens* effectively reduced the incidence of sheath blight. In the study of Inglis and Lawrence (2001), the effects of *S. marcescens* (Bizio) 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. Lysyk *et al*. (2002) showed that *S. marcescens* caused mortality of adult *Stomoxys calcitrans* (Diptera: Muscidae). Inglis *et al*. (2000) observed that *S. marcescens* is pathogenic to southwestern corn borer larvae under controlled environmental conditions. A highly chitinolytic strain of *S. marcescens* was found to suppress the growth of *Botrytis* spp. *in vitro*. In a greenhouse setting, *S. marcescens*

has been shown to control *Botrytis cinerea*, *Rhizoctonia solani* and *Fusarium oxysporum*, all pathogens of cyclamen. Similarly, *S. marcescens* controlled growth of *Sclerotinia minor*, the casual agent of basal drop disease, in lettuce grown in green house. The *chiA* and *chiB* genes from *S. marcescens* have been transformed into other bacterial species like either *Pseudomonas fluorescens* or *E. coli* in an attempt to improve their ability to control fungal plant pathogens or create new biocontrol agents (Brurberg *et al*., 2000). For instance, the gene coding for the major chitinase of *S. marcescens*, *chiA*, was cloned under the control of the *tac* promoter into the broad-host-range plasmid pKT240 and the integration vector pJFF350. *P. fluorescens* carrying *tacchiA* either on the plasmid or integrated into the chromosome is an effective biocontrol agent of the phytopathogenic fungus *R. solani* on bean seedlings under plant growth chamber conditions (Downing and Thomson, 2000). Furthermore, chitinase ChiA partially purified after cloning into *E. coli* was found to reduce disease caused by *Sclerotium rolfsii* in beans and *R. solani* in cotton. Several transgenic plants overexpressing plant, fungal or bacterial chitinases have been described. 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).

1.5. *Bacillus thuringiensis* and Cry Toxins

Bacillus thuringiensis is a gram-positive, rod-shaped, aerobic, and spore-forming bacterium closely related to the omnipresent soil bacterium *Bacillus cereus*. The vegetative cells are 1 μm in width, 5 μm long and have short hair-like flagella. The species is distinguished from *B. cereus* by its ability to produce a protein crystal during sporulation.

B. thuringiensis was first isolated by Japanese bacteriologist S. Ishiwata from disease silkworm *Bombyx mori* (L.) larvae in 1901. In the same year, E. Berliner in Germany recorded the first scientific description of the bacterium. In 1916, Aoki and Chigasaki found that its activity was due to a toxin present in sporulated cultures, but not in young cultures of vegetative cells (Joung and Côté, 2000). *B. thuringiensis* is the natural host for a variety of plasmid-encoded parasporal crystals containing one or

more insecticidal crystal proteins (ICPs), also known as delta-endotoxins that are toxic to important coleopteran, lepidopteran, and dipteran insect pests (Slaney *et al*., 1992; Malvar *et al*., 1994; Kaur, 2000). ICPs include the more prevalent Cry (crystal) proteins, as well as the Cyt (cytolytic) proteins produced by some *B. thuringiensis* strains. Cry proteins are solubilized upon ingestion and in most cases, cleaved by proteolytic enzymes to active toxins which bind to specific sites on the microvillar brush-border membranes in the gut of susceptible insects. Subsequent insertion of toxin into the membrane leads to disruption of osmotic balance and lysis of epithelial cells, which may be rapidly lethal or may cause starvation and ultimate death (Kaur, 2000). *B. thuringiensis* produces various virulence factors other than the crystal proteins, including α-exotoxins, β-exotoxins, hemolysins, enterotoxins, chitinases and phospholipases. The spore itself contributes to pathogenicity, often synergizing the activity of the crystal proteins. All of these factors might have a role in insect pathogenesis under natural conditions, helping the bacterium to develop in the dead or diseased insect larvae (de Maagd *et al*., 2001).

B. thuringiensis strains have been isolated worldwide from a variety of habitats including soil, insects, stored grain products, phyllospheres, and other miscellaneous habitats. There is enormous genetic diversity among *B. thuringiensis* strains. Most strains are naturally multigenic, harboring from 2 to 12 *cry* genes. Most *cry* genes are located on low copy number, high molecular weight (>30 KDa) plasmids. Many of these plasmids are self-transmissible to other *Bacillus* species by conjugal transfer. Some *cry* genes are located on chromosomes. Often, *cry* genes are flanked by transposable elements, presumably involved in amplification of *cry* genes and creation of diversity (Kaur, 2000).

In an effort to overcome a somewhat confusing situation, a classification of crystal proteins and their genes was proposed (Joung and Côté, 2000). This classification is based on the crystal protein structure and on the host range. More than 14 distinct crystal protein (*cry*) genes are described. The genes specify a family of related insecticidal (Cry) proteins, and are divided into four major classes: Lepidoptera-specific (I), Lepidoptera- and Diptera-specific (II), Coleoptera-specific (III), and Diptera-specific (IV) genes. A number of subclasses, based on insecticidal and

structural properties, are also recognized within each class. More recently a newer classification system based solely on amino acid identity was proposed. The new classification allows closely related toxins to be ranked together and removes the necessity for researches to bioassay each new toxin against a growing series of organism (Joung and Côté, 2000; de Maagd *et al*., 2001). New nomenclature has defined four hierarchical ranks. Romen numerals, which comes after the Cry or Cyt words, have been exchanged for Arabic numerals in the primary rank, it is followed by other three hierarchical rank consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry23Aa1), depending on its place in a phylogenetic tree. Proteins with the same primary rank often affect the same order of insect; those with different secondary and tertiary ranks may have altered potency and targeting within an order. The quaternary rank was established to group "alleles" of genes coding for known toxins that differ only slightly, either because of a few mutational changes or an imprecision in sequencing. The proteins, less than 45% sequence identity differ in primary rank (Cry1, Cry2, etc.) and 78 % and 95% identity constitute the borders for secondary and tertiary rank, respectively (Crickmore *et al*., 1998).

Cry3A is a Coleopteran-active delta-endotoxin produced by some strains of *B. thuringiensis*, including EG2158 and *B. thuringiensis* subsp. *tenebrionis*. It is toxic to larval Coleoptera at concentrations 10-100x same as the effective concentration of potent Cry1-toxins on larval Lepidoptera. Cry3A is a 67 kDa protein and is produced as a rhomboid crystal. There is also a 70 kDa form of Cry3A produced by *B. thuringiensis* strains; however, this form is unstable. Cry3A forms cation-selective channels in planar lipid bilayers similar to the channels formed by Cry1Ac, suggesting that the mode of action of Cry3A is similar to that of the Lepidopteran-active Cry1Ac delta-endotoxin. Not all larval Coleoptera are equally susceptible to Cry3A, just as not all larval Lepidoptera are equally susceptible to Cry1Ac. Differential susceptibility is most likely due to the complex interaction of the physiological differences between insect species and the different toxins. Given the understanding of the steps theoretically required for toxicity, it should be possible to delineate where in this scheme insect responses differ, and thereby account for the differences in susceptibility (Slaney *et al*., 1992).

The specific toxicity of ICPs against target insects is the basis for the use of *B. thuringiensis* as a biopesticide in agriculture, forestry and mosquito control since 1961. With the increased use of *B. thuringiensis* in insect control, efforts to find new *B. thuringiensis* strains have also intensified. Novel strains have been found in diverse habitats. The advantages of *B. thuringiensis* over synthetic pesticides include lack of polluting residues, high specificity to target insects, safety to non-target organisms such as mammals, birds, amphibians and reptiles as well as low costs of development and registration. Despite the ecological benefits, the use of *B. thuringiensis* biopesticides has lagged behind that of synthetic chemicals. Biopesticides constitute only 1% of the pesticide market share out of which *B. thuringiensis* products comprise 95% of biopesticides. Of the large number of *B. thuringiensis* products registered for use for over 50 crop species, many are conventional products based on unimproved natural *B. thuringiensis* strains, predominantly the *B. thuringiensis* subsp. *kurstaki* HD-1. Such products suffer from limitations of narrow host range, low persistence on plants and inability of foliar application to reach the insect feeding inside the plants. Nevertheless, there is considerable market potential for *B. thuringiensis* biopesticides due to good discovery rate of novel *B. thuringiensis* strains, amenability of these strains to genetic improvement by recombinant DNA technology and low developmental cost *vis a vis* synthetic insecticides (Kaur, 2000). Several approaches and techniques have recently been used to genetically manipulate *B. thuringiensis* strains to improve their insecticidal properties and contribute to their development as biopesticides. Conjugation was first used to construct recombinant strains with new combinations of genes. However, this procedure is only applicable to *cry* genes carried by conjugative plasmids and does not allow the association of genes on plasmids belonging to the same incompatibility group.

Expansion of the insecticidal host range of an anti-Lepidopteran *B. thuringiensis* strain has been obtained by insertion of an anti-Coleopteran toxin gene into a resident plasmid by homologous recombination. The efficiency and economic production of *B. thuringiensis* products greatly benefit from the construction of engineered *B. thuringiensis* strains with a broader activity spectrum and producing larger amounts of each of the crystal delta-endotoxins in the strain (Sanchis *et al*., 1996). The natural

and recombinant strains of *B. thuringiensis* that have been used as commercial biopesticides are shown in Table 1.3.

Table 1.3. *B. thuringiensis* biopesticides based on natural or novel/recombinant strains (Kaur, 2000).

Trade name	*Bt* subsp. strain	Current producer	Specificity
Natural Strains			
Dipel	*kurstaki* HD-1	Abbott	Lepidoptera
Florbac	*aizawai*	Abbott	Lepidoptera
Costar	*kurstaki* SA-12	Thermo trilogy	Lepidoptera
Javelin	*kurstaki* SA-11	Thermo trilogy	Lepidoptera
Bactimos	*israelensis*	Abbott	Diptera
Vectolex GC	*B. sphaericus*	Abbott	Diptera
Acrobe	*israelensis*	American cyanamide	Diptera
Novodor	*tenebrionis*	Abbott	Coleoptera
Novel/Recombinant Strains			
Condor	*kurstaki* EG 2348	Ecogen	Lepidoptera
Maatch	*kurstaki* CryIA & *aizawai* CryIC	Mycogen	Lepidoptera
M-Peril	*kurstaki* CryIAc	Mycogen	Lepidoptera
Able	*kurstaki* M-200	Thermo trilogy	Lepidoptera
M-Trak	*tenebrionis* Cry3A	Mycogen	Coleoptera

1.6. Important Pests Belonging to Coleoptera Order

Yield losses due to phytophagous insects still account for 20-30% of the total agricultural production despite the widespread use of synthetic chemical insecticides.

These losses result from the adaptation or evolution of insect populations to selection pressures exerted by insecticide treatments. Resistance is a natural consequence of the frequent and prolonged use of an insecticide on a relatively geographically isolated insect population (Haffani *et al*., 2001). Coleoptera order includes some of the important insect pests. For instance, the genus *Sitona* includes the pea leaf weevil, *Sitona lineatus*, a significant insect pest of peas in the Pacific Northwest, Europe, Australia and the Middle East. A number of other *Sitona* species are pests of *Trifolium* spp, *Medicago* spp, *Lens culinaris* and *Vicia faba*. As adults, all *Sitona* spp feed on foliage, whereas their larvae feed on legume root systems (Bezdicek *et al*., 1994). The hazelnut beetle (*Balaninus nucum* L., Coleoptera: Curculionidae) is the single greatest source of damage to hazelnut fruits throughout the world. It causes approximately 30–40% of the total economic damage to hazelnut products per year in Turkey (Sezen and Demirbağ, 1999). The major insect pest of stored tobacco is the cigarette beetle, *Lasioderma* serricorne (F.) (Coleoptera: Anobiidae), which is the most widespread and destructive pest. Damage to tobacco is caused by the insect larvae that eat the stored leaf and contaminate the product with excreta and body oils (Kaelin *et al*., 1999). The Andean potato weevil, *Premnotrypes vorax*, an insect of the order Coleoptera, is a major cause of damage to potato crops in the Andean regions of South America. In Colombia, 10-30% of the potato harvest is lost due to this insect (Gomez *et al*., 2000). The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) is a major potato insect pest in Europe, North and Central America, which to date has developed resistance to a wide variety of synthetic chemical insecticides. Alternative approaches for effective control of this pest rely on the use of new insecticidal molecules with a totally different mode of action in order to circumvent the problem of resistance developed to conventional insecticides.

1.7. Combined Use of Chitinases and Cry Toxins as Biocontrol Agent

While high levels of Cry proteins have been recorded in *cry*-carrying bacteria, the native *cry* genes were poorly expressed as plant nuclear transgenes and consequently their larvicidal activity was limited. The relatively low level of *cry* gene expression in transgenic plants is presumably due to a high A+T content, the presence of transcription pretermination signals, and differences in codon usage (Regev *et al*.,

1996). Despite the success of conventional *B. thuringiensis*-based products, they have several disadvantages as bioinsecticides. In the case of the sugarcane borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae), a widespread sugarcane pest which causes considerable crop loss in the cane-growing areas of South Africa and Swaziland, these include instability in the environment and on the surface of sugarcane as well as the difficulty in reaching the internal regions where the larvae feed (Downing *et al*., 2000).

An additional conceptual strategy may increase the efficacy and potential future of *B. thuringiensis* Cry toxins in insect control (Regev *et al*., 1996). The introduction of both Cry and ChiA into bacteria or plants offers great potential for increasing the insecticidal activity in transgenic systems (Downing *et al*., 2000). Reports have shown that co-application of *B. thuringiensis* delta-endotoxins and bacterial chitinases significantly increased the insecticidal effect of the former against insect larvae (Regev *et al*., 1996; Barboza-Corona *et al.* 1999; Downing *et al*., 2000; Arora *et al*., 2003; Lin and Xiong, 2004; Lertcanawanichakul *et al*., 2004). Similarly, application of low concentrations of a mixture of spore crystal suspension from *B. thuringiensis* subsp. *entomocidus* and chitinolytic bacteria resulted in a significant synergistic insecticidal effect against *Spodoptera littoralis* larvae (Regev *et al*., 1996). Sampson and Gooday (1998) showed that addition of *S. marcescens* chitinase A enhanced the activity of two strains of *B. thuringiensis* against hosts from different orders of insects. It was suggested that the chitinolytic bacteria affect the larval peritrophic membrane which normally forms a cylindrical sheet separating the gut epithelium from the lumen. The peritrophic membrane consists of a network of chitin embedded in a protein-carbohydrate matrix and provides a physical barrier against mechanical damage and invasion of microorganisms. Isolated peritrophic membranes were shown to limit penetration of dissolved delta-endotoxin *in vitro*. Thus, increasing the level of endochitinases in the larval midgut may elevate the larvicidal effect as a result of peritrophic membrane perforation and increase accessibility of the delta-endotoxin molecules to the epithelial membranes (Regev *et al*., 1996). The potential of *B. thuringiensis* chitinases as synergistic agents is based on their ability to degrade chitin under alkaline conditions which are normally found in the midgut of susceptible

insects (Barboza-Corona *et al.* 1999). Until now, the synergistic effect between *B. thuringiensis* chitinases and ICPs has not been quantitatively tested, mainly due to the low levels of endogenous expression of the chitinases. This is an important reason to clone the endogenous chitinase genes and over-express them in *E. coli* so that the synergistic effect can be determined. So far, genes coding for endogenous chitinases have been cloned from *B. thuringiensis* serovar *israelensis, kurstaki, pakistani, kenyae* and *sotto* (Lin and Xiong, 2004). 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 by Downing *et al*. (2000), requiring lower levels of Cry1Ac7 protein expression. This is advantageous, since lower expression may enable the bacteria to compete better in the environment with a diminished risk of generation of resistant larval populations resulting from exposure to high levels of Cry protein.

1.8. Present Study

The present study focused on cloning of chitinase A (*chiA*) gene from a local isolate of *S. marcescens* (Bn10) and expression in an anti-Coleopteran *B. thuringiensis* producing Cry3Aa delta endotoxin. Recombinant *B. thuringiensis* cells expressing *chiA* were expected to have increased larvicidal activity by the synergistic effect of Cry3Aa toxin and chitinase enzyme, and gain ability to kill adult forms of coleopteran insect pests besides larvae. The cloned chitinase gene was sequenced and translated and its amino acid sequence was aligned with other known chitinases. Chitinase production by *S. marcescens* Bn10 under different cultural conditions and the effects of certain parameters on enzyme activity were also investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Plasmids

The sources and characteristics of bacterial strains are listed in Table 2.1. Plasmids used in cloning experiments are given in Table 2.2. The structures of plasmid vectors and the size markers are presented in Appendix A.

2.2. Culture Media

The composition and preparation of culture media are given in Appendix B.

2.3. Solutions and Buffers

The composition of solutions and buffers are given in Appendix C.

2.4. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are listed in Appendix D.

2.5. Growth Conditions and Maintenance of Bacterial Strains

B. thuringiensis, *B. subtilis* 168, *S. marcescens* Bn10 and *E. coli* DH5α strains were grown in Luria Broth (LB) medium and stored on Luria agar (LA) plates. The cultures were stored at 4 ^{o}C and subcultured monthly. Cultures grown in Luria Broth until mid-log phase were covered with 20% glycerol for long term storage at –80 ^{o}C.

Table 2.1. Sources and characteristics of the strains.

Strain	Characteristics	Source and Reference
B. thuringiensis Mm2	Local isolate	Prof. Dr. Zihni Demirbağ, Department of Biology, KTU; Kurt *et al*., In press
B. subtilis 168; standard strain	*trp*C2, *thy*A1, *thy*B1	Dr. Daniel Zeigler, BGSC
S. marcescens Bn10	Local isolate	Prof. Dr. Zihni Demirbağ, Department of Biology, KTU; Sezen *et al*. (2001)
E. coli DH5α	F' ϕd*lac*ZΔ(*lac*ZY A-argF)U169 *sup*E44λ^{-} *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection; Hanahan (1983)
B. thuringiensis 3023	Local isolate	Prof. Feruccio Gadani, Philip Morris Europe S.A., Neuchatel, Switzerland; Kaelin *et al.* (1994)

LB and LA media were supplemented with the appropriate antibiotics, whenever necessary. The concentrations of antibiotics included in media were as follows: Ampicillin, 100 μg/mL; chloramphenicol, 25 μg/mL for *E. coli* and *B. thuringiensis*, 5 μg/mL for *B. subtilis*; erythromycin, 25 μg/mL.

Table 2.2. Plasmids used in cloning.

Plasmid	Size	Markers	Source and Reference
pNW33N	3.8 kb	*cat* (Cm^r)	Bacillus Genetic Stock Center (*E. coli* strain ECE136)
pHT315	6.5 kb	*erm* (Ery^r), *lac*Z	Prof. Didier Lereclus (Unite Genetique Microbienne et Environnement INRA, France); Arantes and Lereclus, 1991.
pGEM®-T	3.0 kb	*amp* (Amp^r), *lac*Z	Promega Inc. (Madison, WI)

2.6. Plasmid Isolation

Plasmid DNA from the cells of *B. subtilis* and *B. thuringiensis* were isolated by using the following procedure:

1 mL of overnight bacterial culture was added to 10 mL of LB and grown until late exponential phase. 3 mL of culture was precipitated in a 2 mL Eppendorf tube at 13,000 rpm for 1 min. Supernatant was removed carefully and the pellet was washed twice with 750 µL of SET buffer (Appendix C). The cells were spinned down after each wash for 2 min. 500 µL of SET buffer containing 5 mg/mL lysozyme was added to the pellet and suspended by pipetting. The mixture was incubated at 37 ^oC for 10 min. 390 µL of freshly prepared alkaline SDS (390 µL of sterile water + 50 µL 20% SDS + 20 µL 10 M NaOH) was added to the mixture and mixed by inverting the tube gently until the suspension was clear. 390 µL of ice cold K-Acetate (3 M, pH 4.7) was added immediately and mixed by inverting the tube gently several times. Precipitate was removed by centrifugation at 13,000 rpm for 5 min. Supernatant was transferred to a new Eppendorf tube and extracted with 900 µL of ice cold phenol:chloroform: isoamyl alcohol (24:24:1). The phases were separated by centrifugation at 13,000 rpm for 2 min. Aqueous phase was transferred to a new Eppendorf tube and the extraction step was repeated. Equal volume of cold isopropanol was added to the supernatant and mixed by inverting the tube. After incubation of the mixture at -20 ^oC for 10 min,

DNA was collected by centrifugation at 13,000 rpm for 5 min. Isopropanol was removed carefully and 70% ethanol was added to the pellet without mixing. After centrifugation at 13,000 rpm for 2 min, supernatant was removed. The pellet was dried and dissolved in 50 µL of TE buffer or dH_2O.

