MICROBIAL DEGRADATION OF LOBSTER SHELLS TO EXTRACT CHITIN DERIVATIVES FOR PLANT DISEASE MANAGEMENT

by

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To my mother

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Abstract

Chitinolytic microorganisms are an environment safe approach to utilize lobster processing wastes and extract chitin derivatives from lobster shells. Two microbes, "S223" and "S224" isolated from soil samples exhibited significant levels of deproteinisation, demineralisation of lobster shells and chitinolysis among ten microorganisms screened. Their culture conditions were optimized for factors influencing efficient degradation of lobster shells and chitinase was purified (~30 kDa) from crude extract by affinity chromatography. Morphological observations and 16S rDNA sequencing resolved that the isolates belong to *Streptomyces* genus. The digested lobster shell extracts induced disease resistance in Arabidopsis, which was determined by the increased levels of enzyme activity and expression of defence related genes (*PR1* > 500 fold, *PDF1*.2 > 40 fold) upon *Pseudomonas syringae* and *Botrytis cinerea* infection. The study strongly suggests that soil microbial diversity aid in the sustainable bioconversion of lobster shells and the chitin derivatives extracted could be applied in plant protection.

Keywords: Lobster shells, chitin, microbes, degradation, chitinase, Arabidopsis, disease

LIST OF ABBREVIATIONS AND SYMBOLS USED

% – Percentage

°C – degree Celsius

$ – dollar

AAS – Atomic Absorption Spectroscopy

ANOVA – Analysis of variance

BLAST – Basic Local Alignment Search Tool

CAD – Canadian Dollar

CDA – Chitin deacetylase

cDNA – complimentary Deoxyribonucleic acid

cm – centimetre(s)

DNA – Deoxyribonucleic acid

ET – Ethylene

GlcNAc – N-Acetylglucosamine

g – gram(s)

h – hour(s)

HR – Hypersensitive Response

ICS1 – Isochorismate synthase gene 1

ISR – Induced Systemic Resistance

JA – Jasmonic acid

kDa – kiloDalton(s)

kg – kilogram(s)

km – kilometre(s)

L – litre

LSE – Lobster shell extract

LSP – Lobster shell powder

mg – milligram(s)

min – minute(s)

mL – millilitre(s)

NCBI – National Center for Biotechnology Information

NOD factors – Nodulation factors

NPR1 – Non-expressor of *PR* genes 1

nm – nanometre(s)

P – probability value

PAL – Phenylalanine ammonia lyase

PDF1.2 – Plant defensin gene 1.2

PDA – Potato dextrose agar

ppm – parts per million

PR – Pathogenesis related

Pst DC3000 – *Pseudomonas syrinage pv. tomato* DC3000

qPCR – Real time Polymerase Chain Reaction

rRNA – ribosomal Ribonucleic acid

RNA – Ribonucleic acid

RT-PCR – Reverse Transcriptase Polymerize Chain Reaction

SA – Salicylic acid

SAR – Systemic Acquired Resistance

SAS – Statistical Analysis System

SDS-PAGE – Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SE – standard error

SEM – standard error mean

U – units

v/v – volume/volume

w/v – weight/volume

YEME – Yeast Extract Malt Extract

α – Alpha, level of significance

β – Beta

γ – Gamma

λ – Lambda, wavelength

μg – microgram(s)

μL – microlitre(s)

μmoles –micromole(s)

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CHAPTER 1

INTRODUCTION

The lobster industry contributes to a significant part of Atlantic Canada's economy through local markets and exports. In 2012, around $70 million CAD value of processed lobster was exported from Nova Scotia and New Brunswick (Report of the Maritime Lobster Panel, 2013). In recent years, over half of the landed volume of Canadian lobsters is processed. Canada is the largest worldwide exporter of processed lobster with approximately 68 million kg of lobster being processed each year (Report of the Maritime Lobster Panel, 2013).

The processing procedure involves cooking the whole lobster first and then separating meat from the shells (Waterman, 1991). Lobsters are also frozen immediately without cooking and canned for export. The processing inevitably generates a large amount of waste shells, which presents financial and environmental challenges to the lobster processors for waste management.

The lobster shell is composed of calcium carbonate, chitin, proteins and traces of other minerals and organic compounds (Stirn, 2012). Chitin constitutes about 75% of organic fraction of the exoskeleton (Brimacombe and Webber, 1984). Chitin and its deacetylated derivative, chitosan have diverse chemical and biological applications (Muzzarelli and Muzzarelli, 2005). Chitin is industrially extracted through acid, alkali and heat treatments of crustacean shells, which are hazardous to the environment (Percot et al., 2002).

Earlier studies have shown the possibility of using microorganisms to extract chitin after deproteinisation and demineralisation of crustacean shells (Yang et al., 2000; Oh et al., 2007; Sini et al., 2007). Lactic acid fermentation improved ensilation of crustacean shells with added whey, lignocellulose and starch (Fagbenro, 1996). Successive microbial demineralisation and

deprotenisation of crab and shrimp shells yielded a liquid fraction rich in proteins and minerals and an insoluble chitin fraction, which was retained in the sediment (Jung et. al., 2007; Xu et al., 2008).

Chitinolytic microorganisms play an important ecological role in biogeochemical cycles by recycling chitin, the second most abundant polysaccharide in nature (Muzzarelli, 1997). The application of chitinolytic microorganisms would be a prospective eco-friendly alternative to utilize lobster processing wastes and extract chitin derivatives from the shells (Wang et al., 2002). This approach would reduce management costs and provide a sustainable method for biodegradation of lobster shells discarded from the processing plants.

The chemical composition of crustacean shells holds potential source as a raw material for agricultural amendments (Kandra et al., 2012). Chitin and chitosan derived from lobster shells can be used in plant protection as elicitors of immune responses against pests and pathogens. The induced disease resistance is primarily due to the activation of innate plant defence pathways that are mediated primarily by phytoalexins and pathogenesis related proteins (Benhamou, 1996; El-Hadrami et al., 2010).

Adding value to what is normally a by-product through the extraction of bioactive compounds from crustacean shells including lobster will provide commercial benefits to the processing industries. It is obvious that for both economic and environmental reasons that wherever possible, appropriate technology should be developed to prevent pollution and convert the biomaterials into valuable products. Hence, there is a significant interest in bioconversion of crustacean shell waste.

The objectives of this study are stated at the end of next chapter, Literature review.

CHAPTER 2

LITERATURE REVIEW

2.1. LOBSTER INDUSTRY

Atlantic lobster (*Homarus americanus*) is Canada's most profitable fishery resource and iconic Canadian seafood that is exported around the globe (Fisheries and Oceans Canada, 2012). As of 2011, there are almost 10,000 licensed lobster enterprises employing about 30,000 harvesters across Quebec and Atlantic Canada, the major lobster producing regions of the country (Fisheries and Oceans Canada, 2012). Lobster is the most valuable seafood in Canada, generating over $1 billion CAD (2011) in exports. The majority (78%) of lobster exports are marketed to the United States. Other key destinations include the European Union (UK, Belgium and France) and Asia (China and Japan). Lobster is also exported to an additional 50 other countries (Fisheries and Oceans Canada, 2012).

Canadian lobster landings remain at one of the highest levels recorded in 100 years, with an upward trend over recent decades. Annual lobster landings average 50,000 to 55,000 tonnes (Fisheries and Oceans Canada, 2012). Lobster landings were 74,790 tonnes in 2012 and 66,500 tonnes in 2011 and the landed values were $662.8 million and $619.7 million respectively. Canada is the leading lobster processing nation in the world with approximately 68 million kg (150 million pounds) of lobster processed each year, which is half of the landed volume (Report of the Maritime Lobster Panel, 2013). Lobster fishing is most active in the Gulf of Maine, Bay of Fundy, coastal Nova Scotia and southern Gulf of St. Lawrence (Fisheries and Oceans Canada, 2012). Lobsters are caught in baited traps placed at the bottom of the sea. Most of the fishing take place within 15 km off-shore and in shallow waters less than 40 metres deep, although some harvesters fish much farther out in waters up to 200 metres deep.

Lobster populations in Canada are healthy and sustainably managed. Conservation measures to protect lobster populations are regulated by the sustainability framework of Atlantic lobster (Fisheries and Oceans Canada, 2012). These measures include protection of egg-bearing females, maximum size limit, allowing maximum catch of 720 tonnes per season for deep-basin fishing and harvesters are not permitted to fish farther than 92 km from shore (Report of the Maritime Lobster Panel, 2013).

2.1.1. Lobster processing

Lobster processing facilities are located close to the shores where fishermen bring their catch from the sea. If lobsters are quick frozen raw, the flesh upon subsequent cooking is often soft and shrunken, and very difficult to remove from the shell without breakage. Lobsters are precooked alive in boiling salt water (2 - 3% salt) for easy removal of shells while preserving the flavour in further processing (Waterman, 1991). In most commercial operations the lobsters are lowered into the water in some form of open mesh wire basket and covered. Factors like boiling temperature, cooking time, container volume, quantity of lobsters and hygiene affect the processing and adverse conditions result most likely in toughening of the meat, hardening of the shell or food poisoning. When cooking is complete, the lobsters are left to cool and drain before shelling or wrapping (Waterman, 1991). Lobster meat is either canned in salt water or stored at -30 °C to -18 °C. Lobsters are also frozen immediately without cooking (cryogenic freezing). In 2012, around $70 million CAD value of processed lobster was exported from Nova Scotia and New Brunswick. Lobster processing is generally very labour intensive and hundreds of employees work in processing plants. Lobster processors therefore are very important economic engines for many small coastal communities (Report of the Maritime Lobster Panel, 2013).

2.1.2. The Atlantic lobster

Homarus americanus is distributed in the northwest Atlantic from North Carolina to the waters of Newfoundland and southern Labrador. Lobsters are among the largest marine crustaceans, sometimes growing 60 cm in length and weigh over 18 kilograms. They are also the longest living, capable of a life span up to 50 years (Fisheries and Oceans Canada, 2012). The largest populations are found in the Gulf of Maine and the southern Gulf of St. Lawrence. Lobsters are active hunters, feeding on a variety of animals, including crab, shellfish, marine worms, starfish, sea urchins and fish. When outside their burrows, juveniles are prey for many fish species. Larval and post-larval lobsters are prone to predation by crabs and finfish species. Lobsters become less vulnerable to predation as they grow, except during molting periods when they shed their hard outer shell (Fisheries and Oceans Canada, 2012).

2.1.3. Lobster shell

"Light weight and flexible, yet strong: the properties of lobster shell derive from a sophisticated structure of chitin and calcium carbonate," was the description about lobster shells by Helge Fabritius, a scientist investigating its structure and composition at the Max-Plank Institute for iron research in Dusseldorf, Germany (Stirn, 2012).

He further explained the arrangement of the shells as follows: N-Acetylglucosamine units form long chains of chitin (Figure 2.1), twelve to eighteen chitin chains join to form a chitin fibril and the fibrils are covered with a thin layer of proteins and arrange parallel to each other in the epithelium. The fibrils are stiffer in the longitudinal direction and the fibrous layers in the endothelium are twisted by a certain angle forming an extremely resistant material. The epithelium is pervaded by tiny pores and serves as a transport system for minerals and nutrients.

In lobster shell, calcium carbonate is arranged between the chitin fibrils in the form of tiny spherules. The type of crystal lattice and the number of calcium carbonate particles determine the hardness of the shell. The other minerals present in the shell are magnesium and phosphorus. The thin layer on the surface of epicuticle is made up of calcite, a resilient compound that protects the lobster from attack and wear. In the regions below, the exoskeleton has more amorphous calcium carbonate, with high solubility. X-ray diffraction, confocal and electron microscopy are being used to study the structure and strength of the shells, yet the complete resolution is still a challenge (Stirn, 2012).