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were mostly used for isolation of *E. coli* plasmid DNA as specified by the manufacturers. *E. coli* plasmid DNA was also prepared by the plasmid miniprep method described by Hopwood *et al*. (1985). Each strain was grown as a patch on selective medium, LB agar containing 100 µg/mL ampicillin. About 1 square cm of cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µL cold STE solution containing 2 mg/mL lysozyme (Appendix C). Each tube was mixed by vortexing to disperse the cells and the toothpick was discarded. The tubes were then incubated on ice for 20 min. 3/5 volume of lysis solution (Appendix C) was added to each tube and vortexed immediately. The mixture was incubated at room temperature for 10 min to lyse the cells and then at 70 °C for 10 min to denature DNA. Then, tubes were cooled rapidly in cold water. An equal amount of phenol-chloroform (water saturated, Appendix C) was added, vortexed hard until a homogeneous and milky white mixture was obtained. Finally, the samples were spinned for 5 min at 13,000 rpm to separate phases. 10 µL of supernatant was loaded directly on an agarose gel for electrophoresis.

2.7. Genomic DNA Isolation

The procedure of Hintermann (1983) was used for the isolation of genomic DNA from *S. marcescens* Bn10. The organism was grown in 50 mL of LB medium until mid-log phase. The cells were harvested by centrifugation at 10,000 x g for 10 min and resuspended in 5 mL TSE buffer (Appendix C). The mixture was incubated at 37 ^{o}C for 15 min with occasional mixing until clearing occurs. After addition of 100 µL of 5 mg/mL solution of proteinase K, 0.5 mL of 10% SDS was added. The solution was mixed vigorously and incubated at 65 ^{o}C for 25 min. After cooling to room temperature, 5 mL of phenol:chloroform solution (1:1) was added and vortexed hardly

until the phases were very well mixed. The phases were separated by spinning at 10,000 rpm for 10 min. The organic phase (bottom) was removed and phenol:chloroform extraction step was repeated. The aqueous phase was extracted with chloroform again and after phases were separated, the chloroform (bottom) was removed completely and the tube was spinned at 10,000 rpm for 10 min to pellet the remaining interphase. The supernatant was removed carefully to a fresh tube; RNAse to a final concentration of 20 µg/mL was added and incubated at 37 ^{o}C for 1 h. 5 M NaCl to a final concentration of 1 M and 50% PEG 6000 to a final concentration of 10% were added to the supernatant which was then mixed by inversion. After 30 min on ice or o/n incubation at 4 ^{o}C, the mixture was centrifuged at 3,000 rpm for 5 min. The supernatant was removed and the pellet was dissolved in 5 mL of TE buffer. 1/10 volume of 3 M unbuffered sodium acetate and 3 volumes of ethanol were added and mixed. The mixture was incubated at -20 ^{o}C overnight. The precipitate was spinned down at 10,000 rpm for 10 min the pellet was dried and dissolved in 1 mL of TE buffer. Concentration of genomic DNA was then checked either by performing agarose gel electrophoresis or spectrophotometrically. To determine the concentration and purity of DNA, it was diluted and its optical density (OD) was read at 260 nm and 280 nm. Samples having an OD_{260}/OD_{280} ratio < 1.8 should be re-extracted with phenol:chloroform and then chloroform to remove the protein contamination or should be discarded. To calculate the concentration of DNA, the equation used was "concentration (µg/mL) = A_{260} x 50 x dilution factor".

2.8. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus. 0.8% agarose gel was prepared in TAE buffer (Appendix C) and ethidium bromide was added to give a 0.5 µg/mL final concentration in melted agarose gel. Electrophoresis was performed at 90 Volts for 45-60 min. The DNA bands were visualized on a shortwave UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. *Pst*I digested lambda DNA marker (Appendix A) was used to determine the molecular weights of DNA bands.

2.9. Ligation Reactions

Ligation of PCR products to pGEM®-T vector was performed as follows: 5 µL 2X ligase buffer, 1 µL (55 ng/µL) pGEM®-T vector, 500 ng insert DNA, 1 µL T4 DNA ligase (3 Unit/µL) was mixed and volume was completed to 10 µL with H_2O. Ligation was carried out as overnight incubation at 4 ^oC. When the vectors pNW33N or pHT315 were used, vector and insert DNA were mixed in 1:7 ratio and incubated at 45 ^oC for 10 min and on ice for 5 min after which ligase buffer and the enzyme were added. The mixture was incubated overnight at 16 ^oC.

2.10. Restriction Enzyme Digestion

Restriction enzyme was added in a suitable buffer to the DNA to introduce 2 Units per µg of DNA. The mixture was incubated at a temperature appropriate for that restriction enzyme for 3-5 h. The sample was stored at -20 ^oC when needed.

2.11. Transformation of Bacteria

2.11.1. Transformation of *E. coli* DH5α cells

The procedure developed by Inoue and his colleagues (1990) was used for competent cell preparation and transformation. 250 mL SOB medium (Appendix B) in a 2 L Erlenmeyer flask was inoculated with freshly grown *E. coli* cells and incubated at 28 ^oC with shaking at 200-250 rpm until OD_{600} reaches to 0.6. The flask was placed on ice for 10 min. Cooled cells were harvested at 2,500 x g for 10 min at 4 ^oC. The pellet was suspended in 80 mL of ice cold TB buffer. After incubation on ice for 10 min, it was centrifuged and pellet was suspended in 20 mL TB buffer. DMSO was added with gentle swirling to a final concentration of 7 %. The solution was incubated for 10 min on ice and 0.4 mL aliquots were dispensed into Eppendorf tubes. These tubes were immersed in liquid nitrogen and stored at -80 ^oC.

E. coli competent cells were also prepared according to the protocol described by Sambrook *et al*. (1989) with slight modifications. In a 250 mL flask, 5–50 mL of LB broth (Appendix B) was inoculated with *E. coli* from a fresh LB agar plate and incubated overnight with shaking at 37 °C to obtain a stationary phase culture. 300 µL from this seed culture was inoculated into a fresh flask containing 50 mL LB broth. The culture was incubated for 2–2.5 h at 37 °C with vigorous shaking (300 rpm) in an orbital shaker to obtain an exponentially growing culture. Then the culture was split into two sterile pre-chilled 40 mL screwcap centrifuge tubes asepticaly and stored on ice for 10 min. After centrifuging at 4,000 rpm for 10 min at 4 °C, supernatants were decanted and each pellet was resuspended in 5 mL of ice-cold 10 mM $CaCl_2$ by vortexing. The cells were spun down at 3,000 rpm for 10 min at 4 °C. Finally, supernatants were decanted and each pellet was resuspended gently in 1 mL ice-cold 75 mM $CaCl_2$. The competent cells were stored at – 80 °C.

For transformation, competent *E. coli* cells were kept on ice for 15 min. 10 µL of ligation products or 0.5 µg of appropriate plasmid DNA was added to the cells and mixed gently. The mixture was incubated on ice for 30 min. After a heat shock at 42 ^{o}C for 90 sec, it was incubated on ice for 5 min. 900 µL of LB was added to the mixture and incubated at 37 ^{o}C for 80 min by gentle agitation (100 rpm). The cells were microfuged at 4,000–5,000 rpm for 10 min and resuspended in 500 µL saline solution. Transformed cells were plated on selective medium containing appropriate antibiotic (100 µg/mL ampicillin or 25 µg/mL chloramphenicol). For blue – white colony selection, they were plated on LB agar media containing 80 mg/mL X-gal, 0.5 mM IPTG and 100 µg/mL ampicillin.

2.11.2. Preparation and Transformation of *Bacillus subtilis* 168 Competent Cells

Preparation and transformation of *B. subtilis* competent cells were performed as described by Klein *et al.* (1992). 3 mL of overnight culture was prepared in HS medium (Appendix B) by incubation at 37 ^{o}C and shaking at 350 rpm. 0.5 mL of it was then transferred into 20 mL of LS medium (Appendix B) and incubated at 30 ^{o}C with shaking at 100 rpm until OD_{600} of cultures was reached to 0.55. 1 mL of

competent cells was transferred into 2 mL Eppendorf tube and 1 µg of DNA was added. Cells were then incubated at 37 ^{o}C for 2 h with shaking at 350 rpm and were collected by centrifugation at 4,000 rpm for 10 min. Cells were resuspended in 100 µL of sterile saline and plated out onto selective LA plates containing 5 µg/mL chloramphenicol. Plates were incubated at 37 ^{o}C for 16 h.

2.11.3. Protoplast Transformation of *B. thuringiensis*

Transformation of *B. thuringiensis* cells was performed according to the method of Pragai *et al*. (1994). *B. thuringiensis* to be transformed was grown overnight on LA agar plus 1% insoluble starch. Cells were inoculated from the plate into 55 mL of YTA broth in a 250 mL flask to OD_{550} 0.1. They were shaken for 10–15 min at 37 ^{o}C and then half of the culture was taken and put it in another flask (prewarmed at 37 ^{o}C). Bacteria were grown to OD_{550} of 1. Culture was centrifuged in two sterile 50 mL tubes at room temperature for 10 min at 16,000 rpm. The supernatant was discarded and the pellet was resuspended (both tubes) in 4.5 mL of P solution. Lysozyme was added to a final concentration of 2 mg/mL. The lysozyme was prepared freshly in 20 mg/mL in P solution (Appendix C) and filter sterilized with a 0.45-µm filter. 500 µL lysozyme was added to 4.5 mL of cells. Tubes were incubated at 37 ^{o}C with no shaking for 30–45 min and protoplasting was checked under the microscope. When >99% of cells were protoplasts, they were spinned down at 2,000–4,000 rpm for 20 min. The speed for pelleting depended on the stickiness of the strain used. It was washed once with 5 mL of P solution and cells were resuspended in 1–5 mL of P solution. The resuspension volume may be varied depending on the number of transformations to be done. 10–15 µL of the plasmid DNA (high concentrations of plasmid were used) was added into 1 mL of cells and mixed by gentle pippetting. The mixture was pippetted into centrifuge tubes containing 1 mL 60% PEG 6000 and mixed gently for 3 min by rocking. 5 mL of ART liquid media was added and mixed. The mixture was spinned at 3,000 rpm for 20 min at room temperature. The pellet was resuspended in 1 mL of ART and incubated for 90 min at 30 ^{o}C, 100 rpm. The soft agar was microwaved and placed at 55 ^{o}C (no antibiotic in soft agar). 10 mL of soft agar was distrubuted in tubes, the transformed protoplasts were added (100–200 µL)

and poured onto large ART plates with appropriate antibiotic. The plates were dried for 15 min under hood, inverted and incubated at 30 ^{o}C. Colonies took 3–8 days to appear. When grown, they were picked to LA plates with appropriate antibiotic.

2.11.4. Electroporation

The electroporation of bacteria was performed as described by Lereclus *et al*. (1989) with modifications. *B. thuringiensis* cells were grown with shaking in 1 L of Brain Heart Infusion (BHI) at 37 ^{o}C to an A_{600} of 2. The cells were washed once in 100 mL of 4 ^{o}C water. The pellet was resuspended in 10 mL of cold sterile PEG 6000 (40%, w/v). The cell concentration was about 10^{10} cells/mL. This suspension was frozen in aliquots and stored at -80 ^{o}C. Cell aliquots of 300 µL were mixed with 1 µg of plasmid DNA (10 µL in 10 mM, Tris-HCl, pH 7.5; 1 mM, EDTA) in 0.2 cm electroporation cuvettes (Bio-Rad), at 4 ^{o}C. The Bio-Rad MicroPulser^{TM} apparatus was set to StA program (1.8 kV, 2.5 msec). The cuvette was placed in the safety chamber and the pulse was applied once. Following electroporation, the cells were diluted 1 in 1 in 2 mL of BHI medium and incubated with shaking at 37 ^{o}C for 1 h. After this expression period, the cells were plated on BHI-agar containing erythromycin (25 µg/mL).

2.12. Primer Design

Primers to amplify chitinase A gene of *S. marcescens* Bn10 were designed according to the sequence of *S. marcescens* endochitinase (*chiA*) gene (NCBI accession number AF544462). In order to amplify the promoter region of *cry*3Aa11 gene promoter of *B. thuringiensis* subsp. *tenebrionis* Mm2, the primers were designed according to the nucleotide sequence of *cry*3Aa gene of *B. thuringiensis* subsp. *tenebrionis* strain NB176 and *B. thuringiensis* LM79 (the GeneBank acsession numbers U10985 and L03393, respectively).

Table 2.3. Primers used in PCR amplification. Restriction enzyme sites were underlined.

Gene name	Primer name	Nucleotide sequence	Size of the PCR products
chiA	chiF	5' aggatccatgcgcaaatttaataaaccgctg 3'	1706 bp (with chiR)
chiA	chiR	5' caagcttttattgaacgccggcgctgtt 3'	1706 bp (with chiF)
chiA	chiAHF	5' aagcttatgcgcaaatttaataaaccgctg 3'	1704 bp (with chiABR)
chiA	chiABR	5' ggatccttattgaacgccggcgctgtt 3'	1704 bp (with chiAHF)
*cry3*Aa11 promoter	crypF	5' gagctctcgaaacgtaagatgaaacctt 3'	638 bp (with cryp7R) 642 bp (with cryp11R)
*cry3*Aa11 promoter	cryp7R	5' gatggatcctcctccctttctt 3'	638 bp (with crypF)
*cry3*Aa11 promoter	cryp11R	5' gatggatccttcttcctccctttctt 3'	642 bp (with crypF)
chiA and the promoter of *cry3*Aa11	chiproXF	5' tctagatcgaaacgtaagatgaaacctt 3'	2342 bp (with chiAER)
chiA and the promoter of *cry3*Aa11	chiAER	5' gaattcttattgaacgccggcgctgtt 3'	2342 bp (with chiproXF)

2.13. Polymerase Chain Reactions (PCR)

PCR mixture contained 5 µL 10X PCR buffer, 5 µL 2mM dNTP mix, 50 pmols of each primer, 5 µL of 10X $MgCl_2$, 2 Units of *Taq* polymerase, 0.1 µg template DNA and 22 µL sterile dH_2O to complete the volume to 50 µL. The sequences of primers and the size of products are given in Table 2.3. Table 2.4 shows primers, templates and PCR conditions used for the amplification of the sequences/gene of interest.

Table 2.4 Amplified sequences/genes and PCR conditions.

Product	Primers used	Template DNA	PCR conditions
*cry3*Aa11 promotor region (Distance between Shine Dalgarno and ATG is 7 bp)	crypF and cryp7R	0.1 µg total DNA of *B. thuringiensis* Mm2	Initial denaturation: 3 min at 95 °C Denaturation: 1 min at 94 °C Annealing: 1 min at 65 °C Extension: 1 min at 72 °C Number of cycles: 35 Final extention: 10 min at 72 °C
*cry3*Aa11 promotor region (Distance between Shine Dalgarno and ATG is 11 bp)	crypF and cryp11R	0.1 µg total DNA of *B. thuringiensis* Mm2	Initial denaturation: 3 min at 95 °C Denaturation: 1 min at 94 °C Annealing: 1 min at 65 °C Extension: 1 min at 72 °C Number of cycles: 35 Final extention: 10 min at 72 °C
chiA	chiF and chiR	0.1 µg genomic DNA of *S. marcescens* Bn10	Initial denaturation: 3 min at 94 °C Denaturation: 1 min at 94 °C Annealing: 1 min at 63 °C Extension: 1 min at 72 °C Number of cycles: 40 Final extention: 10 min at 72 °C
chiA	chiAHF and chiABR	0.1 µg pNW33NP7C	Initial denaturation: 3 min at 94 °C Denaturation: 1 min at 94 °C Annealing: 1 min at 63 °C Extension: 1 min at 72 °C Number of cycles: 40 Final extention: 10 min at 72 °C
chiA with the promoter region of *cry3*Aa11	chiproXF and chiAER	0.1 µg pNW33NP7C	Initial denaturation: 3 min at 94 °C Denaturation: 1 min at 94 °C Annealing: 1 min at 58 °C Extension: 2.5 min at 72 °C Number of cycles: 40 Final extention: 10 min at 72 °C

Reaction mixtures were separated in 0.8% agarose gel. Desired bands from PCR products were cut from the gel and extracted by using Qiagen Gel Extraction Kit.

2.14. Sequencing Reactions

DNA sequencing was carried out either in Molecular Biology and Biotechnology Research and Development Center of Middle East Technical University (Ankara, Turkey) and Iontek Inc. Laboratories (İstanbul, Turkey) by the Chain Termination Method with dye-labelled dideoxy terminators of Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham). Perkin Elmer-ABI Prism 377 Automated Sequencer was used in either case.

Deduced nucleotide and amino acid sequence data were compared with the National Center for Biotechnology Information (NCBI) database using the BLAST search at the web site (http://www.ncbi.nlm.nih.gov/BLAST).

2.15. Endochitinase Activity Assay

2.15.1. Preparation of Colloidal Chitin

Colloidal chitin was prepared according to the method described by Roberts and Selitrennikoff (1988). 10 g sample of crab-shell chitin was dissolved in 175 mL of cold concentrated HCl and the mixture was placed at 4 ^{o}C for 24 h with rapid stirring. The mixture was added into 1 L cold ethanol and placed at -20 ^{o}C for 24 – 48 h. The resulting chitin suspension was filtered through a Whatman No. 1 filter paper and washed with dH_2O until the pH becomes neutral. The washed chitin was dried at 90 ^{o}C for overnight and crumbled in a porcelain mortar.

2.15.2. Preparation of Chitin Plates

The method of Roberts and Selitrennikoff (1988) was used to prepare chitin plates. 100 mg of colloidal chitin was homogenized in 20 mL dH_2O by the aid of a glass homogenizer. 1.6 g of Nutrient Broth (NB) and 2 g of agar were added into 80 mL dH_2O. Chitin suspension was mixed with NB+agar solution. The mixture was

autoclaved, desired antibiotic was added if needed and 20 mL of this medium was poured into disposable petri plates.

2.15.3. Preparation of Endochitinase Enzyme

Endochitinase was obtained as in Roberts and Selitrennikoff (1998). The medium containing 1.6% Bacto Nutrient Broth and 0.2% colloidal chitin was used to grow the bacteria at 30 ^{o}C for overnight at 200 rpm. An appropriate antibiotic was added whenever necessary. Culture was centrifuged at 12,000 rpm for 15 min. The pellet was discarded and the supernatant was used as endochitinase source. Enzyme solution was kept at -20 ^{o}C.

2.15.4. Qualitative Determination of Enzyme Activity on Agar Plates

Chitin plates were used qualitative determination of chitinase activity on agar plates. A bacterial colony was transferred as a small spot onto a chitin plate by using a toothpick. The plate was incubated at 30 ^{o}C up to 10 days. Presence of chitin degradation around the colonies indicated the production of chitinase enzyme.

2.15.5. Fluorometric Determination of Endochitinase Activity

The method of Brants and Earle (2001) was used with slight modifications to determine the endochitinase activity fluorometrically**.** 4-methylumbelliferyl-β-D-N, N', N''-triacetylchitotrioside [4-MU-β-$(GlcNAc)_3$] solution was prepared as a substrate at a concentration of 25 μg/mL in 0.1 M potassium phosphate buffer (pH 6.6). 15 μL of enzyme solution was added into 200 μL of the substrate and incubated at 30 ^{o}C for 20 min. 30 μL of aliquots were added into 1.9 mL of 0.2 M Na_2CO_3 solution to terminate the reaction. Fluorescence after the enzyme activity was measured by Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Chrompack International, BV, Netherlands) at 360 nm excitation and 460 nm emission wavelengths. The calibration curve shown in Figure 2.1 was used to determine the activity of endochitinases produced by parental or recombinant strains. The specific

activity was reported as Unit (nanomoles of MU released per minute) per mg of total extracellular protein.