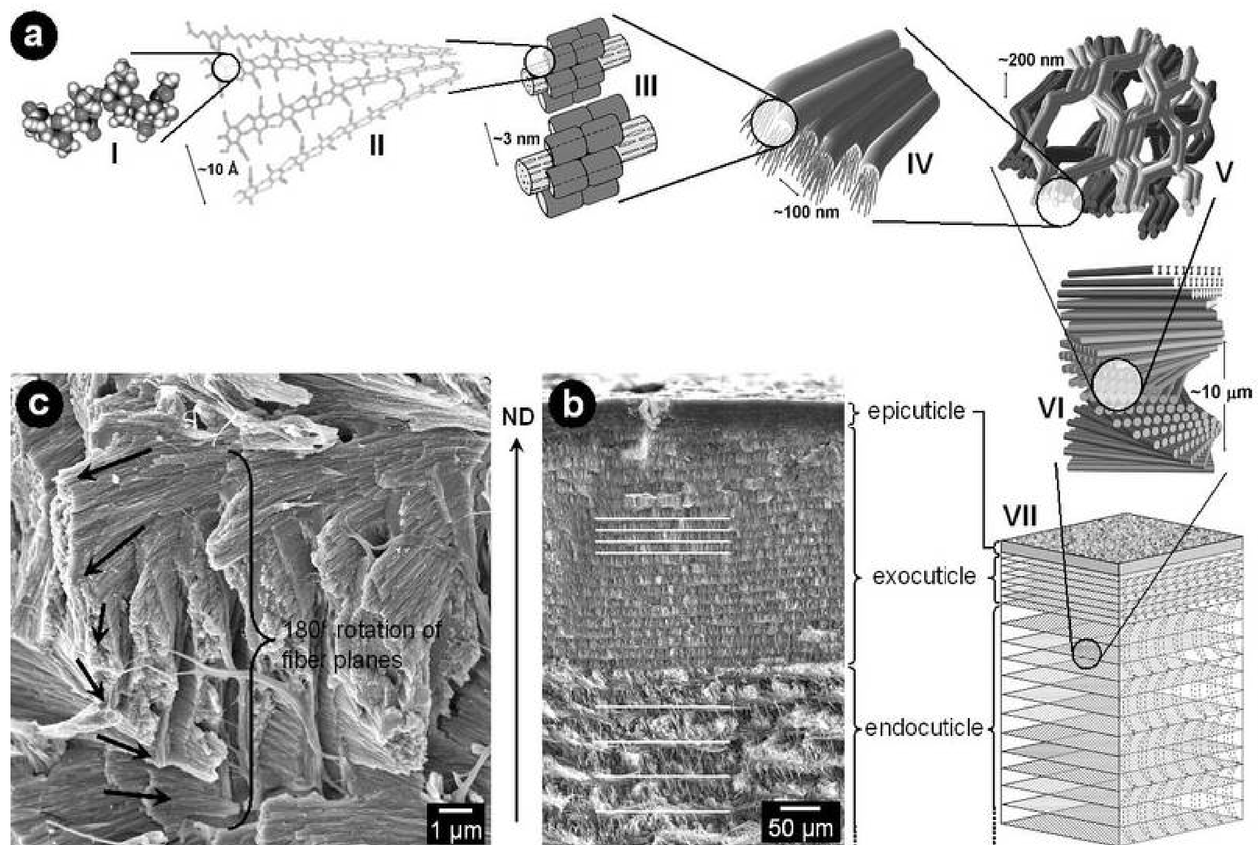

Figure 2.1. Microstructure of lobster cuticle **(a)** (I) Hierarchical organization starting with the N-Acetylglucosamine molecules (II) forming anti-parallel chains of α-chitin (III) Eighteen to 25 chitin molecules form nanofibrils wrapped with proteins (IV) which cluster to form chitin protein fibers (V) that are arranged in horizontal planes (VI) form the typical twisted plywood structure (VII) of the three-layered cuticle **(b)** SEM micrograph of a cross section through the cuticle showing the epi-, exo- and endocuticle **(c)** Superimposed planes gradually rotate around the normal axis of the cuticle (©Max-Planck-Institut für Eisenforschung, GmbH, Germany)

In *Homarus americanus* 60-75% of organic weight of the cuticle is made up of chitin (Brimacombe and Webber, 1984). The chitin is present in association with other organic compounds such as proteins, carotenoids and other polysaccharides. N-Acetylglucosamine units react with α-amino acids, peptides, and cuticular proteins to form stable complexes by covalent linkages. However, these complexes dissociate when subjected to alkaline pH and after protein removal, the residual amino acids present were aspartic acid, serine and glycine, which are thought to be predominantly involved in the chitin-protein linkage (Brine and Austin, 1981).

Carotenoids bind with the proteins, giving colour to the crustacean shells. They include β-carotein, lutein, astaxanthin and their derivatives. Carotenoids are combined with chitin-amino groups by carbonylamino or Schiff's base linkages. The pigments can be removed from the shells by digesting with organic solvents and organic acids (Fox, 1973).

2.1.4. Lobster shell waste

Lobsters are processed for their meat and contribute to the economy, but leave behind a large volume of shells. Most commonly, crustacean waste is dumped in the sea, soil or landfills (Kandra et al., 2012). When the production is commercialized due to increasing consumer demand for fishery and aquaculture products and with a decline in natural fishery resources, it is no longer environmentally responsible and practical to discard huge amount of wastes from crab, lobster and shrimp processing plants around coastal areas (Morgan and Chuenpagdue, 2003). The shells begin to decay immediately after processing like in any other dead organic material and create an unpleasant smell due to the easily perishable protein content and production of biogenic amines from the decaying material. When the decay could not be prevented, it threatens the surrounding ecosystem by releasing volatile toxic compounds, pose serious risk to coastal

environments and becomes a burden if not discarded properly (Kelleher, 2005). A significant amount of bioactive compounds can be recovered from the waste shells as value added products. Medium and large-scale treatments have been developed to process the waste shells and mix it with other agricultural raw materials to produce manure and animal feed (Kandra et al., 2012).

2.2. CHITIN

Chitin is a natural polymer of N-Acetylglucosamine (GlcNAc) with β-1,4 glycosidic linkages similar to cellulose. It is the second most abundant renewable polymer in nature next only to cellulose (Muzzarelli, 1997). Chitin is utilized as a structural component in many species. Chitin is primarily present in the crustacean exoskeleton (lobster, crab, shrimp etc.,), insect cuticle, worms, fungi and mushrooms. In nature, 10^9 tons of chitin is recycled every year (Jeuniaux, 1971) and the major part of chitin production is from the marine ecosystem (Arcidiacono and Kaplan, 1992).

2.2.1. History

Chitin is well known from ancient times and has been found in the shell of 100 mega years old Pterygotus (ancient scorpion). Henri Braccanot, a French professor in Natural History discovered this polymer in 1811 from a fungal species of *Agaricus*, and called it “Fungine” (Jeuniaux, 1996). In 1823, Odier found the same polysaccharide in insect cuticle and named it chitin - a Greek term for tunic or envelope (Muzzarelli and Mozzarelli, 2009). In 1843, Lassaigne demonstrated the presence of nitrogen in chitin (Jeuniaux, 1996).

2.2.2. Properties

Chitin is not readily soluble in water due to its intermolecular hydrogen bonds (Minke and Blackwell, 1978). However, chitin derivatives such as chitosan and carboxymethyl chitin are

relatively water-soluble. One of their most important features is the flexibility to be shaped into different forms such as beads, sponges, fibers, hydrogels, and membranes (Mano et. al., 2007). The origin of chitin affects its crystallinity, purity, polymer chain arrangement and dictates its properties (Rinaudo, 2006). Chitin contains 6–7% nitrogen and in its deacetylated form, chitosan contains 7–9.5% nitrogen.

There are three forms of chitin distinguished on the basis of chitin-chain arrangement: α, β, and γ chitin. The N-Acetylglucosamine chains in α-chitin are aligned in anti-parallel fashion that gives rise to strong hydrogen bonding and makes the α-chitin form more stable than others (Sikorski et al., 2009). β- chitin is characterized with parallel chains of N-Acetylglucosamine units and γ -chitin is made up of a three-chain GlcNAc unit cell, where two of them are facing 'up' - one chain is facing 'down'. The α-chitin is widely distributed and mainly obtained from crustacean shells. The commercial sources of chitin are shrimp, Antarctic krill, crab, and lobster shells (Shahidi and Synowiecki, 1991; Muzzarelli, 1997).

2.2.3. Applications

Chitin is biodegradable, biocompatible and biosorbtive, with anti-bacterial and wound-healing abilities with low immunogenicity (Muzzarelli, 2009). There are many reports on biomedical applications of chitin (Jolles and Muzzarelli, 1999). Chitin is a natural polymer and has great potential in agriculture, biotechnology and in the pharmaceutical industries (Muzzarelli and Muzzarelli, 2005). Applications of chitin in different fields such as agriculture, tissue engineering, food technology, microbiology, material science, drug delivery systems, wastewater treatment and nanotechnology have been reported (Khoushab and Yamabhai, 2010).

2.2.4. Purification

Chitin is commercially produced from crustacean shells with acid and alkali treatments. Chitin is closely associated with proteins, calcium, lipids and carotenoids. All these compounds must be removed to get highly polymeric chitin (Percot et al., 2002). For separating chitin from crustacean exoskeleton 4% NaOH and 4% HCl are used. 50% NaOH and high temperature of 70- 90 °C are required for deacetylation of chitin into chitosan (Figure 2.1). The disadvantage of chemical purification is that the use of strong acid and alkali causes certain degree of depolymerisation and affect intrinsic properties of the purified chitin (Percot et. al., 2002).

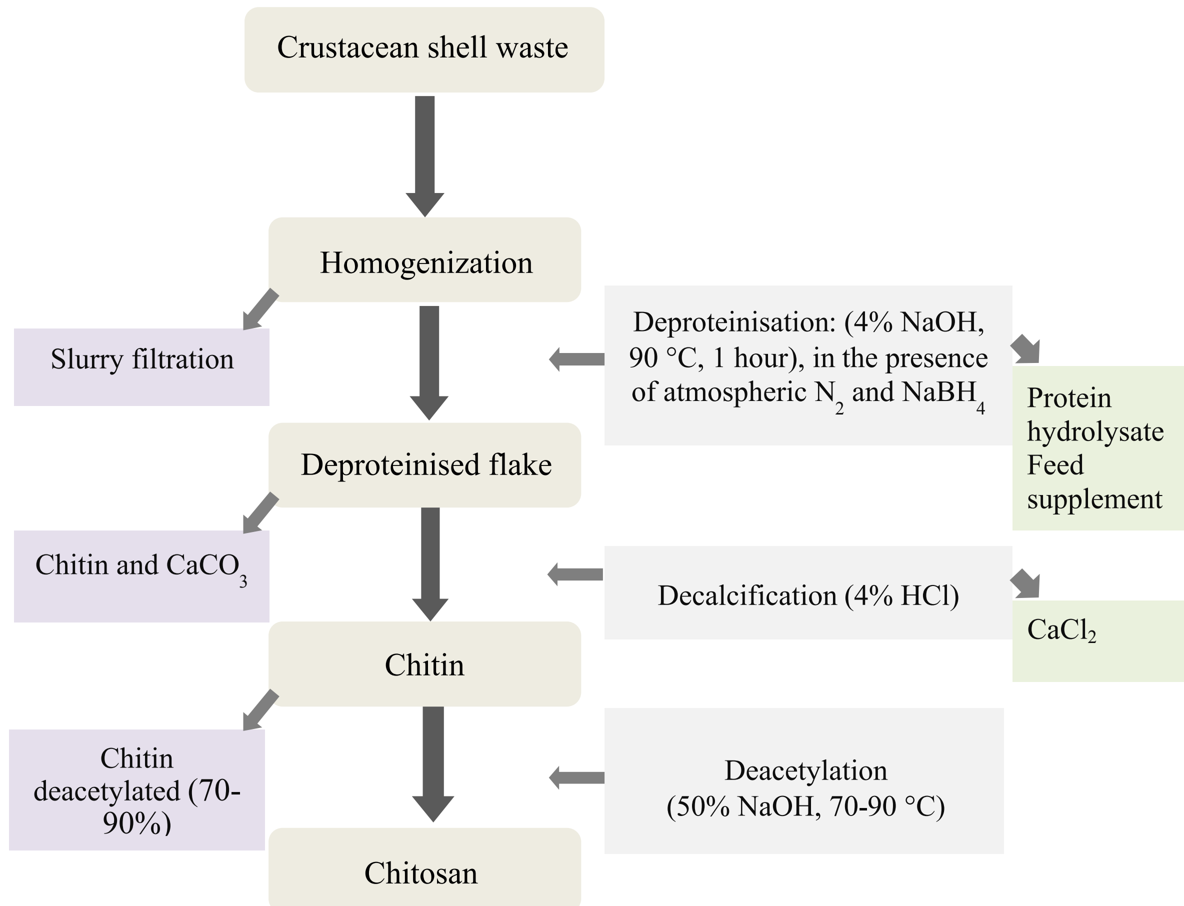

Figure 2.2. Chemical synthesis of chitin and chitosan (adapted from Percot et al., 2002).