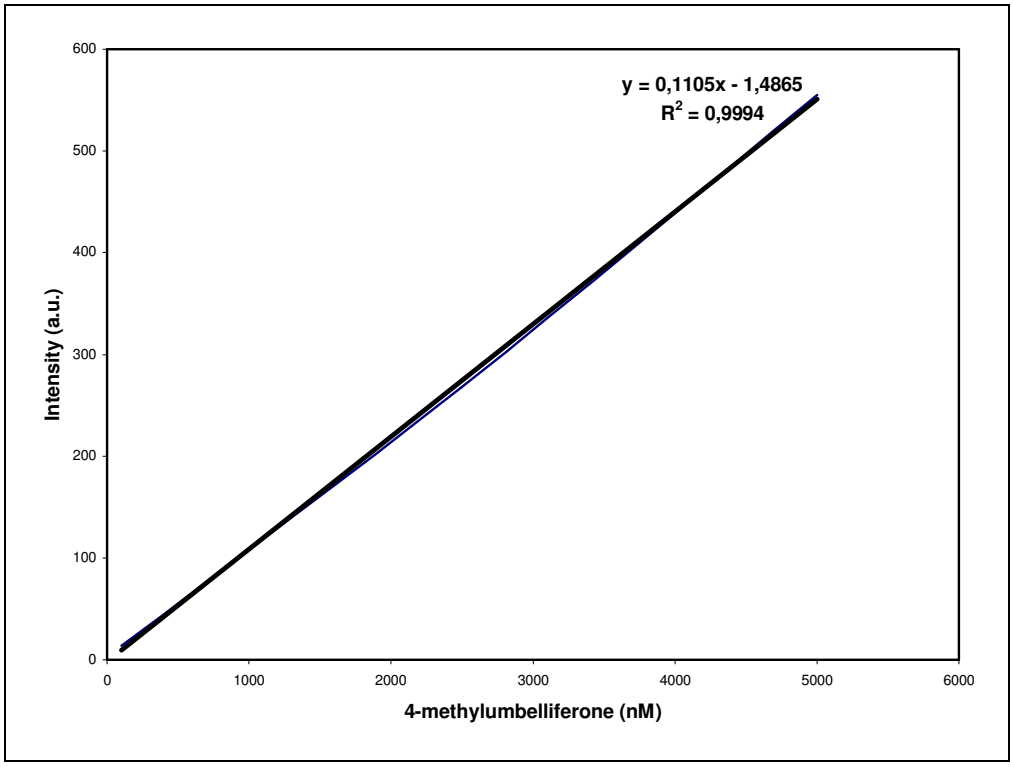


Figure 2.1. Calibration curve for fluorometric endochitinase assay.

2.16. Polyacrylamide Gel Electrophoresis

2.16.1. Native PAGE and Activity Staining

For native PAGE, proteins were separated on 4.5% stacking and 12% separating polyacrylamide slab gels (Laemmli, 1970) under nondenatured (native) conditions (Table 2.5).

Table 2.5. Preparation of native polyacrylamide gels.

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	**4.5%**	**12%**
Acrylamide/bis	650 µL	2 mL
dH_2O	3100 µL	1715 µL
5 mM 4-MU$(GlcNAc)_3$ (for activity staining only)	-	10 µL
1.5 M Tris-HCl, pH 8.8	-	1250 µL
0.5 M Tris-HCl, pH 6.8	1250 µL	-
10% Ammonium persulphate (fresh)	25 µL	25 µL
TEMED	5 µL	2.5 µL
TOTAL MONOMER	5 mL	5 mL

10 µL of 5 mM 4-MU-β-$(GlcNAc)_3$ was added to separating gel. Electrophoresis was run in electrophoresis buffer which was prepared as in Walker (1994) (Appendix C) at 15 mA for approximately 1 h at 4 ^oC. After electrophoresis, the gel was cut into two. One half was incubated at 30 ^oC for 20 min in 0.1 M phosphate buffer pH 6.6 and the bands were visualized under UV light. The other half was stained with silver nitrate by the method of Blum *et al.* (1987) (Table 2.7).

2.16.2. SDS-PAGE

For SDS-PAGE (Laemmli, 1970; Table 2.6) electrophresis was run at 20 mA at the beginning until the samples reached to the separating gel, then the current was increased to 40 mA and the electrophoresis was continued until the loading dye reached to the end of the gel.

Table 2.6. Preparation of SDS-polyacrylamide gels.

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	**4.5%**	**12%**
Acrylamide/bis	1.3 mL	4 mL
dH_2O	6.1 mL	3.35 µL
1.5 M Tris-HCl, pH 8.8	-	2.5 mL
0.5 M Tris-HCl, pH 6.8	2.5 mL	-
10% (w/v) SDS	100 µL	100 µL
10% Ammonium persulphate (fresh)	50 µL	50 µL
TEMED	10 µL	5 µL
TOTAL MONOMER	10 mL	10 mL

2.17. Staining of Polyacrylamide Gels

2.17.1. Coomassie Blue R-250 Staining

After electrophoresis, the gel was soaked in 200 mL of freshly prepared Coomassie Blue R-250 stain (Appendix C) for 1 h at room temperature. The gel was then destained by keeping it in destaining solution (Appendix C) for at least 24 h.

2.17.2. Silver Staining

Silver staining was performed as in Blum *et al*. (1987) (Table 2.7).

Table 2.7. Silver staining method.

Step	Solutions	Time of treatment
Fixation	50% MeOH 12% AcOH 0.5 mL 37% HCOH/L	1 h.
Washing	50% EtOH	3 X 20 sec.
Pretreatment	$Na_2S_2O_3.5H_2O$ (0.2 g/L)	1 min.
Rinse	dH_2O	3 X 20 sec.
Staining	$AgNO_3$ (2 g/L) 0.75 mL 37% HCOH/L	30 min.
Rinse	dH_2O	2 X 20 sec.
Development	Na_2CO_3 (60 g/L) 0.5 mL 37% HCOH/L $Na_2S_2O_3.5H_2O$ (4 mg/L)	10 min.
Rinse	dH_2O	2 X 20 min.
Stop	50% MeOH 12% AcOH	10 min.

2.18. Determination of Protein Concentration

Protein concentrations were measured by the Bradford quantification method (1976). The assay is based on the observation at 595 nm when the absorbance is maximum for an acidic solution of Coomassie Brilliant Blue G-250 while binding to a protein. Assay reagent was made by dissolving 100 mg of Coomassie Blue G-250 in 50 mL of 95% ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Watman No. 1 filter paper. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 10,15, 20, 30 and 50 µL of 1 mg/mL standard protein, BSA were added to tubes and volumes were adjusted to 500 µL with water. 500 µL of distilled water was added into a tube as reagent blank. 4.5 mL of assay

reagent was added to each tube and mixed gently, but thoroughly. A standard curve of absorbance versus micrograms protein was prepared (Figure 2.2) and the amounts of proteins were determined from the curve.

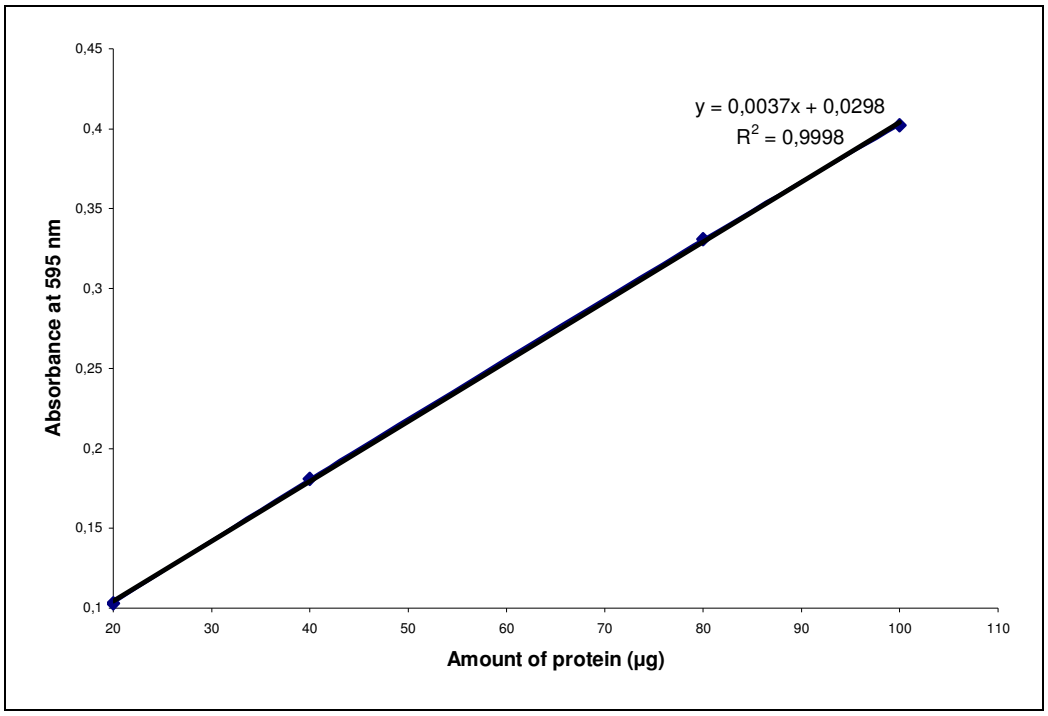


Figure 2.2. Calibration curve for quantification of protein concentrations.

2.19. Determination of Chitinase Production by Parental and Recombinant Strains

The medium containing 1.6% (w/v) Nutrient Broth (Merck) and 2 g/L colloidal chitin was used to grow bacterial strains at 30 ^{o}C for 16 h. The cultures were centrifuged at 6,000 rpm for 10 min and the supernatants were used as chitinase sources. Total extracellular proteins were run on SDS-PAGE after estimation of protein concentration by the method of Bradford (1976). Enzyme activity determinations were made as described in Section 2.15.5.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of Chitinase A (*chiA*) Gene from *S. marcescens* Bn10 in *E. coli*

3.1.1. PCR Amplification and Insertion of *chiA* Gene into pGEM®-T vector

The primers chiF and chiR (Table 2.3) designed according to the sequence of *S. marcescens* endochitinase (*chiA*) gene (GeneBank Accession No. AF454462) were used in order to amplify the *chiA* gene of *S. marcescens* Bn10. The expected 1706 bp gene product belonging to the *chiA* ORF (open reading frame) was obtained at the end of the PCR (Figure 3.1).

In order to check if the product is the correct fragment, 1706 bp band was purified from the gel and sequenced. It was compared to known chiA sequences in NCBI database using the BLAST search. The amplified fragment showed significant homology with the *chiA* gene of *S. marcescens*. The gene was next cloned in pGEM®-T vector to be transformed into *E. coli* cells (Table 2.2).

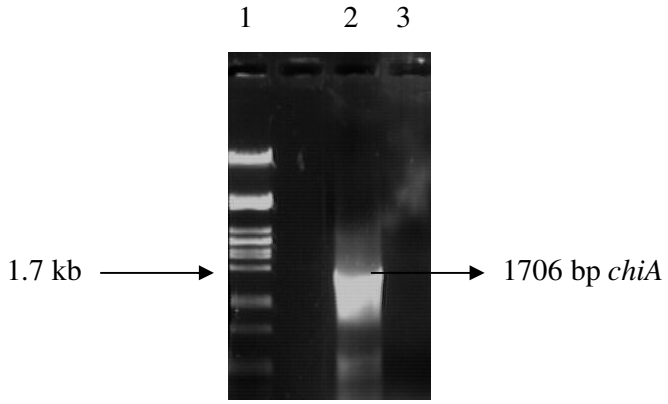


Figure 3.1. Amplification of *chiA* gene from the genomic DNA of *S. marcescens*. Lane 1: Marker [*Pst*I digested Lambda DNA (Appendix A)], Lane 2: 1706 bp PCR product obtained with the primers chiF and chiR, Lane 3: Negative control (no template).

For cloning in, the PCR product was purified from the gel and ligated into pGEM®-T vector.

3.1.2. Transformation of *E. coli* with Recombinant Plasmid and Verification of Cloning in *E. coli*

The ligation product was introduced into *E. coli* DH5α competent cells and resulting recombinants were selected on X-gal + IPTG + ampicillin containing LA plates. Plasmids isolated from putative white recombinant colonies were used for the verification of the cloning by PCR and restriction enzyme digestion. Figure 3.2 and 3.3 show the expected 1706 and 1702 bp products obtained from the PCR reaction and restriction enzyme digestion, respectively. For restriction digestion, the enzymes used were *Bam*HI and *Hin*dIII for each one recognition site has been placed to flank the gene when designing the primers.

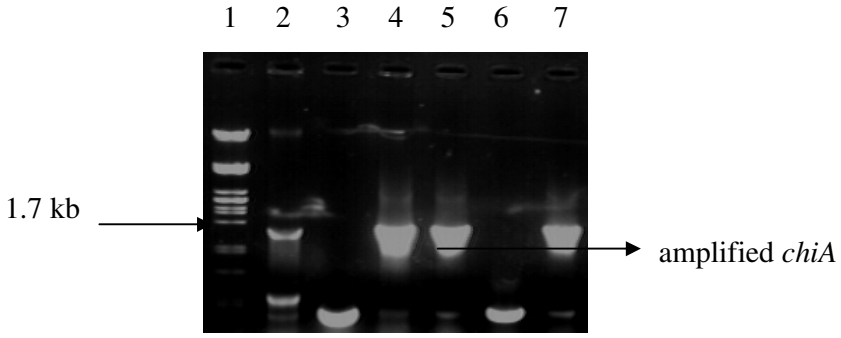


Figure 3.2. Verification via PCR of cloning of *chiA* into pGEM®-T vector. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Positive control (*S. marcescens* genomic DNA as the template), Lanes: 3, 4, 5, 6, 7: PCR products from putative recombinants.

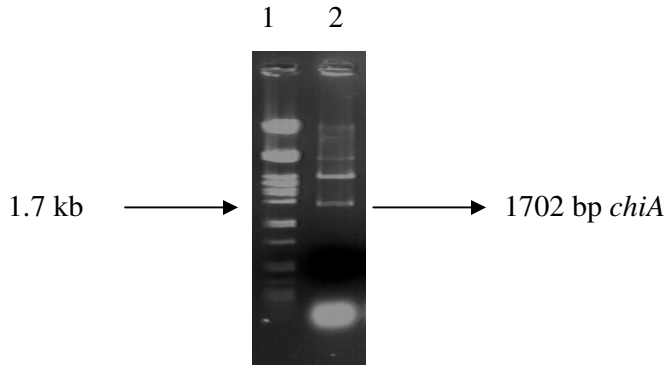


Figure 3.3. Restriction enzyme digestion of recombinant pGEM®-T. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: *Bam*HI and *Hin*dIII digested pGEM®-T carrying *chiA*.

3.1.2.1. Cloning of *chiA* Gene into *E. coli-Bacillus* Shuttle Vector pNW33N

For cloning of *chiA* into *E. coli–Bacillus* shuttle vector pNW33N, both this vector and pGEM®-T vector carrying *chiA* gene were digested with *Bam*HI and *Hin*dIII restriction enzymes. Linearized vector and *chiA* gene were ligated after purification from the agarose gel and transferred into *E. coli* DH5α competent cells. Recombinants were selected on the basis of their chloramphenicol resistance (Cm^r). Plasmids were

isolated from Cm^r cells and used as template for amplification of *chiA* by PCR to verify recombination (Figure 3.4). The recombinant plasmid pNW33N carrying *chiA* was named as pNW33NC.

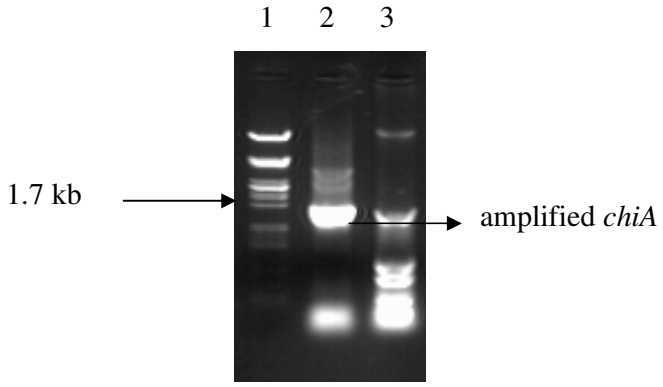


Figure 3.4. Verification via PCR of cloning of *chiA* gene into pNW33N. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: PCR product (putative recombinants as the template), Lane 3: Positive control (*S. marcescens* genomic DNA as a template).

3.2. Cloning of Chitinase A (*chiA*) Gene of *S. marcescens* Bn10 in *B. subtilis* as an Intermediate Host

3.2.1. Cloning of Promoter Region of *cry3*Aa11 Gene into pNW33NC

The promoter site of *cry3*Aa11 gene of *B. thuringiensis* Mm2 was placed in the upstream region of *chiA* carried by pNW33NC for a better expression in *B. thuringiensis* cells. Previously, Ruan and his collaques (2002) cloned the melanin (*mel*) gene of *Pseudomonas maltophila* under the control of the *cry3*A promoter in pHT3101 and the gene was successfully expressed in *B. thuringiensis* 171 cells.

Although the crystal genes of most of the *B. thuringiensis* strains exist on plasmids, some may be localized in their chromosome (Kaur, 2000). Because of that reason, the total DNA of *B. thuringiensis* Mm2 was isolated and used for amplifying promoter region of *cry3*Aa11. 642 bp and 638 bp DNA products were obtained at the end of

PCR reactions performed by using two different sets of primers, namely crypF-cryp11R and crypF-cryp7R, respectively (Figure 3.5 and Figure 3.6). Reverse primers cryp11R and cryp7R were designed so as to leave 11 bp and 7 bp between Shine Dalgarno (SD) sequence of *cry*3Aa11 promoter and start codon of *chiA* cloned in pNW33NC, respectively.

The distance between SD and ATG codon is generally 7 bp in the genes of the members of the genus *Bacillus*. We left these 7 bases since the distances between SD and start codon as well as the sequences around SD might be species specific and important for transcription.

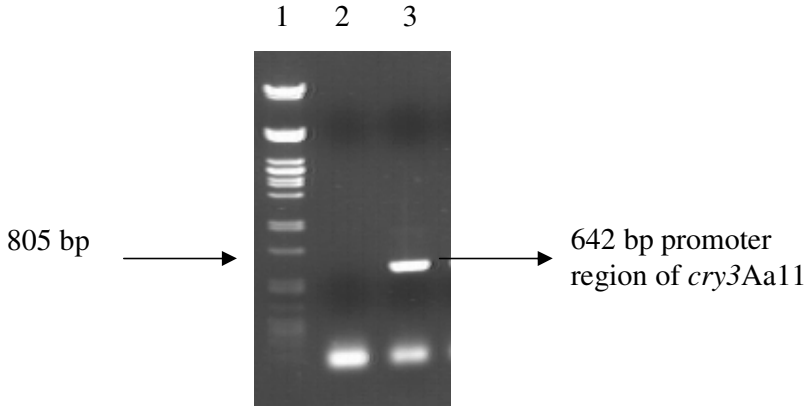


Figure 3.5. Amplification of the promoter region of *cry3*Aa11 via PCR by using the primers crypF-cryp11R. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Negative control (no template), Lane 3: Amplified 642 bp promoter sequence of *cry3*Aa11 gene.

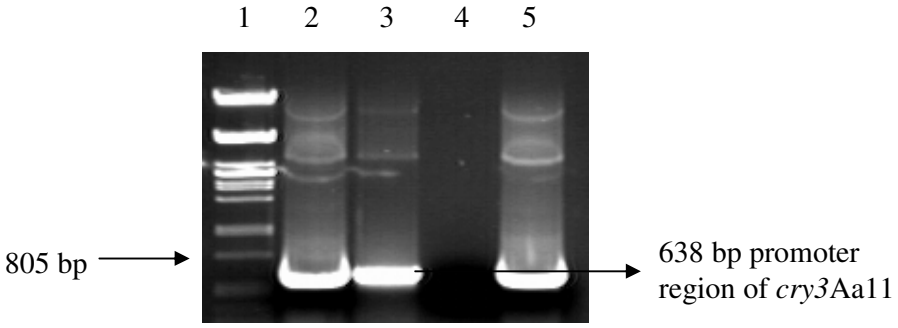


Figure 3.6. PCR amplification of the promoter region of *cry3*Aa11 by using the primers crypF-cryp7R. Lane 1: Marker (*Pst*I digested Lambda DNA), lanes 2-3: 638 bp PCR product for *cry*3Aa11 promoter, Lane 4: Negative control (no template), Lane 5: 642 bp PCR product of *cry3*Aa11 gene promoter using the primers crypF and cryp11R as positive control.