The use of chemicals results in high production costs and effluent treatments. Even though industrial production methods are efficient in recovering chitin, other biomolecules in the shells like proteins, lipids and carotenoids are rendered useless during the process (Healy et al., 2003; Rao and Stevens, 2005). Acid ensilage with weaker organic acids has been reported to stabilize carotenoids and assist their further recovery (Sachindra et. al., 2007).

2.3. BIOLOGICAL DEGRADATION OF CRUSTACEAN SHELL WASTE

Biological methods would be an alternative approach to overcome the problems of chemical extraction. Extensive research has been carried out on the biological degradation of shellfish wastes particularly from shrimps.

2.3.1. Recovery of protein by enzymatic hydrolysis method

Proteolytic enzymes, like proteases and alkaline proteases could be used for the deproteinisation of crustacean shells and replace the alkali treatment. Certain proteolytic enzymes such as trypsin (Synowiecki and Al-Khateeb, 2000) and alcalase (Guerard et al., 2007) have been used to extract protein from the shell waste. Application of sodium sulfite, alcalase (a commercial protease), Triton X-100, and their combination in association with mild chemical treatments improved the quality and quantity of the protein as well as chitin extraction (Guerard et al., 2007). The enzymatic deproteinisation process has limitations due to residual small peptides directly attached to chitin molecules. However, using chemical reagents in combination with proteolytic enzymes significantly reduced the proteinaceous fraction of the precipitate (Mizani and Aminlari, 2007). Since commercial enzymes are expensive, this method is still a financial challenge and an alternative economic technology for extracting chitin and proteins from lobster shells should be developed.

2.3.2. Microbial digestion of crustacean shells

Besides the application of enzymes, proteolytic microorganisms have been used for the deproteinisation of chemically demineralised shells (Jung et. al., 2007). This method yielded a liquid fraction rich in proteins, minerals, and astaxanthin and an insoluble chitin fraction. Deproteinisation of the shell waste and subsequent liquefaction of the shell proteins was chiefly due to the proteolytic enzymes produced by the added bacteria and microbiota present in the biowaste (Rao et al., 2000). It resulted in a fairly clean liquid fraction with a high content of soluble peptides and free amino acids.

Deproteinisation of shrimp waste for chitin production using mechanical (Jung et al., 2007), enzymatic (Valdez-Pena et al., 2010) and microbial processes have been reported (Oh et al., 2000; Yang et al., 2000). Degradation of shrimp (*Metapenaeopsis dobsoni*) shell in jaggery broth using *Bacillus subtilis* showed that the level of acid produced as well as the proteolytic activity, allowed shell demineralisation and deproteinisation (Sini et al., 2007). About 84% of the protein and 72% of the minerals were removed from the shrimp shell after digestion.

A *Pseudomonas aeruginosa* K-187 strain isolated from soil samples of northern Taiwan secreted protease and chitinase/lysozyme when cultured in a medium containing shrimp and crab shell wastes as the sole carbon sources (Wang and Chio, 1998). 92% demineralisation and 63% deproteinisation of crab shell waste was achieved after seven days incubation at 30 °C with *P. aeruginosa* F722 (Oh et al., 2007).

Two bacterial species, *Bacillus cereus* and *Exiguobacterium acetylicum* (Sorokulova et al., 2009) were tested for the degradation of shrimp shell waste. The protein content was reduced from 18.7 to 5.3% with *B. cereus* and to 7.3% with *E. acetylicum* incubation. Degradation of crab (*Chionoecetes opilio*) shell wastes was investigated (Jo et al., 2008) using *Serratia*

marcescens FS-3 isolated from sea-side soil (in the southwestern area of Korea), which exhibited strong protease activity. Deproteinisation yield of 73% from squid pen shells was carried out by protease producing *Bacillus sp*. TKU004 (Wang et al., 2006) in the preparation of β-chitin.

Organic acids (lactic and acetic) produced during cheese and whey fermentation were used to demineralise northern pink shrimp (*Pandalus borealis*) shell wastes that were deproteinised by *Aspergillus niger* ATCC 16513 (Mahmoud et al., 2007). This study recommended the use of lactic and acetic acids in place of hydrochloric acid for demineralisation of shell wastes. Biological demineralisation by using enzymes such as alcalase, probiotics in milk curd and microbial processes have also been reported for chitin production (Synowiecki and Al-Khateeb, 2000; Bautista et al., 2001; Prameela et al., 2011). In these approaches, deproteinisation and demineralisation occur simultaneously but often incomplete (Jung et al., 2007).

2.3.3. Lactic acid fermentation

Fermentation of shell wastes with affordable technology was carried out for many years (Fagbenro, 1996). It involves ensilation of crustacean shells and a low-cost *in situ* production of lactic acid from by-products such as whey, lignocellulose and starch. Lactic acid is formed from the anaerobic breakdown of glucose, lowering the pH and activation of proteases, which improves the ensilation and suppresses the growth of contaminants. Chitin is usually retained in the sediment and the protein-rich liquid could be separated from it (Xu et al., 2008). Lactic acid reacts with the calcium carbonate component of the shells and the precipitate of calcium lactate formed can be removed by washing (Bautista et al., 2001).

Fermentation of shrimp (*Penaeus monodon*) waste with lactic acid bacteria for chitin extraction has been reported with added carbohydrates, such as lactose or cassava extract (Jung et al., 2005). Chitin was recovered from head and carapace of shrimp *Parapenaeus longirostris* using *Lactobacillus helveticus* (Adour et al., 2008). *L. plantarum* fermented raw heads of African river prawn (*Macrobrachium vollenhovenii*) using cane molasses (Fagbenro, 1996). Lactic acid bacteria fermentation has also been reported in prawn waste (Healy et al., 1994), scampi waste (Zakaria et al., 1997), and crayfish exoskeleton (Bautista et al., 2001). Chemical pre-treatment of shrimp shell waste using low concentrations of HCl (0.5 M) and NaOH (0.4 M), followed by lactic acid fermentation was studied (Cira et el., 2002).

The recovery of chitin from prawn (*Nephrops norvegicus*) shell waste using lactic acid fermentation was reported by Healy et al., (1994). In that study, HCl was used to demineralise the shells before incubating for deproteinisation with bacterial inoculum (*Streptococcus faecium* M74, *L. plantarum*, *L. salivarius*, and *Pediococcus acidilactici*) with lactose for 7 days. Protein depletion of approximately 40% was achieved irrespective of the fermentation conditions (aerobic or anaerobic). In another study where no chemical treatment was employed, 90% demineralisation of prawn shells were achieved Healy et al., (1994). *L. paracasei* strain A3 was used in the microbial treatment of minced scampi waste supplemented with glucose (Zakaria et al., 1997). After five days of batch culture at 30 °C, the solid fraction contained 17.5% chitin (on dry mass basis).

Crayfish (*Procambarus clarkii*) exoskeleton was also used to recover chitin (Cremades et al., 2001). The waste was fractionated by floatation and sedimentation into proteinaceous and chitinous fractions respectively. Deproteinisation and demineralisation processes supplemented with dextrose yielded chitin fraction by semi-solid state fermentation with *L. paracasei*.

Demineralisation of crayfish exoskeleton by using immobilized cells of *L. pentosus* 4023 in fed-batch fermentation was also studied (Wang and Chio, 1998). Red crab (*Chionoecetes japonicus*) shell wastes were fermented by *L. paracasei* ssp. *tolerans* KCTC-3074 and the results were compared with chemical treatment using lactic acid (Jung et al., 2005). The results suggested that fermentation with lactic acid bacteria could be an alternative for crab shell demineralisation, even though efficiency was lower than chemical treatment.

2.3.4. Co-culturing for chitin extraction

Chitin extraction from *Penaeus monodon* and *Crangon crangon* shells was investigated in a two-step process involving anaerobic deproteinisation by proteolytic bacteria and autochitinous microbes of Indonesian shrimp shells followed by lactic acid fermentation using *Lactobacillus casei* MRS1 (Xu et al., 2008). Chitin was recovered from prawn waste using lactic acid producing bacterium, *L. lactis*, and protease-producing marine bacterium, *Teredinibacter turnerae* (Aytekin and Elibol, 2010). Both bacteria were tested for degradation activities individually and in co-culture. The yield (95.5%) was highest during co-fermentation process.

Serratia marcescens FS-3 and *L. paracasei ssp. tolerans* KCTC-3074 were co-fermented to extract chitin from red crab shell wastes (Jung et al., 2006). The method resulted in 52.6% deproteinisation and 97.2% demineralisation showing that co-fermentation is applicable in a one-step extraction of crude chitin from shells. Chitinase-deficient but proteolytic microbial cultures were isolated from shrimp shell waste and the two most efficient *Bacillus licheniformis* strains were employed in fermenting shrimp shells for 48 h (Waldeck et al., 2006). The fermentation product was demineralised with 0.9% lactic acid for 3 h and the sediment was oven dried. The method achieved 99% deproteinisation and 98.8% demineralisation. Although microbial chitin extraction was not able to recover the purest form of chitin but when compared to the chemical

methods it is promising an environmental friendly alternative.

2.4. CHITINOLYTIC ENZYMES

The vast annual production of chitin in nature is balanced by an equal rate of recycling. In the sea it is chiefly by free-living bacteria and those in association with animal guts and in the soil chiefly by fungi and bacteria (Gooday, 1990a). Microorganisms degrade chitin with chitinases and are known as chitinolytic. Chitinases are synthesized by a vast array of organisms including those which are not composed of chitin. Chitinases also play an important ecological role in biogeochemical cycles by breakingdown chitin and generating carbon and nitrogen sources.

The most studied pathway in the chitinolytic system is the hydrolysis of the glycosidic bonds of chitin (Davis and Eveleigh, 1984). Chitinases are classified into two major categories and there may not always be a clear distribution between the activities of these two enzymes (Davis and Eveleigh, 1984) since their action is dependent on the nature of the substrate. Endochitinases (EC 3.2.1.14) randomly hydrolyze internal β-1,4-linkages of chitin, releasing soluble oligosaccharides of N-Acetylglucosamine, such as tetraacetylchitotetrose, triactylchitotriose and the dimer diacetylchitobiose. Exochitinases are divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of the chitin microfibril and 1,4-β-N-Acetylglucosaminidases (EC 3.2.1.30), which generate monomers of N-Acetylglucosamine by cleaving the oligomeric products (diacetylchitobiose) of endochitinases and chitibiosidases (Sahai and Manocha, 1993). An alternative system for degrading chitin is via deacetylation to chitosan (Gooday et al., 1991). A third possible pathway of deamination of aminosugars is still being explored. The mechanism of chitinolytic enzymes is shown in Figure 2.2.