PCR product corresponding to the promoter regions of *cry3*Aa11 of *B. thuringiensis* Mm2 (Figure 3.6) was extracted from the gel and subcloned into pGEM®-T vector. pGEM®-T carrying *cry3*Aa11 promoter region was sequenced in Molecular Biology and Biotechnology Research and Development Center of Middle East Technical University (Ankara, Turkey). BLAST search was performed using the NCBI database for the sequence obtained. The sequence showed significant homology with the promoter site of the previously sequenced *cry3*Aa gene (GeneBank Accession Number AJ237900; Kurt *et al*., in press)

Isolated pGEM®-T carrying *cry*3Aa11 promoter region and pNW33NC were digested with *Bam*HI and *Sac*I restriction enzymes, the bands of interest were extracted from the gel and used for ligation. After transferring the plasmid into *E. coli* DH5α competent cells, recombinants were selected on the basis of their chloramphenicol resistance. Cloning was verified by PCR and digestion with *Bam*HI and *Sac*I restriction enzymes by using the plasmids isolated from putative recombinants manually (Figure 3.7).

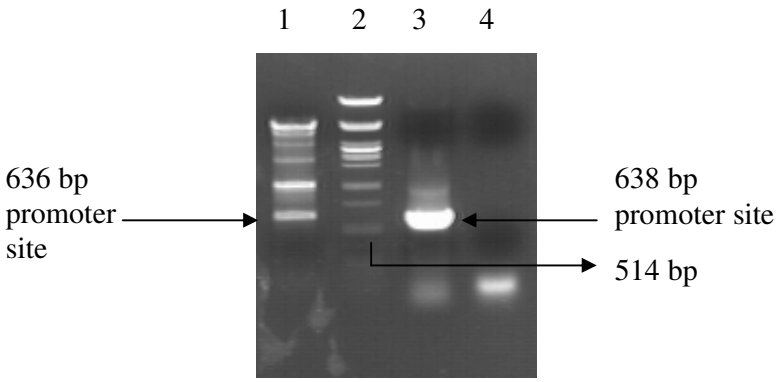


Figure 3.7. Verification of cloning of 638 bp promoter region in pNW33NC. Lane 1: Digestion product of pNW33NP7C, Lane 2: Marker (*Pst*I digested Lambda DNA), Lane 3: PCR product for 638 bp promoter region, Lane 4: Negative control (no template).

Recombinant pNW33NCs having a distance of 11 bp and 7 bp between SD sequence of *cry3*Aa11 and the start codon of *chiA* gene were named as pNW33NP11C and pNW33NP7C, respectively.

3.2.2. Transformation of *B. subtilis* 168 with the Recombinant Plasmids pNW33NP11C and pNW33NP7C

Our ultimate goal was to introduce the cloned genes into *B. thuringiensis* for obtaining a more potent strain industrially promising in insect control. As it is relatively easier to transform, we used *B. subtilis* 168 as an intermediate host to check if the cloned genes were expressed in a gram-positive background. The putative recombinants were selected on LA plates containing 5 µg/mL chloramphenicol and their plasmids were isolated. Figure 3.8 and 3.9 show the result of PCR performed by using primers for *cry3*Aa11 promoter site. Expected bands belonging to *cry*3Aa11 promoter was amplified when pNW33NP11C and pNW33NP7C isolated from the putative recombinant colonies were used as the templates.

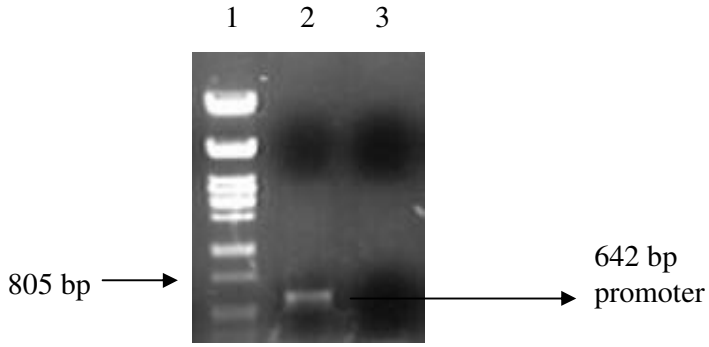


Figure 3.8. Verification of transformation of *B. subtilis* with the recombinant pNW33NP11C carrying 642 bp promoter region. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: PCR product for 642 bp promoter region by using primers crypF and cryp11R, Lane 3: Negative control (no template).

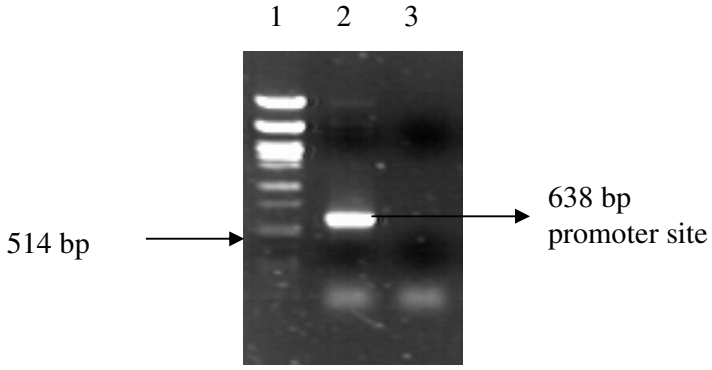


Figure 3.9. Verification of transformation of *B. subtilis* with the recombinant pNW33NP7C carrying 638 bp promoter region. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: PCR product for 638 bp promoter site by using primers crypF and cryp7R, Lane 3: Negative control (no template).

3.2.3. Endochitinase Activity of Parental and Recombinant *E. coli* and *B. subtilis* Strains as Compared to That of the Native Producer *S. marcescens* Bn10

Endochitinase activity of the recombinant *B. subtilis* 168 and *E. coli* DH5α cells carrying pNW33NP11C or pNW33NP7C as well as the parental strains were assayed by using the fluorescent substrate 4-MU-β-$(GlcNAc)_3$ as described in section 2.15.5.

Table 3.1. Endochitinase activities of the parental and recombinant *E. coli* and *B. subtilis* strains carrying pNW33NP7C or pNW33NP11C as compared to that of *S. marcescens* Bn10 as the gene source.

Bacteria	Recombinant plasmids	Specific activity (U/min/mg)	Relative activity (%)
S. marcescens Bn10	-	15905	100
B. subtilis 168	-	281	1.7
B. subtilis 168	pNW33NP11C	5381	33.8
B. subtilis 168	pNW33NP7C	6261	39.3
E. coli DH5α	-	265	1.6
E. coli DH5α	pNW33NP11C	2804	17.6
E. coli DH5α	pNW33NP7C	2185	13.7

As can be seen in Table 3.1, endochitinase activity of *B. subtilis* 168 carrying pNW33NP11C or pNW33NP7C was measured as 5381 and 6261 U/min/mg which corresponded to 33.8% and 39.3% of the endochitinase activity of *S. marcescens* Bn10, respectively. It was observed that increasing the number of bases between SD and ATG codon from 7 to 11 bases, decreased the activity only by 5%. Expression level of recombinant chitinase in *E. coli*, on the other hand, was much lower than that in *B. subtilis*.

3.3. Cloning and Expression of *S. marcescens chiA* Gene in *B. thuringiensis*

3.3.1. Transformation of *B. thuringiensis* 3023 with pNW33NP7C

The recombinant plasmid pNW33NP7C was transferred into *B. thuringiensis* 3023 cells by protoplast transformation. However, neither PCR nor restriction digestion by using the plasmids obtained from putative recombinant *B. thuringiensis* colonies did not confirm transformation. No sign of expression of the *chiA* gene cloned in pNW33N could be obtained in *B. thuringiensis* 3023. Since the results suggested rearrangement and instability of the recombinant plasmid in *B. thuringiensis* cells we decided to clone *chiA* into another shuttle vector, pHT315 which has been

successfully used in a couple of gene expression studies with *B. thuringiensis* as a host (Grandvalet *et al*., 2001; Juárez-Pérez *et al*., 2002; Fedhila *et al*., 2003).

3.3.2. Subcloning of *chiA* into *E. coli–B. thuringiensis* Shuttle Vector pHT315

In order to be able to insert (*chiA*) gene under the *cry3*Aa11 promoter into the multiple cloning site of pHT315, the gene cloned on pNW33NP7C was amplified by using the primers chiproXF–chiAER and chiAHF–chiABR having the appropriate restriction enzyme sites at their 5' regions (Table 2.3) (Figures 3.10 and 3.11).

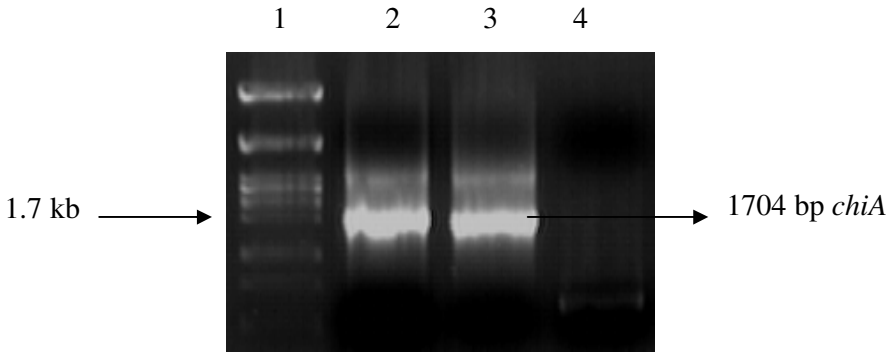


Figure 3.10. Amplification of *chiA* gene by using the primers chiAHF and chiABR. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Positive control (pGEM®-T carrying *chiA* as template), Lane 3: Amplified 1.7 kb chitinase gene, Lane 4: Negative control (no template).

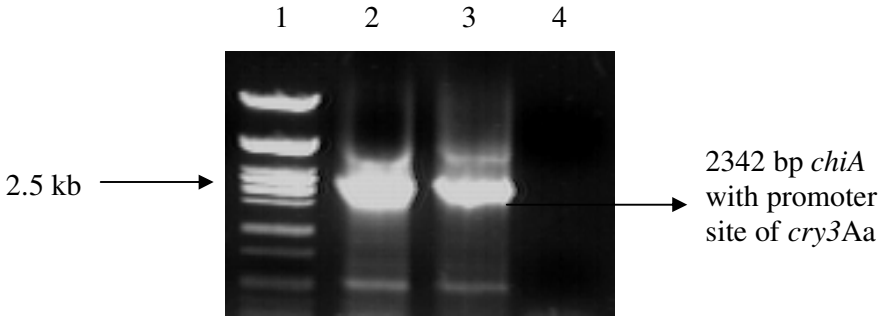


Figure 3.11. Amplification of the *chiA* gene along with promoter region of *cry3*Aa11 gene using the primers chiproXF-chiAER. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Positive control (pNW33NP7C as a template), Lane 3: Amplified *chiA* gene together with *cry*3Aa promoter, Lane 4: Negative control (no template).

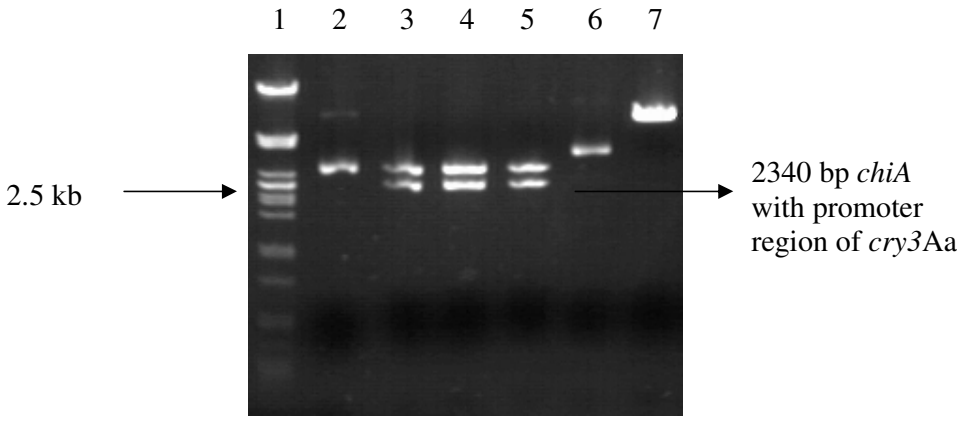


Figure 3.12. Digested and undigested pHT315 and recombinant pGEM®-T vector. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Undigested pGEM®-T carrying *chiA* and the promoter region, Lanes 3-4-5: pGEM®-T carrying *chiA* and *cry*3Aa promoter digested with *Sac*I and *Eco*RI, Lane 6: Undigested pHT315, lane 7: pHT315 digested with *Sac*I and *Eco*RI.

Recombinant pGEM®-T vectors carrying *chiA* with and without promoter were released from pGEM®-T by double digestion with *Bam*HI-*Hin*dIII and S*ac*I-*Eco*RI, respectively and cloned into pHT315 linearized with the same enzymes (Figure 3.12). Ligation products were introduced into *E. coli* DH5α competent cells and the recombinants were selected on LA plates containing ampicillin + X-gal + IPTG. Plasmids were isolated from putative recombinants and cloning was verified by performing restriction enzyme digestion (Figures 3.13 and 3.14). Recombinant pHT315 carrying *chiA* with and without promoter were named as pHT315PC and pHT315C, respectively.

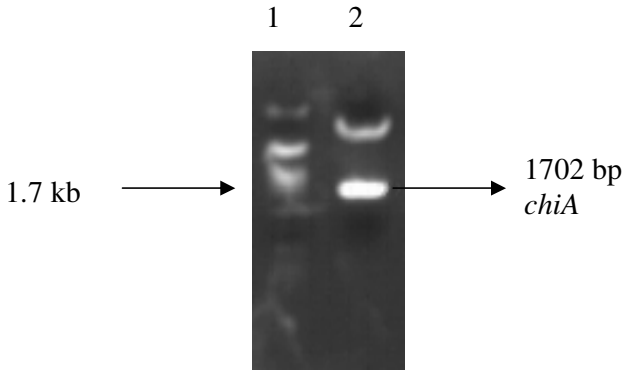


Figure 3.13. Double digestion of pHT315C with *Hin*dIII and *Bam*HI for verification of successful cloning. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Digested pHT315C.

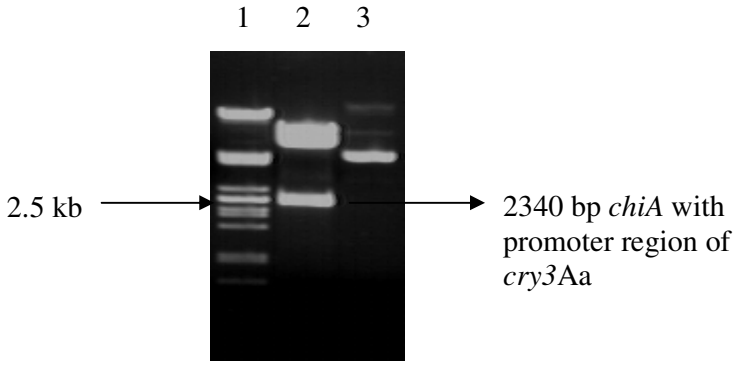


Figure 3.14. Double digestion of pHT315PC for verification of successful cloning. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: pHT315PC digested with *Sac*I and *Eco*RI, Lane 3: Undigested pHT315PC.

3.3.3. Transformation of *B. subtilis* 168 with pHT315C and pHT315PC

The recombinant plasmids pHT315C and pHT315PC were isolated using Qiagen plasmid isolation kit and introduced into *B. subtilis* 168. Recombinant *B. subtilis* cells were selected on Luria agar plates containing erythromycin. Plasmids were isolated from putative recombinants and verification of transformation was performed by PCR and restriction enzyme digestion (Figure 3.15). The chitinase production of *B. subtilis* 168 carrying pHT315PC was determined qualitatively on chitin-containing agar plates

as clear zone around the colony (Figure 3.16b). No zone was observed around the parental strain *B. subtilis* 168 (Figure 3.16a).

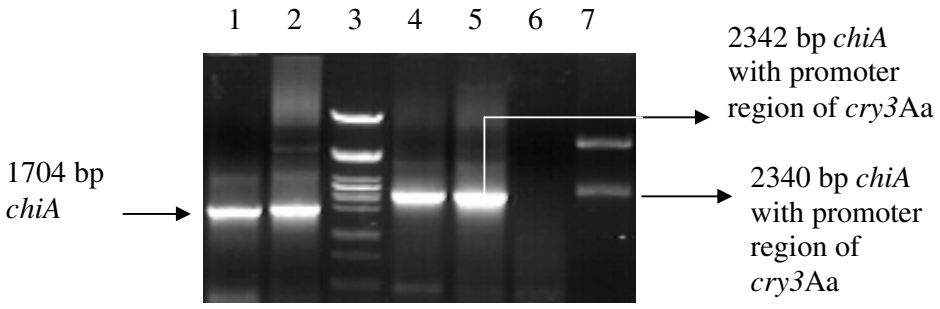
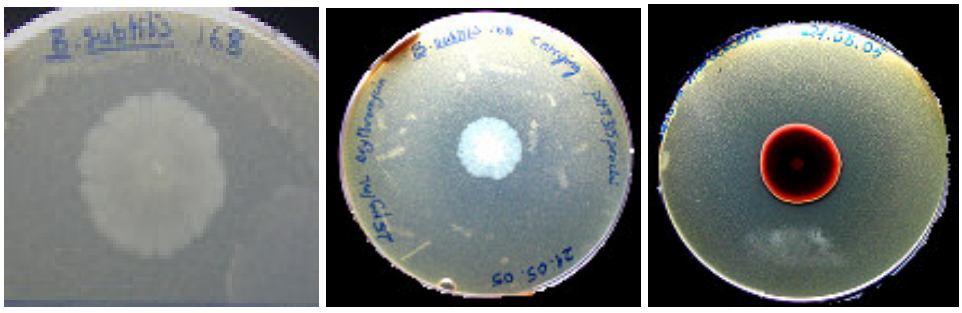


Figure 3.15. Verification of transformation of *B. subtilis* cells with pHT315PC. Lanes 1-2: Amplification of chitinase gene by using pHT315C from *E. coli* and *B. subtilis*, respectively as the template, Lane 3: Marker (*Pst*I digested Lambda DNA), Lanes 4-5: Amplification of chitinase gene together with the promoter of *cry3*Aa11 by using pHT315PC from *E. coli* and *B. subtilis*, respectively as the template, Lane 6: Negative control (no template), Lane 7: Double digestion of pHT315PC, isolated from *B. subtilis*, with *Sac*I and *Eco*RI.



(a) (b) (c)

Figure 3.16. Qualitative determination of chitinase activity of *B. subtilis* 168 carrying pHT315PC on colloidal chitin containing agar plate. (a) *B. subtilis* 168, (b) *B. subtilis* 168 carrying pHT315PC, (c) *S. marcescens* Bn10. The chitin degradation around the bacterial colony is seen as a clear zone.

3.3.4. Transformation of *B. thuringiensis* 3023 with pHT315C and pHT315PC

For expression of *chiA* in an anti-Coleopteran strain, *B. thuringiensis* 3023 serovar *morrissoni* was selected as a Cry3Aa overproducer host on the basis of our earlier findings (Kurt *et al.*, in press). We also showed earlier that it harbours no plasmid

DNA, a property which is useful to prevent any plasmid incompatibility (Kurt, 2005). In order to introduce the recombinant plasmids pHT315C and pHT315PC into *B. thuringiensis* 3023, the plasmids were isolated from recombinant *B. subtilis* 168 cells using Qiagen plasmid isolation kit. pHT315C was introduced into *B. thuringiensis* 3023 by protoplast transformation method whereas electroporation was used for pHT315PC. Recombinants were selected on agar plates containing erythromycin. Plasmids were isolated from putative recombinants and verification was performed by PCR using these plasmids as the templates (Figure 3.17 and Figure 3.18).