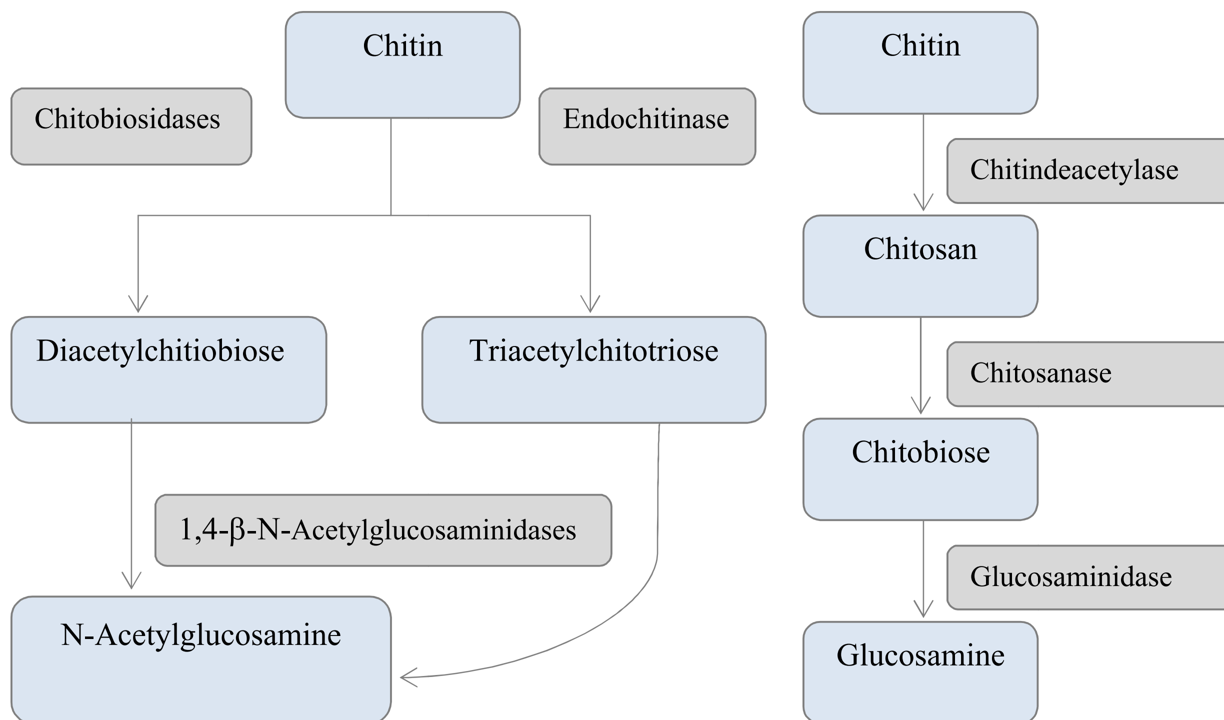

Figure 2.3. Mechanism of chitin breakdown by chitinolytic enzymes.

2.4.1. Classification of chitinases

Based on amino acid sequence similarity of chitinases from various organisms, five classes of chitinases have been proposed. Chitinases from classes I, II and IV are of plant origin and make up the family 19 glycosyl hydrolases (Hamel et al., 1997). Three classes of plant chitinases have been proposed based on the primary structure of the protein (Shinshi et al., 1990): Class I chitinases are enzymes with a N-terminal cysteine-rich domain of approximately 40 amino acids and a highly conserved main structure, separated by a short glycine/ proline rich region to the catalytic domain (Perrakis et al., 1994). Most of the class I chitinases contain a carboxy-terminal signal peptide that is essential for targeting into the plant cell vacuole.

Class II chitinases lack the N-terminal cysteine-rich domain and the carboxy-terminal vacuolar targeting signal, indicating that these chitinases do not bind chitin and are secreted to

the apoplast but have a high amino acid sequence identity to the main structure of class I chitinases. Collinge et al., (1992) proposed Class IV chitinases, identified mainly in dicotyledons, which comprises a group of extracellular chitinases and also contain cysteine-rich regions. Class III chitinases are mainly of fungal and plant origin (Shinshi et al., 1990). They are grouped in family 18 glycosyl hydrolases (Henrissat, 1992) along with Class V chitinases, which are structurally different from family 19 (Meins et al., 1992). The bifunctional lysozyme/chitinase enzyme is included in Class III. Class V is mainly comprised of bacterial chitinases.

2.4.2. Bacterial chitinases

Bacteria produce chitinases to meet nutritional needs by digesting N-Acetylglucosamine macromolecules. The possible end products of chitin digestion are acetate, ammonia and fructose-6-phosphate (Comb and Roseman, 1956, 1958). Many bacteria can use chitin as sole source of carbon, and chitin can be used as an enrichment medium for their isolation from soil. They usually produce several chitinases, to hydrolyze the diversity of chitins found in nature. Bacterial chitinases range from 20 to 60 kDa while the molecular weight of chitinases can be anywhere between 20 to 90 kDa. When grown in liquid cultures most of the chitinolytic bacteria secrete chitinases in to the medium (Gooday, 1990a).

Wolkin and Pate (1985) concluded that chitin digestion requires moving cell surfaces, presumably involving enzymatic contact between bacterium and substrate. Chitinase production by bacteria has been shown to be inducible by chitin oligomers and low levels of N-Acetylglucosamine (Monreal and Reese, 1969). Chitinases can be active over a range of temperatures and pH (Gooday, 1986) where optimum conditions depend on the source of origin. The thermostable chitinase, which is active at 80 °C, has been isolated from *Streptomyces*

thermoviolaceus OPC-520 (Tsujibo et al., 1993)

Chitinolyitc bacteria are widespread in all productive habitats. Chitinases secreted by marine bacteria play important role in recycling of chitin in marine ecosystem (Watanabe et al., 1997). Chitinases of marine organisms isolated so far share only little homology with other chitinases (Cohen-Kupiec and Chet, 1998). The mechanism of chitin breakdown by marine bacteria has been reviewed by Keyhani and Roseman (1999) and *Vibrio furnissii* is one such marine bacteria earlier studied for chemotaxis of chitin in the ocean floor (West and Colwell, 1984). The bacteria recognize and attach to chitin substrates, synthesize enzymes (chitinolysis take place in sediments and water) and utilize the carbon and nitrogen containing products. Adhesion to chitin involves various proteins on the bacterial surface and formation of biofilms (Vogan et al., 2008). Bacterial degradation of chitin has been extensively studied in a wide range of marine bacteria including *Vibrio furnissii* (Bassler et al., 1991; Li and Roseman, 2004), *V. harveyi* (Svitil et al., 1997), *Salinivibrio costicola* (Aunpad and Panbangred, 2003), *V. cholera* (Li and Roseman, 2004; Kirn et al., 2005), *Alteromonas sp.* strain O-7 (Orikoshi et al., 2005) and *V. alginolyticus* (Suginta, 2007). Chitinase gene cluster of marine bacterium, *Alteromonas sp.* strain O-7 was cloned and sequenced (Tsujibo et al., 2002).