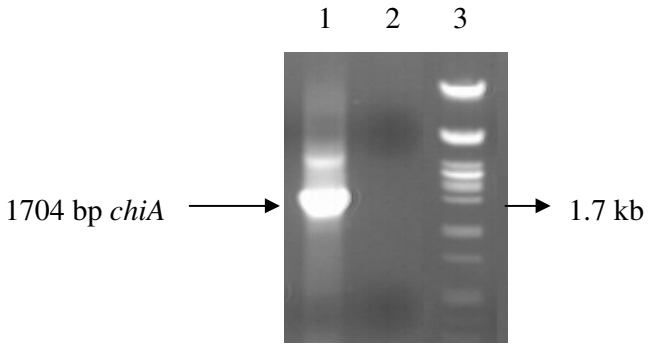
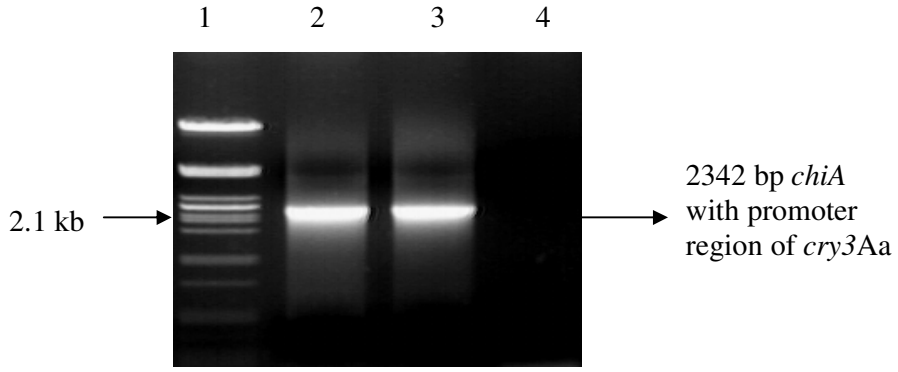


Figure 3.17. Verification via PCR of transformation of *B. thuringiensis* 3023 with pHT315C. Lane 1: PCR product for amplification of *chiA* using pHT315C from *B. thuringiensis* 3023 as template, Lane 2: Negative control (no template), Lane 3: Marker (*Pst*I digested Lambda DNA).



Fgure 3.18. Verification via PCR of transformation of *B. thuringiensis* 3023 with pHT315PC. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: Positive control (pHT315PC from *B. subtilis* 168 as the template), Lane 3: PCR product from amplification of the promotor region of the *cry3*Aa11+*chiA* using pHT315PC from *B. thuringiensis* 3023 as the template, Lane 4: Negative control (no template).

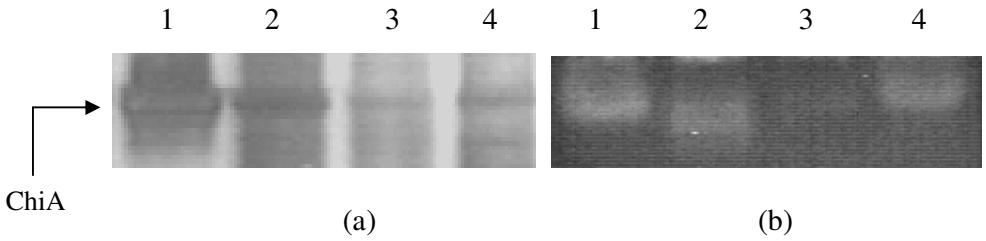


Figure 3.19. Native-PAGE (a) and activity staining (b) analysis of chitinase produced by *B. thuringiensis* 3023 carrying pHT315C or pHT315PC. Lane 1: ChiA produced by *S. marcescens* Bn10, Lane 2: ChiA produced by recombinant *B. thuringiensis* 3023 carrying pHT315PC, Lane 3: ChiA produced by recombinant *B. thuringiensis* 3023 carrying pHT315C, Lane 4: Chitinase produced by parental strain *B. thuringiensis* 3023.

Native-PAGE was first performed for determination of the chitinase production by parental and recombinant cells of *B. thuringiensis* 3023. The result showed that *B. thuringiensis* 3023 carrying pHT315PC produced higher amount of chitinase than *B. thuringiensis* 3023 carrying pHT315C and the parental strain on native gel (Figure 3.19a). Endochitinase activity of the related bands was confirmed by activity staining on polyacrylamide gel (Figure 3.19b).

Chitinase activity of both parental and recombinant strains of *E. coli*, *B. subtilis* and *B. thuringiensis* were also determined quantitatively by fluorometric measurement. The results are documented in Figure 3.20 and Table 3.2. It appeared that *lac*Z promoter found in the vector did not work in *B. subtilis* and *B. thuringiensis* since no expression was observed without *cry*3Aa11 promoter in them. Relative activity of chitinase was 31.7% in *B. thuringiensis* 3023 recombinants transformed with pHT315PC having *chiA* gene under the *cry3*Aa11 promoter.

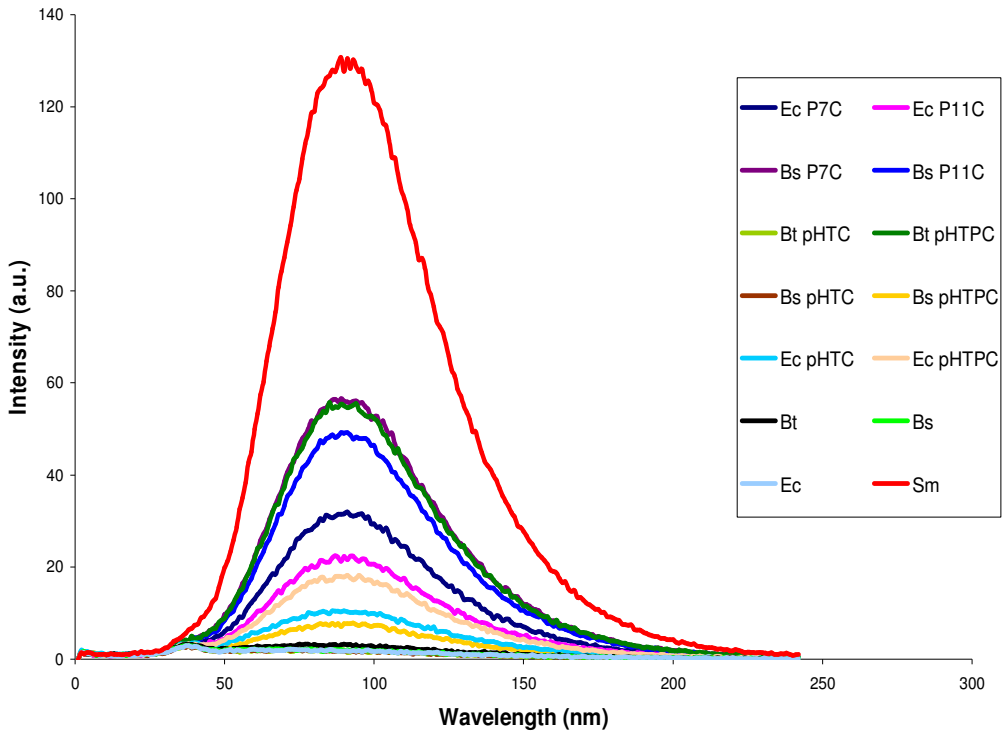


Figure 3.20. Fluorometric measurement of endochitinase activities of the parental and recombinant strains. Ec P7C: *E. coli* carrying pNW33NP7C, Ec P11C: *E. coli* carrying pNW33NP11C, Bs P7C: *B. subtilis* carrying pNW33NP7C, Bs P11C: *B. subtilis* carrying pNW33NP11C, Bt pHTC: *B. thuringiensis* carrying pHT315C, Bt pHTPC: *B. thuringiensis* carrying pHT315PC, Bs pHTC: *B. subtilis* carrying pHT315C, Bs pHTPC: *B. subtilis* carrying pHTPC, Ec pHTC: *E. coli* carrying pHT315C, Ec pHTPC: *E. coli* carrying pHT315PC, Bt: *B. thuringiensis*, Bs: *B. subtilis*, Ec: *E. coli*, Sm: *S. marcescens* Bn10.

The data showed that the pHT315 vector works much better in *B. thuringiensis* as compared to pNW33N which ensured a beter expression in *B. subtilis* (Table 3.1 and Figure 3.20). pNW33N was derived from a *Staphylococcus aureus* plasmid, namely pC194, and an *E. coli* plasmid, pUC19. The vector also contains the origin of replication of *Geobacillus stearothermophilus* plasmid pTHT15 (Mee and Welker, 2003). pHT315 was constructed by three-way ligation of *erm* gene, pUC19 and *ori* from *B. thuringiensis* plasmid pHT1030 (Arantes and Lereclus, 1991). Being more stable in the presence of a *B. thuringiensis ori*, pHT315 worked more efficiently than pNW33N.

Table 3.2. Endochitinase activities of the parental and recombinant strains carrying pHT315C or pHT315PC.

Vector	Cloned fragment	Strain	Specific activity (U/min/mg)	Relative activity (%)
-	-	*S. marcescens* Bn10	15905	100
-	-	*B. thuringiensis* 3023	797	5.0
pHT315C	*chiA*	*B. thuringiensis* 3023	297	1.8
pHT315PC	*chiA*+ *cry3*Aa11 promoter	*B. thuringiensis* 3023	5056	31.7
-	-	*B. subtilis* 168	281	1.7
pHT315C	*chiA*	*B. subtilis* 168	273	1.7
pHT315PC	*chiA*+ *cry3*Aa11 promoter	*B. subtilis* 168	788	4.9
-	-	*E. coli* DH5α	265	1.6
pHT315C	*chiA*	*E. coli* DH5α	824	5.1
pHT315PC	*chiA*+ *cry3*Aa11 promoter	*E. coli* DH5α	1280	8.0

3.4. Characterization of Chitinase A Gene (*chiA*) of *S. marcescens* Bn10

The nucleotide sequence of *chiA* gene of the local isolate *S. marcescens* Bn10 was determined and compared with the known chitinase gene sequences in BLAST. The gene starts with ATG codon and terminates at position 1690 with a TAA stop codon (Figure 3.21). Figure 3.21 shows the secondary structure elements in the crystal structure of *S. marcescens* ChiA and completely conserved regions in chitinase enzymes from different organisms (Suginta *et al.*, 2004). *N*-terminal domain, hinge domain, catalitic region and small alpha and beta domains were marked on predicted amino acid sequence of *S. marcescens* Bn10 *chiA* according to Suginta *et al.* (2004). Computer-aided analysis (DNASTAR, EditSeq) indicated that *chiA* gene of *S. marcescens* Bn10 product is a 563 residue protein with a calculated molecular mass of 60.9 kDa. The mean G+C content of the gene is 58.75%. The sequence was submitted to GeneBank (Accession number DQ165083).

Nucleotide sequence of the *chiA* gene of *S. marcescens* Bn10 was compared with 12 different chitinase gene sequences belonging to 3 different species by the help of MegAlign (DNASTAR) program. Its identity to these sequences was found to be 98.6%, 97.1%, 97.0% 96.9%, 96.4%, 96.2%, 95.7%, 95.1% and 93.3% to *chiA* gene of *S. marcescens* BJL200 (GeneBank Accession number Z36294), chitinase (Chi60) gene of *Burkholderia cepacia* (GeneBank Accession number AY040610), *S. marcescens chiA* gene (GeneBank Accession numbers AB015996), *S. marcescens* endochitinase gene (GeneBank Accession number AF454462), *S. marcescens* strain NIMA endochitinase gene (GeneBank Accession number AY566865) and strain ATCC 990 chitinase gene (GeneBank Accession number AY855211), *Enterobacter* sp. NRG-4 *chiA* gene (GeneBank Accession number DQ013365), *Enterobacter* sp *chiA* gene (GeneBank Accession number U35121), *S. marcescens* 56 kDa *chiA* gene (GeneBank Accession number L01455), *S. marcescens chiA* gene (GeneBank Accession number AY433954), *S. marcescens chiA* gene (GeneBank Accession number X03657), *S. marcescens chiA1* gene (GeneBank Accession number AF085718), respectively.

The deduced amino acid sequence of chitinase A from *S. marcescens* Bn10 was compared with other bacterial chitinase sequences. The alignment of our ChiA sequence with the sequences of these Chi proteins is shown in Figure 3.22. Amino acid sequence of ChiA of the local isolate showed 99.3%, 99.1%, 98.9%, 98.6%, 98.2%, 94.8%, 93.2% and 91.5% identity to *chiA* gene of *S. marcescens* BJL200 (GeneBank Accession number Z36294), chitinase (Chi60) gene of *Burkholderia cepacia* (GeneBank Accession number AY040610), *S. marcescens chiA* gene (GeneBank Accession numbers AB015996 and AF454462), *S. marcescens chiA* gene (GeneBank Accession number AY433954), *Enterobacter* sp. NRG-4 *chiA* gene (GeneBank Accession number DQ013365), *Enterobacter* sp *chiA* gene (GeneBank Accession number U35121), *S. marcescens* strain NIMA endochitinase gene (GeneBank Accession number AY566865) and strain ATCC 990 chitinase gene (GeneBank Accession number AY855211), *S. marcescens* 56 kDa *chiA* gene (GeneBank Accession number L01455), *S. marcescens chiA* gene (GeneBank

Accession number X03657), and *S. marcescens chiA1* gene (GeneBank Accession number AF085718), respectively (Table 3.3 and Figure 3.22).

ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG
1 M R K F N K P L L A L L I G S T L C S A

GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC
2 A Q A A A P G K P T I A W G N T K F A I

***N*-terminal domain**

GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC
3 V E V D Q A A T A Y N N L V K V K N A A

GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA
4 D V S V S W N L W N G D **T** G T T A K **V** L

TTAAATGGCAAAGAGGCGTGGAGCGGCCCTTCAACCGGTTCTTCCGGTACGGCGAATTTT
5 L N G K E A W S G P S T G S S G T A N F

AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC
6 K V N K G G R Y Q M Q V A L C N A D G C

AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG
7 **S** A S D A T E I V V A D T D G S H L A P

TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTG
8 L K E P L L E K N K P Y K Q N S G K V V

Hinge region

GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG
9 G S Y F V E W G V Y G R N F T V D K I P

Catalitic domain

GCGCAGAACTTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC
10 A Q N L T H L L Y G F I P I C G G N G I

AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC
11 N D S L K E I E G S F Q A L Q R S C Q G

CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG
12 R E D F K V S I H D P F A A L Q K A Q K

GGCGTTACCGCCTGGGATGACTCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAA
13 G V T A W D D **S** Y K G N F G Q L M A L K

CAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG
14 Q A H P D L K I L P S I G G W T L S D P

TTCTTCTTCATGGGTGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC
15 F F F M G D K V K R D R F V G S V K E F

CTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA
16 L Q T W K F F D G V D I D W **E** F P G G K

GGCGCCAACCCGAACCTGGGCAGCCCGCAGGACGGGGAAACCTATGTGCTGCTGATGAAG
17 G A N P N L G S P Q D G E T Y V L L M K

GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAATATGAACTGACC
18 E L R A M L D Q L S A E T G R K Y E L T

TCCGCCATCAGCGCCGGCAAGGACAAGATCGACAAGGTGGCTTACAACGTCGCGCAGAAC
19 S A I S A G K D K I D K V A Y N V A Q N

TCGATGGATCACATTTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC
20 S M D H I F L M S Y **D** F Y G A F D L K N

CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG
21 L G H Q T A L N A P A W K P D T A Y T T

GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAAATCGTCGTCGGC
22 V N G V N A L L A Q G V K P G K I V V G

ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC
23 T A M Y G R G W T G V N G Y Q N N I P F

ACCGGTACCGCCACCGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC
24 T G T A T G P V K G T W E N G I V D Y R

Small α+β domain

CAAATCGCTGGCCAGTTCATGAGCGGCGAGTGGCAGTACACCTACGACGCCACGGCAGAA
25 Q I A **G** Q F M S G E W Q Y T Y D A T A E

GCGCCATACGTGTTCAAGCCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCG
26 A P Y V F K P S T G D L I T F D D A R S

GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCTTGGGAG
27 V Q A K G K Y V L D K Q L G G L F S W E

Catalitic domain

GTCGACGCGGACAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC
28 **V** D A D N G D I L N S M N A S L G N S A

GGCGTTCAATAA
29 G V Q *

Figure 3.21. Nucleotide sequence analysis of *chiA* gene of *S. marcescens* Bn10 and predicted amino acid translation. The shifted amino acids are written in bold and black. 6 amino acids that shifted from the consensus residues were Thr^{73}, Val^{79}, Ser^{121}, Ser^{248}, Gly^{484} and Val^{541}. Ser^{248} and Val^{541} are unique to ChiA of *S. marcescens* Bn10 and both belonged to catalytic domain of the protein. The catalytic residue amino acids are written in green.

The chitinase precursor has a typical *N*-terminal secretion signal peptide of 18 residues rich in leucine (27.7%). *N*-terminal signal peptides are cleaved off during secretion of the proteins to the culture medium. They are signatures of *sec*-dependent protein export to the periplasm. The signal peptide is cleaved off by a periplasmic signal peptidase when exported protein reaches the periplasm (Brurberg *et al*., 2000). The secondary structure of the *S. marcescens* Bn10 ChiA was also shown to locate the

positions of an *N*-terminal chitin binding domain connected with a small hinge region which enables a flexibility to the protein while catalytic action and an extra α+β domain that has a role in substrate binding (Suginta *et al.*, 2004). α+β domain makes up one of the walls of the substrate-binding groove in ChiA (Brurberg *et al.*, 2000). The ChiA of *S. marcescens* Bn10 contains the catalytic domain 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 Glu^{315}, acting as a proton donor, and Asp^{391} (Suginta *et al.*, 2004). When the amino acid sequence of *S. marcescens* Bn10 was aligned with the other chitinase sequences, it was found that 6 amino acids were shifted from the consensus residues, Ala^{73} to Thr^{73}, Ile^{79} to Val^{79}, Thr^{121} to Ser^{121}, Pro^{248} to Ser^{248}, Ser^{484} to Gly^{484} and Ile^{541} to Val^{541}. Among these amino acids Pro^{248} and Ile^{541} are conserved in other bacterial chitinases as indicated in Suginta *et al.* (2004). In the present study, Ser^{248} and Val^{541} were found to be unique to ChiA of *S. marcescens* Bn10, both belonging to the catalytic domain of the protein. The other amino acid shifts were found to be located in chitin binding domains of chitinase A. It is often assumed that enzymes cleaving the 4-methylumbelliferyl group from 4-methylumbelliferyl-$(GlcNAc)_3$ have an endo-character. The more open character of the substrate-binding groove in ChiA strongly suggests that the enzyme has an endo-activity (Brurberg *et al.*, 2000).

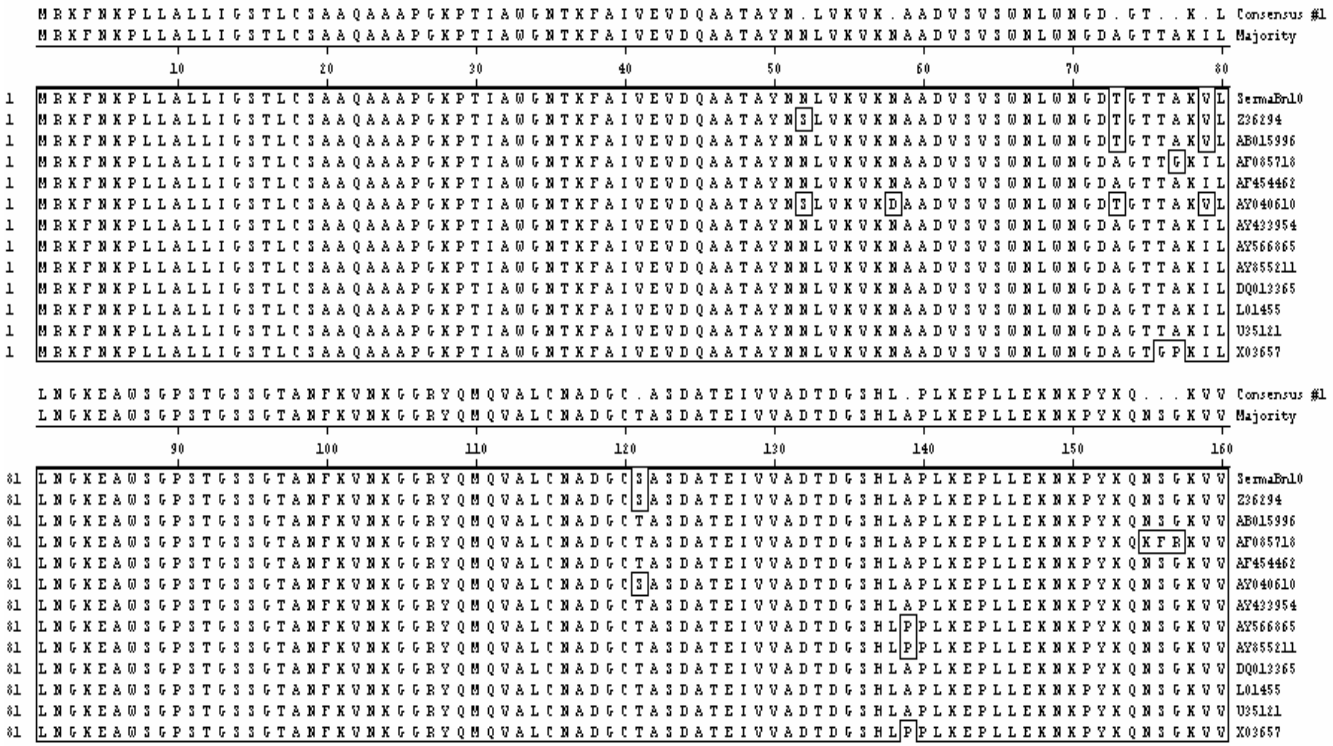


Figure 3.22. Alignment of amino acid translations of known chitinase genes. The amino acids different than the consensus ones are shown in boxes. The names of the strains corresponding to accession numbers are indicated in the text.

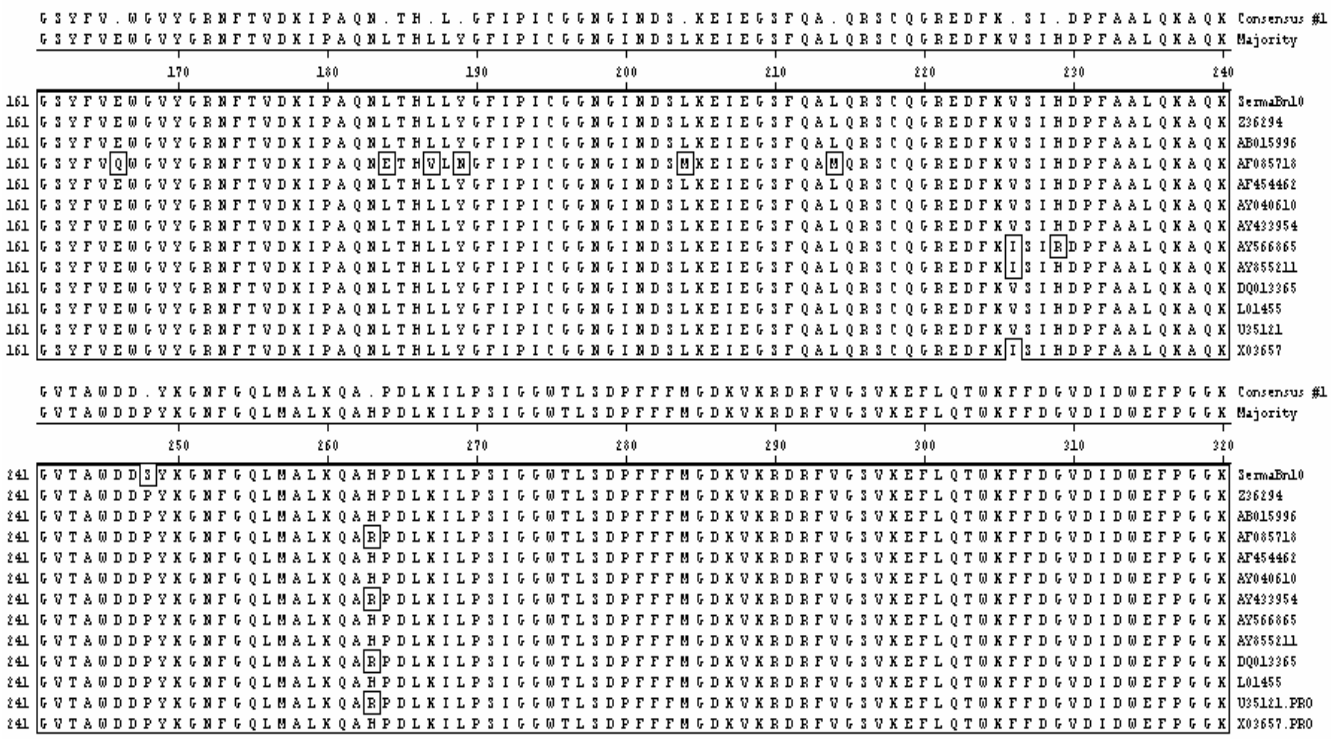


Figure 3.22. (continued)

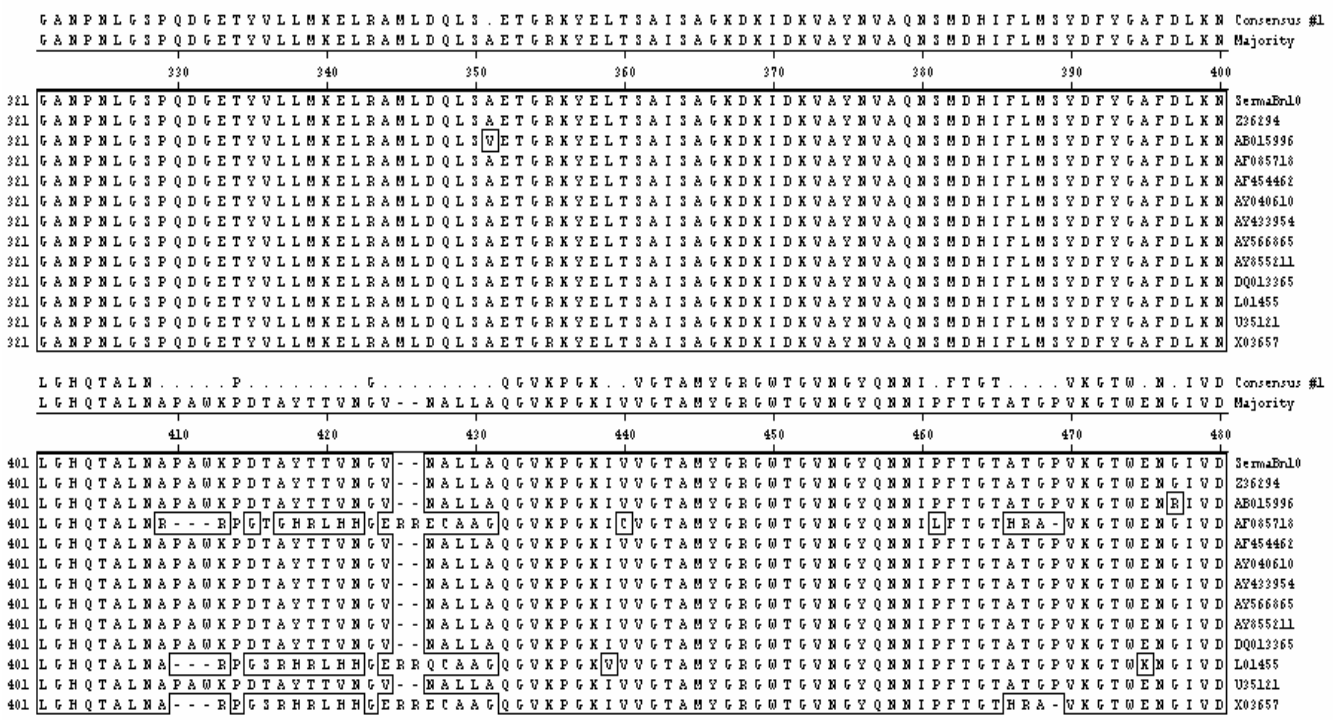


Figure 3.22. (continued)

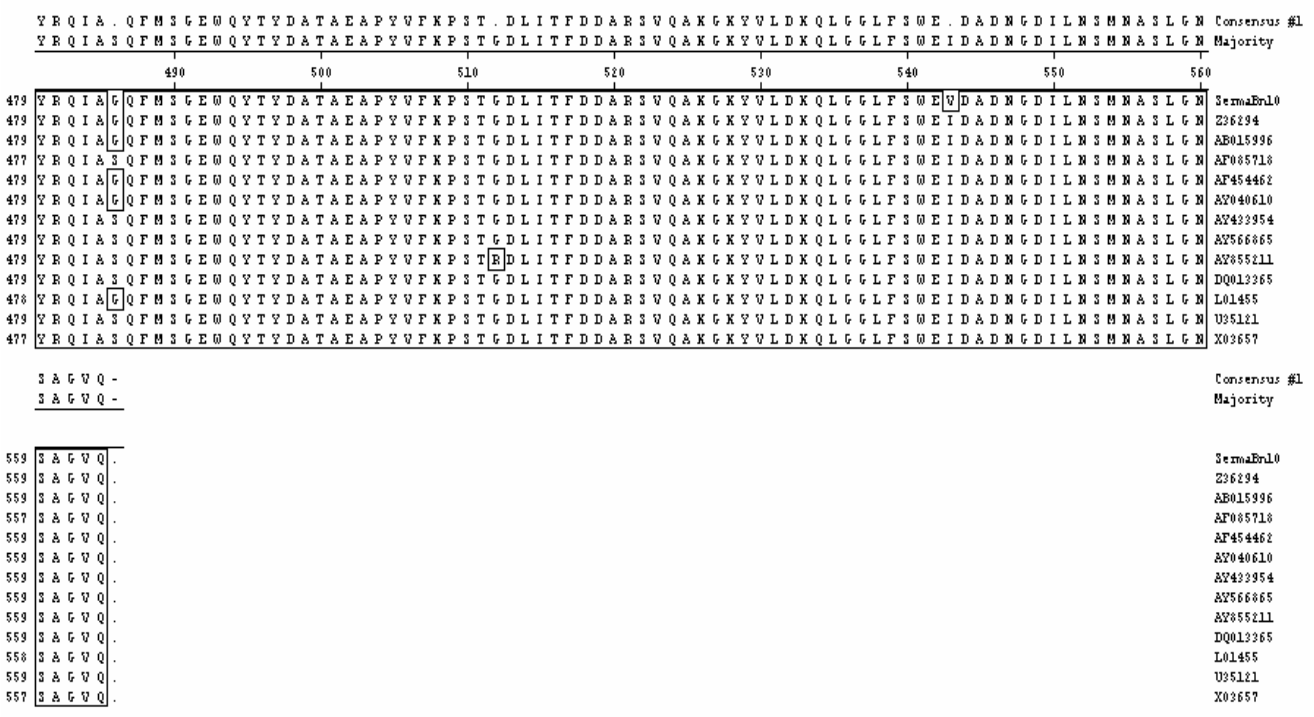


Figure 3.22. (continued)

Table 3.3. Percentage similarity and divergence of chitinase sequences aligned. SermaBn10: *S. marcescens* Bn10, Z36294: *S. marcescens* (BJL200) *chiA* gene for chitinase, AB015996: *S. marcescens* gene for chitinase A precursor, AF085718: *S. marcescens* chitinase (*chiA1*) gene, AF454462: *S. marcescens* endo-chitinase (*chiA*) gene, AY040610: *Burkholderia cepacia* chitinase (Chi60) gene, AY433954: *S. marcescens* chitinase A precursor, AY566865: *S. marcescens* strain NIMA endochitinase gene, AY855211: *S. marcescens* strain ATCC 990 chitinase (*chiA*) gene, DQ013365: *Enterobacter* sp. NRG-4 chitinase (*chiA*) gene, L01455: *S. marcescens* 58 kD chitinase (ChiA) gene, U35121: *Enterobacter* sp. chitinase (*chiA*) gene, X03657: *S. marcescens* gene *chiA* for chitinase A.

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13		
1		99.3	98.9	91.5	98.9	99.1	98.6	98.2	98.2	98.6	94.8	98.6	93.2	1	SermaBn10
2	0.5		99.1	91.6	99.1	99.6	98.8	98.4	98.4	98.8	95.0	98.8	93.4	2	Z36294
3	0.9	0.7		91.6	99.1	98.9	98.8	98.4	98.4	98.8	95.0	98.8	93.4	3	AB015996
4	6.9	6.7	6.7		92.3	91.5	92.7	92.0	92.0	92.7	95.2	92.7	96.4	4	AF085718
5	0.9	0.7	0.7	6.0		98.9	99.5	99.1	99.1	99.5	95.7	99.5	94.1	5	AF454462
6	0.7	0.2	0.9	6.9	0.9		98.6	98.2	98.2	98.6	94.8	98.6	93.2	6	AY040610
7	1.3	1.1	1.1	5.6	0.4	1.3		99.1	99.1	99.8	95.4	99.8	94.1	7	AY433954
8	1.6	1.4	1.4	6.4	0.7	1.6	0.7		99.5	99.1	95.0	99.1	94.5	8	AY566865
9	1.6	1.4	1.4	6.4	0.7	1.6	0.7	0.4		99.1	95.0	99.1	94.5	9	AY855211
10	1.3	1.1	1.1	5.6	0.4	1.3	0.0	0.7	0.7		95.4	99.8	94.1	10	DQ013365
11	4.0	3.9	3.9	4.2	3.1	4.0	3.5	3.9	3.9	3.5		95.4	97.3	11	L01455
12	1.3	1.1	1.1	5.6	0.4	1.3	0.0	0.7	0.7	0.0	3.5		94.1	12	U35121
13	5.2	5.0	5.0	3.5	4.2	5.2	4.2	3.9	3.9	4.2	2.0	4.2		13	X03657
	1	2	3	4	5	6	7	8	9	10	11	12	13		

3.5. Chitinase Production by *S. marcescens* Bn10 Under Different Cultural Conditions

As mentioned earlier, *S. marcescens* Bn10 is a local isolate having a high capacity in terms of chitinase production. Amino acid differences in the catalytic domain of ChiA from *S. marcescens* Bn10 might ensure the high chitinase activity with respect to other strains of *S. marcescens*. Specific activity of chitinase from *S. marcescens* Bn10 was found to be 15905 U/min/mg when 23.3 μg/mL 4-MU-β-$(GlcNAc)_3$ was used as substrate (Table 3.2). Nawani and Kapadnis (2001) found a specific activity of Sephadex G-100 filtrate of chitinase from *S. marcescens* 2170 as 1070 U/min/mg when the substrate was 1% swollen chitin. In the study of Fuchs *et al*. (1986) the specific activity of purified chitinase from *S. marcescens* QMB1466 was reported as

4100 U/min/mg on radioactive chitin. Brurberg *et al*. (1994) expressed the *chiA* gene from *S. marcescens* BJL200 in *E. coli* and the specific activity was found to be 2400 U/min/mg using 4-MU-$(GlcNAc)_2$ as the substrate. Since much higher activity was obtained with chitinase from *S. marcescens* Bn10, as another part of this thesis, enzyme studies for characterization and its production by the local isolate were undertaken.

3.5.1. Effects of Colloidal Chitin Concentration and the Presence of *N*-Acetylglucosamine (GlcNAc) on Chitinase Production

Production of chitinolytic enzymes in bacteria is normally induced by the presence of chitin in the culture medium. Since chitin is insoluble, the microorganisms are unable to utilize it unless it has been hydrolyzed to soluble oligomers of *N*-acetylglucosamine. It has been shown that production of chitinolytic enzymes could be induced by adding only $(GlcNAc)_2$ or $(GlcNAc)_{3-4}$ to the growth medium of *S. marcescens* (Brurberg *et al*., 2000).

In order to determine the effect of colloidal chitin concentration on chitinase production, we prepared a series of growth media containing 0.5 g/L, 1 g/L, 2g/L, 4 g/L, 8 g/L colloidal chitin, respectively. The bacteria were also grown in a chitin-free medium as the control. For the effect of GlcNAc on the production of chitinase, *S. marcescens* Bn10 was grown in a medium containing 2.1 g/L GlcNAc. Cultures were centrifuged and supernatants were used as the chitinase sources. Proteins contained in supernatants were run on SDS-polyacrylamide gels (Figure 3.23). Although a slightly thicker band was obtained in 0.5 g/L chitin containing medium, the effect of varying chitin concentration on chitinase production was not so remarkable. Results of our experiments showed that inclusion of GlcNAc in place of colloidal chitin also stimulated chitinase production (Figure 3.23).

Our results were similar to the results obtained by the Wen *et al*. (2002) They found that the induction level of chitinase production by *Bacillus* NCTU2 was somewhat insensitive to changes in physical and chemical conditions. For example, glucose (5

mM), GlcNAc (5 mM) or tryptone (2%) did not change chitinase production by *Bacillus* NCTU2. Although the presence of colloidal chitin was essential, chitinase induction was not affected when chitin was added at concentrations higher than 0.5%. The other studies suggested that the production of chitinase by *S. marcescens* growing on chitin is inhibited by the addition of glucose and GlcNAc. Glucose effect has also been demonstrated with other chitinase-producing bacteria, indicating that the catabolic repression is generally involved in regulating the chitinolytic system of bacteria (Brurberg *et al*.,2000). Watanabe *et al.* (1997) showed that no chitinase activity was detected when *S. marcescens* 2170 was grown in medium containing either glucose or GlcNAc. Consequencely, regulation of the chitin production is not similar in all organisms. Chitinase production by *S. marcescens* Bn10 did depend neither on the concentration or the presence of colloidal chitin nor the presence of GlcNAc. Thus, it seemed that the basal expression level of *chiA* gene is quite high in *S. marcescens* Bn10 which is slightly induced in the presence of chitin or GlcNAc.

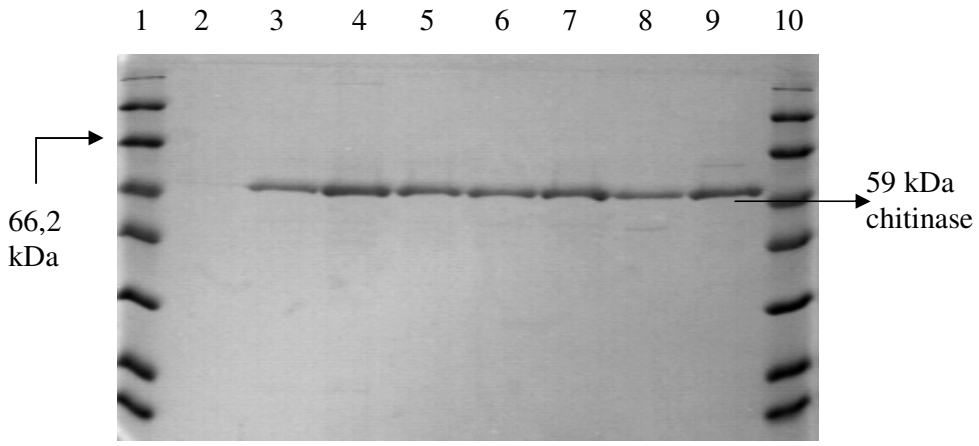


Figure 3.23. Effect of colloidal chitin concentration and the presence of GlcNAc on chitinase production. Lanes 1, 10: Protein molecular weight marker (Appendix A), Lane 2: *S.marcescens* chitinase purchased from Sigma; Total extracellular proteins of *S. marcescens* Bn10 grown in Lane 3: Chitin-free medium, Lane 4: 0.5 g/L, Lane 5: 1 g/L, Lane 6: 2g/L, Lane 7: 4 g/L, Lane 8: 8 g/L colloidal chitin, Lane 9: 2.1 g/L GlcNAc containing media.

3.5.2. Effects of Temperature and pH on Chitinase Production

To determine the effect of temperature on chitinase production, parallel cultures of *S. marcescens* Bn10 was grown at 25 ^{o}C, 30 ^{o}C and 37 ^{o}C for 16 h. For the determination of the effect of pH on chitinase production, cells were grown in a series of media with 5 different pH values of 5.5, 6.5, 7.5, 8.5 and 10, respectively at 30 ^{o}C. Culture supernatants were run on SDS-polyacrylamide gels. The results are presented in Figure 3.24.

The optimum temperature for the production of chitinase by *S. marcescens* Bn10 was found to be 30 ^{o}C and enzyme synthesis decreased at 37 ^{o}C. Optimum pH for chitinase production was shown to be 7.5. Production was fairly good at pH 5.5, 6.5 and 8.5, but no chitinase production was observed at pH 10. Similarly, Green *et al.* (2005) reported that the optimum conditions for chitinase production by *S. marcescens* QMB1466 were pH 7.0 and 32.5 ^{o}C. Wen *et al.* (2002) observed no significant effect of pH and incubation temperature on chitinase production by *Bacillus* NCTU2 in a range of pH 5.5–7.5 and 28–40 ^{o}C, respectively.

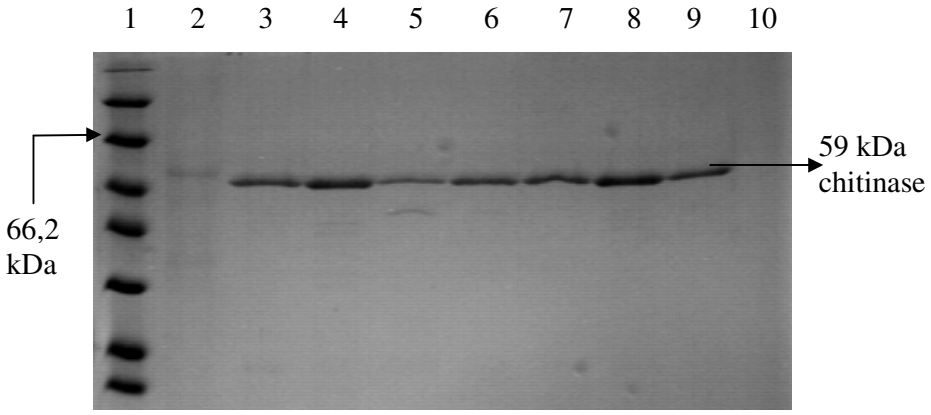


Figure 3.24. Effect of incubation temperature and culture pH on chitinase production. Lane 1: Protein molecular weight markers, Lane 2: *S. marcescens* chitinase purchased from Sigma; Total extracellular proteins of *S. marcescens* Bn10 grown at Lane 3: 25 ^{o}C, Lane 4: 30 ^{o}C, Lane 5: 37 ^{o}C, Lane 6: pH 5.5, Lane 7: pH6.5, Lane 8: pH 7.5, Lane 9: pH 8.5, Lane 10: pH 10.

3.6. Effects of Different Parameters on the Activity of *S. marcescens* Bn10 Chitinase

3.6.1. Effect of Temperature

In order to determine the effect of temperature on the activity of chitinase enzyme produced by *S. marcescens* Bn10, the enzyme was incubated at six different temperatures, 15 ^{o}C, 25 ^{o}C, 30 ^{o}C, 45 ^{o}C, 50 ^{o}C and 65 ^{o}C for 20 min. The specific enzyme activities at different temperatures are presented in Figure 3.25. Interestingly, the highest enzyme activity was obtained at 45 ^{o}C. The enzyme also showed a remarkable activity retaining as much as 91% of the activity at 65 ^{o}C, a quite high temperature for an enzyme from a mesophilic source.

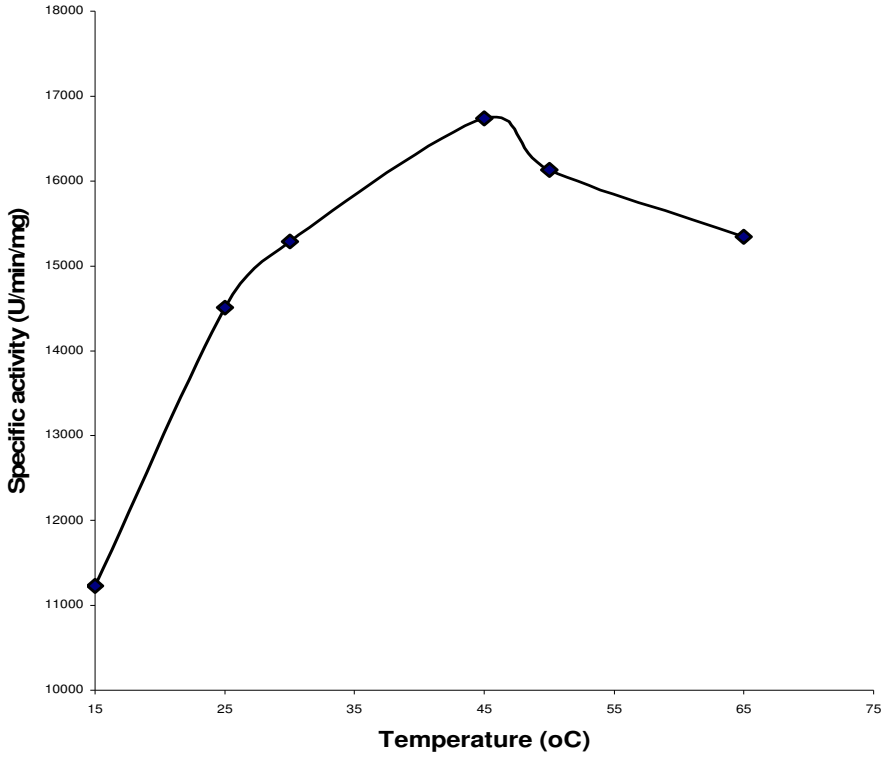


Figure 3.25. Effect of temperature on *S. marcescens* Bn10 chitinase activity.

The effect of temperature on the activity of chitinases from different *S. marcescens* strains have been studied by other researchers. Brurberg *et al*. (1996) reported that the

optimum temperature for the activity of chitinase A from *S. marcescens* is between 50–60 ^{o}C. Gal *et al*. (1998) observed the highest activity of *Serratia marcescens* KCTC2172 chitinase at 45 ^{o}C. The optimum temperature for the activity of chitinase produced by *S. marcescens* NK1 was 47 ^{o}C (Nawani and Kapadnis, 2001). In the present study, the optimum temperature for the activity of chitinase A (ChiA) of *S. marcescens* Bn10 was also found to be 45 °C, being similar to those obtained from other strains of *S. marcescens*.

3.6.2. Effect of pH

In order to investigate the effect of pH on the activity of chitinase produced by *S. marcescens* Bn10, the supernatant of 16h bacterial culture was assayed in the phosphate buffer at a pH range of 3.0 to 11.0. It was found that the chitinase enzyme produced by *S. marcescens* Bn10 was active in the pH range of 4.0 to 9.0. Specific activity of the enzyme was around 18000 unit/min/mg which did not change remarkably within this range. A slightly higher activity was observed at pH 6.0 (Figure 3.26). Gomes *et al*. (2001) found that *Streptomyces* RC1071 endochitinase was stable between the pH 4.0–9.0. Brurberg and his colaques (1996) reported that ChiA of *S. marcescens* has a broad pH optimum around pH 5.0 – 6.0. Nawani and Kapadnis (2001) observed that chitinase of *S. marcescens* NK1 had a pH optimum of 6.2 and was stable in a wide pH range of 3.0 to 10.0. Gal *et al*. (1998) found that *S. marcescens* KCTC2172 chitinase had the optimal reaction pH of 5.5. Taken together, it appears that chitinases from different sources are active within a broad pH range.

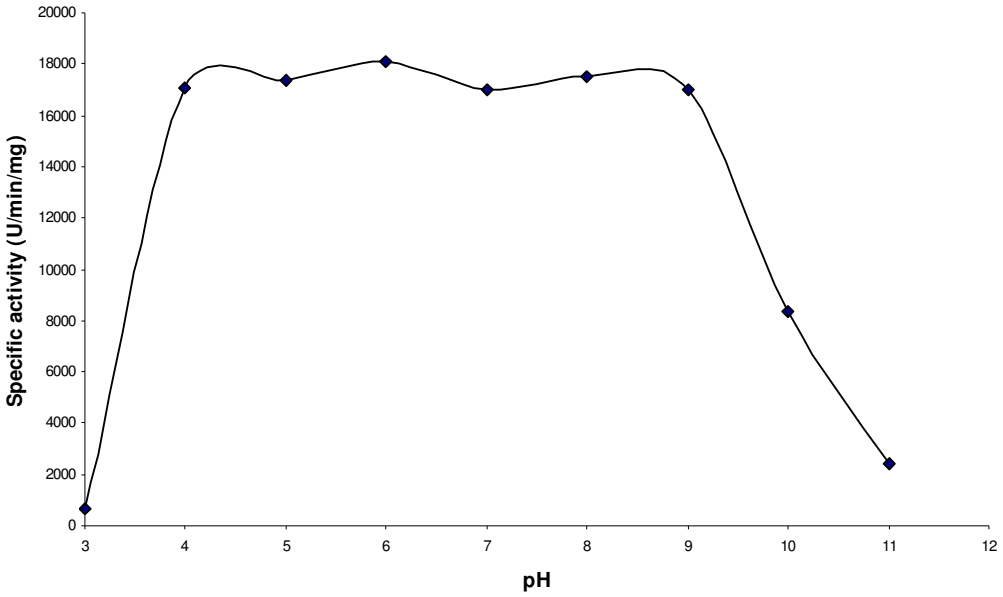


Figure 3.26. Effect of pH on *S. marcescens* Bn10 chitinase activity.

3.6.3. Effect of Substrate Concentration

Six different substrate (4-MU-β-$(GlcNAc)_3$) concentrations of 10 µg/mL, 25 µg/mL, 35 µg/mL, 55 µg/mL, 65 µg/mL and 75 µg/mL were used to investigate substrate concentration effect on the activity of chitinase produced by *S. marcescens* Bn10. The change in the activity of chitinase as a function of substrate concentration is shown in Figure 3.27.

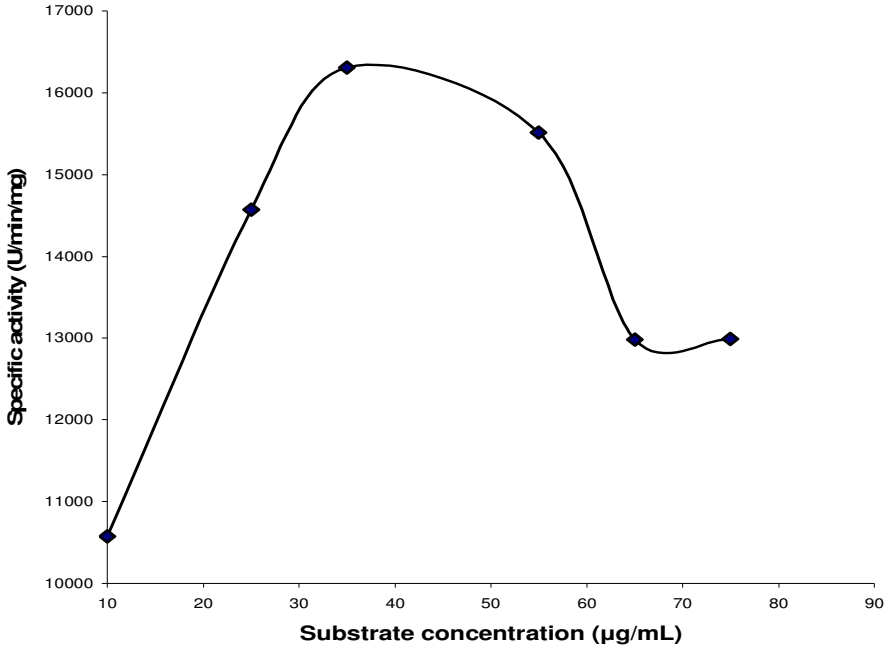


Figure 3.27. Effect of substrate concentration on *S. marcescens* Bn10 chitinase activity.

A sharp increase in the activity of chitinase was observed between the substrate concentrations of 10 to 35 µg/mL 4-MU-β-$(GlcNAc)_3$. Increasing the concentration of substrate above 35 µg/mL, decreased the activity. The extent of inhibiton was about 5% at 55 µg/mL and 20% both at 65 µg/mL and 75 µg/mL 4-MU-β-$(GlcNAc)_3$. There are many documents reporting the inhibition of chitinase activity by its substrate. Brurberg *et al*. (1996) showed the presence of substrate inhibition for ChiA of *S. marcescens* and Fukamizo *et al*. (2001) also reported substrate inhibition of chitinase from *S. marcescens* at a concentration of 40 µg/mL 4-MU-β-$(GlcNAc)_3$. Hoell *et al*. (2005) reported that substrate concentrations higher than 300 µM 4-MU-$(GlcNAc)_2$ inhibited the activity of chitinase produced by *Trichoderma atroviride* strain P1. Conclusively, substrate inhibition appears to be quite common for family 18 chitinases.

3.6.4. Effects of Metal Ions and EDTA

In order to reveal the effects of metal ions on the activity of chitinase produced by *S. marcescens* Bn10, the enzyme activity was determined in the presence of $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ and EDTA each at 10 mM final concentration. The data are tabulated in Table 3.4. The enzyme activity was generally increased in the the presence of metal ions although the positive effect exerted by the metals was not much profound. Co^{2+} and Ca^{2+} increased the activity of *S. marcescens* Bn10 chitinase by 20% and 17%, respectively. A much higher activation by the same concentration of Ca^{+2}, approximately 100%, was observed for the chitinase from *Bacillus* NCTU2 (Wen *et al*., 2002). They also showed that 10 mM Cu^{2+} resulted in a 95% loss in the enzyme activity. However, as opposed to this result, Cu^{+2} increased the activity of *S. marcescens* Bn10 chitinase by 15%. Similar to our results, the presence of other ions, such as Mn^{2+} and Mg^{2+} had no significant effect on the activity of chitinase from *Bacillus* NCTU2. *S. marcescens* Bn10 chitinase was inhibited only by the presence of 10 mM EDTA (4%, data not shown). The inhibitory effect of EDTA was also reported for other chitinases. Souza *et al*. (2003) showed that the activity of endochitinase produced by *Colletotrichum gloeosporioides* was reduced by 35% when incubated with EDTA, a much higher inhibition when compared to our result of 4% obtained for *S. marcescens* Bn10. On the other hand, Gomes *et al*. (2001) reported that 10 mM EDTA reduced the endochitinase activity of *Streptomyces* RC1071 by 5%. Pineapple stem chitinase was not affected by EDTA and metal ions Cu^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+} (Hung *et al*., 2002). There was no significant effect of Fe^{+2} ions on the activity of *S. marcescens* Bn10 chitinase. However, Gomes and his collegues (2001) showed that 10 mM Fe^{2+} exerted 59% inhibition on the chitinase activity of *Streptomyces* RC1071. As can be seen from these literature reports and the results of the present study, the effects of metal ions and EDTA on the activity of chitinases from different sources vary considerably.

Table 3.4. Effects of metal ions on the activity of chitinase produced by *S. marcescens* Bn10.

Metal Ion (10 mM)	Specific activity (U/min/mg)	Relative activity (%)	Metal Ion (10 mM)	Specific activity (U/min/mg)	Relative activity (%)
Ca^{2+}	16826	117	Mg^{2+}	15779	109
Co^{2+}	17220	120	Mn^{2+}	14312	99,5
Cu^{2+}	16603	115	Zn^{2+}	15165	105
Fe^{2+}	15326	106	None (control)	14371	100

CHAPTER 4

CONCLUSION

- Endochitinase activity of *B. subtilis* 168 carrying pNW33NP11C or pNW33NP7C was measured as 5381 and 6261 U/min/mg which corresponded to 33.8% and 39.3% of the endochitinase activity of *S. marcescens* Bn10, respectively. Increasing the number of bases between SD and ATG codon to 11 bases decreased the activity only 5%. Expression level of recombinant chitinase in *E. coli* was much lower than that in *B. subtilis*.

- Fragments for *chiA* gene with and without promoter region of *cry3*Aa11 were cloned in pHT315 vector. 31.7% relative chitinase activity was obtained in *B. thuringiensis* 3023 recombinants transformed with pHT315PC carrying *chiA* with *cry3*Aa11 promoter. *lac*Z promoter found in the vector did not work in *B. subtilis* and *B. thuringiensis*. Being more stable in the presence of a *B. thuringiensis ori*, pHT315 worked more efficiently than pNW33N.

- Computer-aided analysis (DNASTAR, EditSeq) showed that the gene (*chiA*) starts with ATG codon and terminates at position 1690 with a TAA stop codon. The gene product is a 563 residue protein with a calculated molecular mass of 60.9 kDa. The mean G+C content of the gene is 58.75%.

- Nucleotide sequence of the *chiA* gene of *S. marcescens* Bn10 was compared with 12 different chitinase gene sequences belonging to 3 different species. Its

identity to these sequences was found to be 98.6%-93.3% to chitinase genes of *S. marcescens*, *Burkholderia cepacia* and *Enterobacter* sp. The deduced amino acid sequence of chitinase A from *S. marcescens* Bn10 was compared with other bacterial chitinase sequences and a similarity of 99.3%-91.5% was observed for the same chitinase sequences aligned for nucleotide similarity. It was found that 6 amino acids were shifted from consensus residues, Ala^{73} to Thr^{73}, Ile^{79} to Val^{79}, Thr^{121} to Ser^{121}, Pro^{248} to Ser^{248}, Ser^{484} to Gly^{484} and Ile^{541} to Val^{541}. Ser^{248} and Val^{541} were found to be unique to ChiA of *S. marcescens* Bn10, both belonging to the catalytic domain of the protein.

- Although slightly higher production was obtained in 0.5 g/L chitin containing medium, the effect of varying chitin concentration on chitinase production was not so remarkable. Inclusion of GlcNAc in place of colloidal chitin also stimulated chitinase production.

- The optimum temperature for production of chitinase by *S. marcescens* Bn10 was found to be 30 ^{o}C and enzyme synthesis decreased at 37 ^{o}C. Optimum pH for chitinase production was shown to be 7.5. Production was fairly good at pH 5.5, 6.5 and 8.5, but no chitinase production was observed at pH 10.

- The highest enzyme activity was obtained at 45 ^{o}C. The enzyme also showed a remarkable activity retaining as much as 91% of the activity at 65 ^{o}C, a quite high temperature for an enzyme from a mesophilic source.

- The chitinase enzyme produced by *S. marcescens* Bn10 was found to be active in the pH range of 4.0 to 9.0. Specific activity of the enzyme was around 18000 U/min/mg which did not change noticably within this pH range. A slightly higher activity was observed at pH 6.0.

- A sharp increase in the activity of chitinase was observed between the substrate concentrations of 10 to 35 µg/mL 4-MU-β-$(GlcNAc)_3$. Increasing the concentration of substrate above 35 µg/mL decreased the activity.

- Co^{2+} and Ca^{2+} increased the activity of *S. marcescens* Bn10 chitinase by 20% and 17%, respectively. *S. marcescens* Bn10 chitinase was inhibited only by the presence of 10 mM EDTA (4%).

- The bioassay studies on both coleopteran larval and adult insects are going to be performed by Dr. Kazım Sezen (KTU, Trabzon, Turkey) using recombinant strains.

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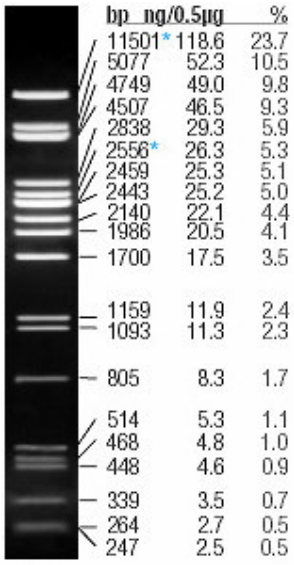
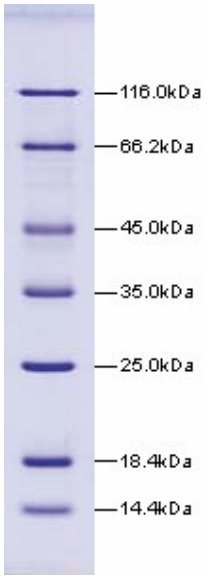
APPENDIX A

MARKERS AND THE STRUCTURES OF PLASMID VECTORS

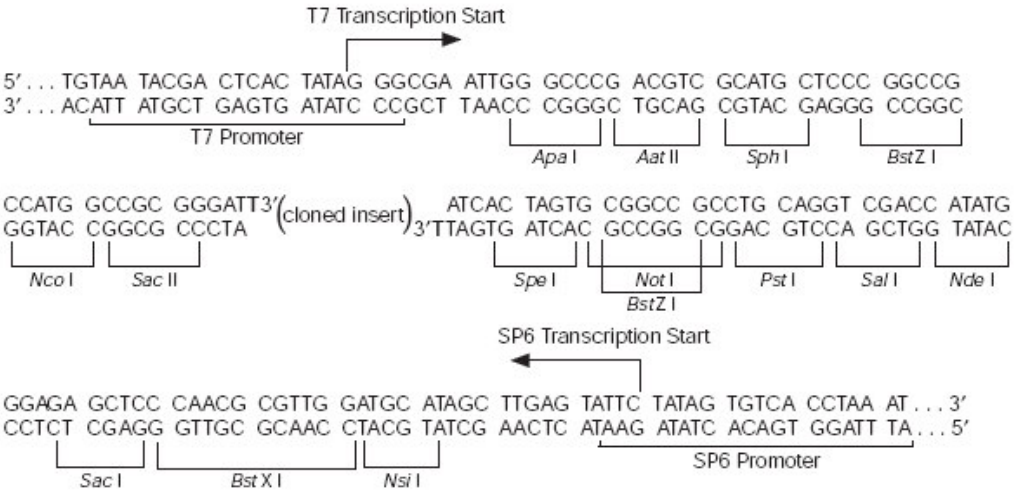


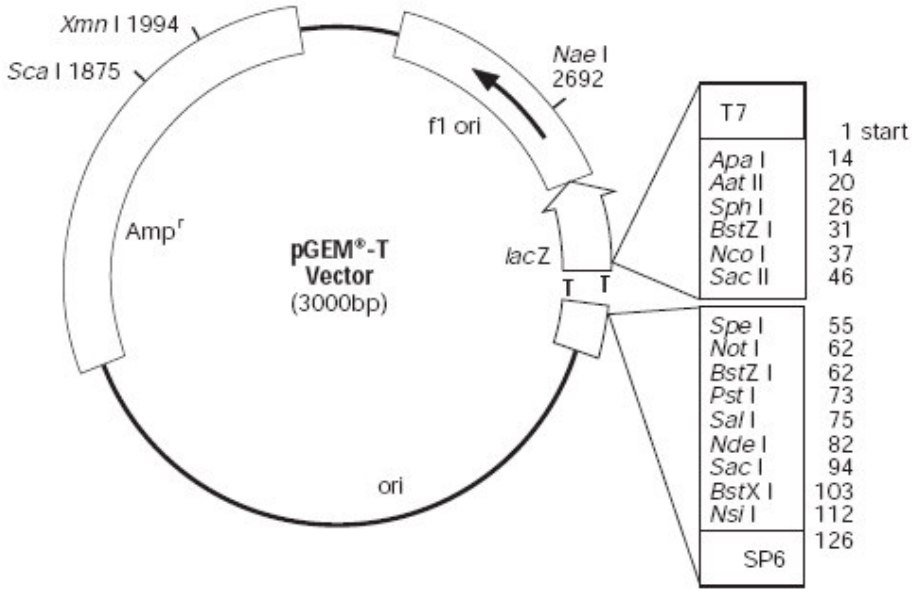
Lambda DNA/*Pst*I Marker



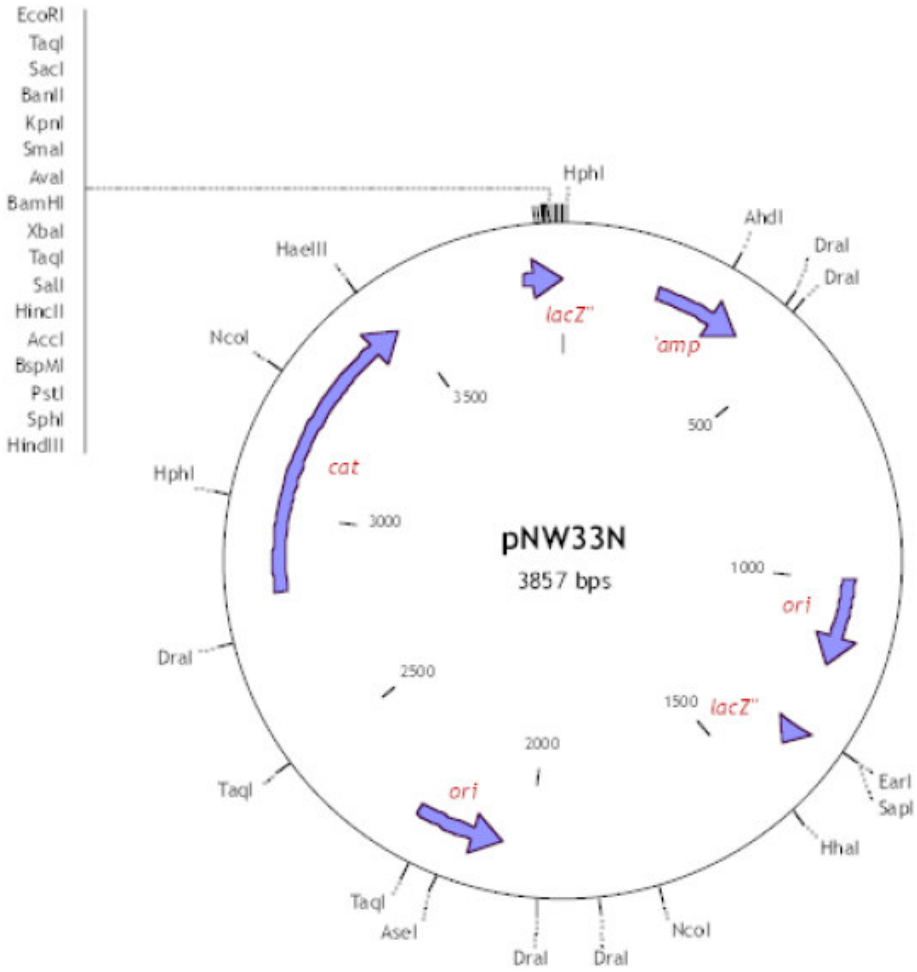
Protein Molecular Weight Marker

pGEM®-T Vector

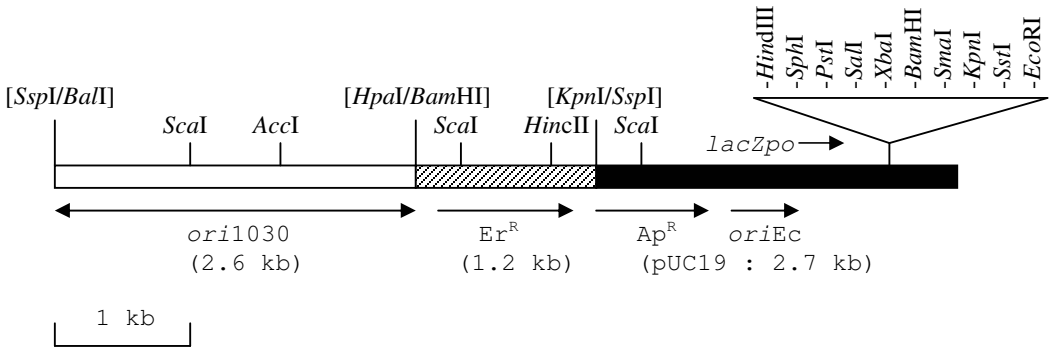




***E. coli – Bacillus* shuttle vector pNW33N**



***E. coli – B. thuringiensis* shuttle vector pHT315**



APPENDIX B

COMPOSITION AND PREPARATION OF CULTURE MEDIA

Luria Broth:

Component:	g/L
Peptone	10.0
Yeast Extract	5.0
NaCl	10.0
or	
Luria Broth	25

pH is adjusted to 7.5

Sterilized at 121 ^{o}C under 1.1 Kg/cm^2 pressure for 15 min. For solid media, agar was added in a concentration of 15 g/L

SOB (for competent cell preparation)

Component:	g/L
Bacto tryptone	20
Yeast Extract	5
NaCl	0.58
KCl	0.185
$MgCl_2$	2.03
$MgSO_4$*	1.2

* autoclaved separately

Components were dissolved in 900 mL dH_2O and 10 mL of 250 mM KCl (18.6 g/L) was added. pH was adjusted to 7.0 with NaOH and volume was completed to 1 L. After autoclaving at 121 ^oC under 1.1 Kg/cm^2 pressure for 15 min., 5 mL of 2 M $MgCl_2$ (188.6 g/L, sterilized by filtration) was added under sterile conditions.

10 X-S-Base

Component:	g/L
$(NH_4)_2SO_4$	20
$K_2HPO_4.3H_2O$	140
KH_2PO_4	60
$Na_3citrate.2H_2O$	10

They were autoclaved together and allowed to cool to 50 ^oC and then supplemented with 1 mL of sterile 1 M $MgSO_4$

HS-Medium

Compenent:	
10 X-S-Base	10 mL
Glucose, 50 % (w/v)	1 mL
Yeast extract, 10 % (w/v)	1 mL
Casamino acid, 2 % (w/v)	1 mL
Arginine 8 % (w/v) + Histidine 0.4 % (w/v)	10 mL
Tryptophane 0.5 % (w/v)	1 mL
Phenylalanine 0.3 % (w/v)	1.5 mL

Total volume was completed to 100 mL with sterile dH_2O.

LS-Medium

Component:	
10 X-S-Base	2 mL
Glucose, 50 % (w/v)	0.2 mL
Yeast extract, 10 % (w/v)	0.2 mL
Casamino acid, 2 % (w/v)	0.1 mL
Tryptophane 0.5 % (w/v)	20 µL

Phenylalanine 0.3 % (w/v)	30 µL
50 mM Spermidine	0.2 mL
1 M $MgCl_2$	50 µL

Total volume was made up to 20 mL with sterile dH_2O.

SOB Medium

Component:	
Tryptone	10 g
Yeast extract	2.5 g
NaCl	0.58 g

Dissolved in 450 mL of dH_2O and 5 mL of 250 mM KCl was added. pH was adjusted to 7.0 with NaOH. Volume was completed to 500 mL with dH_2O and the mixture was autoclaved. Under aseptic conditions, 2.5 mL of 2 M $MgCl_2$ (autoclaved) was added.

ART Media

Component:	per L
NaCl	5 g
Yeast extract	5 g
Tryptone	5 g
Glucose	5 g
Tris	6.0577 g
Sucrose	171.15 g
0.5 M $CaCl_2$	1 mL
1.0 M $MgSO_4$	1 mL
0.5 M $MnSO_4$	1 mL

pH 8.4

ART agar contains 1.8% (w/v) agar

ART soft agar contains 0.8% (w/v) agar

Detailed Preparation of 500 mL

1. Dissolve 3.03 g Tris and 85.575 g sucrose in 250 mL dH_2O. Adjust pH to 8.4 with 5 *N* HCl. Bring to 300 mL. Autoclave for 15 min in a 500-mL bottle.
2. To prepare plate agar, use 9 g of granulated agar in 100 mL dH_2O, use 4 g for soft agar. Autoclave for 15 min in a 500-mL bottle with a stir bar inside.
3. After autoclaving, store bottles at 50 ^oC.
4. To the cooled 300-mL Tris/sucrose solution, add 25 mL of the following sterile stocks: 10% NaCl (filter), 10% yeast extract (autoclave), 10% Bacto-Tryptone (autoclave), and 10% glucose (filter). Then add 1 mL of these filter-sterilized stocks: 0.5 M $CaCl_2$, 1.0 M $MgSO_4$, 0.5 M $MnSO_4$.
5. Add solution from step 4 to the 50 ^oC agar bottle and stir. Add antibiotics, mix, and pour plates. For soft agar, divide into 100-mL sterile bottles.

YTA Media

Component:	g/L
NaCl	5
Difco Bacto-tryptone	10
Difco Bacto-yeast extract	5

Luria Agar

Component:	g/L
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	0.5
Agar	17.5

APPENDIX C

SOLUTIONS AND BUFFERS

Agarose Gel Electrophoresis

TAE Buffer (50X)

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

Loading Buffer (6X)

0.25% bromophenol blue

0.25% xylene cyanol FF

40% (w/v) sucrose in water

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 mL

The solution was filter sterilized and stored at –20 ° C.

Plasmid and Chromosomal DNA Isolation

CTAB/NaCl Solution (10 % CTAB/ 0.7 M NaCl)

4.1 g of NaCl was dissolved in 80 mL of dH2O. Then, 10 g of CTAB (hexadecyl trimethyl ammonium bromide) was added and dissolved with vigorously shaking and gentle heating up to 65 ° C. Final volume was made up to 100 mL with dH_2O.

Phenol-Chloroform Solution (water-saturated, Hintermann, 1981)

Phenol	500 g
Chloroform	500 mL
Distilled water	400 mL

The solution was stored at room temperature, protected from light.

SET Buffer

25 g/L Sucrose

0.05 M Tris-HCl (pH 8.0)

0.5 M EDTA (pH 8.0)

Sterilized at 121 ^{o}C under 1.1 Kg/cm^2 pressure for 15 min.

STE Buffer

10.3% Sucrose

25 mM EDTA pH: 8.0

25 mM Tris-HCl pH: 8.0

2 mg/mL Lyzozyme

TE Buffer

10 mM Tris

1 mM EDTA

pH is set to 8.0 and sterilized at 121 ^{o}C under 1.1 Kg/cm^2 pressure for 15 min.

TSE Buffer

103.0 g/L Sucrose

25 mM Tris-HCl (pH 8.0)

25 mM EDTA (pH 8.0)

2 mg/mL lysozyme

Washing Buffer

0.10 M EDTA (pH 8.0)

0.05 M Tris-HCl (pH 8.0)

0.5 M KCl

Sterilized at 121 ^{o}C under 1.1 Kg/cm^{2} pressure for 15 min.

Protoplast Transformation

P Solution

Components:	g/L
NH_4Cl	1.25
Tris	15
NaCl	0.075
KCl	0.045
$Na_2SO_4.10H_2O$	0.375
$MgCl_2.6H_2O$	5.33
Sucrose	171.15

Preparation: mix all components, adjust pH to 8.4, divide into bottles, and autoclave for 15 min.

PEG Solution:

60% (w/v) PEG 6000 (Serva or Fluka) in P solution

Stir and heat to dissolve, bring to volume, autoclave. Although the PEG tends to fall out of solution over time, it still works.

Polyacrylamide Gel Electrophoresis

Acrylamide/Bis

Acrylamide	146 g
N.N'-Methylene-bis Acrylamide	4 g

Distilled water to 500 mL. Filtered and stored at 4 °C. Protected form light.

Coomassie Blue R-250 Staining

Coomassie blue R-250	0.25 g
Methanol	125 mL
Glacial Acetic acid	25 mL
dH_2O	100 mL

Destaining Solution

Methanol	100 mL
Glacial Acetic acid	100 mL
dH_2O	800 mL

Electrophoresis Buffer (For Native PAGE)

Tris-base	3.0 g
Glycine	14.4 g

Dissolved in 1 L dH_2O and pH was adjusted to 8.3

Running Buffer (5X)

Tris base	15 g
Glycine	72 g
SDS	5 g

Distilled water to 1 L. Stored at 4 °C.

Sample Buffer

dH_2O	3 mL
Tris HCl (0.5 M)	1 mL

Glycerol	1.6 mL
SDS (10%)	0.4 mL
β-mercaptoethanol	0.4 mL
Bromophenol blue (0.5%, w/v)	0.4 mL

Tris-EDTA Buffer (TE) (Maniatis, 1989)

Tris	10 mM
EDTA	1 mM

pH is adjusted to 8.0 with HCl

Tris HCl (1.5 M)

Tris base	54.45 g
dH_2O	150 mL

pH is adjusted to 8.8 with HCl, distilled water to 300 mL and stored at 4 °C.

Tris HCl (0.5 M)

Tris base	6 g
dH_2O	60 mL

pH is adjusted to 6.8 with HCl, distilled water to 100 mL and stored at 4 °C.

TB Buffer (Required for Competent Cell Preparation)

10 mM HEPES

15 mM $CaCl_2.2H_2O$

250 mM KCl

dissolved in 80 mL of dH_2O and pH was adjusted to 6.7 with KOH. 55 mM $MnCl_2.4H_2O$ was added, volume was completed to 100 mL with dH_2O and sterilized by filtration.

X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

X-Gal	20 mg
Dimethylformamide	1 mL

The solution was stored at –20 ° C protected from light.

APPENDIX D

CHEMICALS AND THEIR SUPPLIERS

Chemicals	**Supplier**
Acetic Acid	Merck
Acrylamide	Merck
Agar Bacteriological	Merck
Agarose	Prona
$AgNO_3$	Merck
Ammonium persulfate	AppliChem
Arginine	Sigma
Bovine Serum Albumin	Sigma
Bromophenol Blue	Sigma
$CaCl_2.2H_2O$	Merck
Casamino acid	AppliChem
Cloramphenicol	Sigma
Chloroform	Merck
Coomassie Brillant Blue G-250	Merck
Coomassie Brillant Blue R-250	Sigma
Crab-shell Chitin	Sigma
CTAB	Sigma
dNTPs	MBI Fermentas
Dimethylformamide	Merck
EDTA	AppliChem

Ethanol	Botafarma
Ethidium bromide	Sigma
Formaldehyde	Merck
Glacial Acetic Acid	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
HCl	Merck
HEPES	Sigma
Histidine	Sigma
IPTG	Sigma
Isoamyl alcohol	Merck
Isopropanol	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck
KCl	Merck
Luria Broth	Q-Biogene
Methanol	Merck
Mercaptoethanol	Sigma
$MgCl_2.6H_2O$	Merck
$MgSO_4.7H_2O$	Merck
$MnCl_2.4H_2O$	Merck
$MnSO_4.H_2O$	Merck
4-MU-β-$(GlcNAc)_3$	Sigma
N, N'-Methylene bis-acrylamide	Sigma
Na_2CO_3	Merck
$Na_2SO_4.10H_2O$	Merck
Na_3citrate.$2H_2O$	Merck
N-acetylglucosamine	Sigma
NaCl	Merck
NaOH	Merck
NH_4Cl	Merck

Nutrient Broth	Merck
PEG 6000	Merck
Phenol	Merck
Phenol-chloroform-isoamylalcohol	Sigma
Phenylalanin	Sigma
Potassium acetate	Merck
SDS	Merck
Spermidine	Sigma
Sucrose	Merck
TEMED	Sigma
Tris-base	Merck
Tryptone	Difco
Tryptophane	Sigma
X-Gal	MBI Fermentas
Yeast Extract	Oxoid

Enzymes

*Bam*HI	MBI Fermentas
*Hin*dIII	MBI Fermentas
*Kpn*I	MBI Fermentas
Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
*Sac*I	MBI Fermentas
*Eco*RI	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas
T4 DNA Ligase	MBI Fermentas

Size Markers

***Pst*I digested /Lambda DNA**	MBI Fermentas

The DNA Marker yields the following 29 discrete fragments (in base pairs):

11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 82, 15.

Protein molecular weight marker MIB Fermentas

β-galactosidase (116.2 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease *Bsp*981 (25 kDa), 126 β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were used as the size markers.

Kits

DNA Extraction Kit	Qiagen
pGEM®-T Vector	Promega
Plasmid Isolation Kit	Qiagen