The soil contains many chitinous organisms and chitinolytic bacteria can be isolated readily. *Bacillus amyloliquefaciens, B. thuringiensis*, *Pseudomonas aeruginosa, Serratia marcescens, Streptomyces* and other species were extensively studied for their chitinolytic properties and enzyme characteristics (Wang et al., 2002; Watanabe et al., 2002; Arora et al., 2003). Bacterial chitinases have antimicrobial properties and are used as biocontrol agents against many plant pathogenic fungi and insects, since chitin is a major constituent of fungal cell wall and insect cuticle. (Ordentlich et al., 1988; Hoster et al., 2005; Ajit et al., 2006)

Actinomycetes (filamentous bacteria) are saphrophytic soil microorganisms, which have been explored for their antibiotic production (particularly *Streptomyces*) and extracellular enzymes. Mitsutomi et al., (1995) isolated two novel chitinases from *Streptomyces griseus* HUT 6037. It was then the first report on a chitinase breaking down β-glucosaminidic linkages in chitin. *Streptomyces* produce secondary metabolites in presence of chitin, which are antifungal and relatively lose their potential when grown in a medium without chitin. Chitinase gene from *Streptomyces* species have been isolated and cloned for optimized production of the antimicrobial compunds. (Fujii and Miyashita, 1993; Miyashita et al., 1997).

2.4.3. Fungal chitinases

Similar to bacterial chitinases, fungal chitinases also play role in nutrition, but they are also active in fungal morphogenesis and developmental processes because chitin is a major fungal cell wall component (Sahai and Manocha, 1993). Chitinolytic fungi are readily isolated from soils where they rival or even exceed chitinolytic activities of bacteria. Most common among them are *Mucor*, *Trichoderma*, *Aspergillus*, *Pencillium* and *Verticillium* (Gooday, 1990b).

Entomopathogenic fungi such as *Beauveria bassiana*, *Metarhizium anisophilae* and *Verticillium lacanii* (Smith and Grula, 1983) are of particular interest in agricultural biocontrol. Leger et al., (1986) reported that chitosanase is co-induced with chitinase in *M. anisophilae*. As most fungi, invertebrate pests and pathogens of plants have chitin as an essential structural component, chitinase activity could have an important place in the repertoire of mechanisms for biological control with the use of beneficial microorganisms. Thus the strongly chitinolytic fungus *Trichoderma harzianum* has good potential for the control of a range of soil-borne plant pathogens (Sivan and Chet, 1989). The fungi is being extensively studied for its significance in

agriculture as well as molecular tools for enhancing plant protection (Woo et al., 2001)

2.4.4. Plant chitinases

The proposed role of chitinases in plants is a defence mechanism against chitin-containing pathogens. Much of the research on plant chitinases has focused on their role as pathogenesis related proteins. It has been observed that purified barley chitinases inhibit the growth of fungal hyphae (Leah et al., 1991). Chitinases are synthesized in plants in response to assorted environmental stimuli, such as fungal or insect attack (Busam et al., 1997), osmotic pressure or developmental stage, such as fruit ripening (Clendennen and May, 1997).

Plants respond to attack by pathogenic microorganisms by inducing the expression of a large number of genes encoding diverse proteins, many of which are believed to have a role in immune response (Collinge et al., 1992). Most of these induced genes encode for chitinases but there are no suitable substrates for these in plants whereas chitin is the principle component of fungi and insects, which led to the proposal that the major natural role for chitinase is defence, primarily against pathogens (Mauch et al., 1988). Bacterial peptidoglycan and in some cases chitosan are also alternative substrates for plant chitinases (Bartnicki-Garcia, 1968; Wessels and Sietsma, 1981).

Heterologous chitinase gene expression is observed in various plants that enhance their defence mechanisms against fungal pathogens (Schickler and Chet, 1997). Plants produce chitinases as a major component of their 'pathogenesis-related proteins' induced following attack by potential pathogens or treatment with elicitors like ethylene (Mauch and Staehlin, 1989). The antifugal activity of the plant chitinases can be greater than that of some bacterial chitinases (Roberts and Selitrennikoff, 1988).

Endochitinases are the most extensive type of chitinase studied in plants (Molano et al., 1977) generating soluble low molecular mass oligomers of N-Acetylglucosamine. Synthesis of exochitinase has been reprorted in many plants. Purified plant endochitinases also show some degree of lysozyme (EC 3.2.1.17) activity, they can hydrolyze β-1,4 linkages between N-acetylmuramic acid and N-Acetylglucosamine residues in peptidoglycan (Boller, 1988; Majeau et al., 1990).

In general, plant endochitinases are monomer proteins of 25 – 35 kDa molecular weight, and have either high or low isoelectric points. Chitinase is induced in plants by various factors including pest and pathogen as well as treatment with elicitors, abiotic factors such as heavy metals and plant hormones. Induction of chitinase is often co-ordinated with the induction of specific β-1, 3-glucanases and other pathogenesis related (PR) proteins (Brederode et al., 1991).

Colorimetric, radioactive and gel electrophoresis based assays (Molano et al., 1977; Boller et al., 1983; Trudel and Asselin, 1989) have been developed to measure endo- and exochitinase enzyme activity in plant tissues.

Nodulation (NOD) factors

Immunochemical studies using gold-labelled wheat germ agglutinin and bacterial chitinases revealed abundant N-Acetylglucosamine residues in secondary walls of plants (Benhamou and Asselin, 1989). The data suggested that the N-Acetylglucosamine residues might be present in the form of glycolipids whereas N-Acetylglucosamine polymers analogous to chitin are absent. Legume roots secrete nodulation signals called Nod factors which are lipochitooligosaccharides i.e. β -1, 4 linked oligomers of N-Acetylgulcosamine, with a fatty acid replacing the N -Acetyl group on their non-reducing ends.

In this context, it is interesting to note that rhizobia (*Rhizobium*, *Bradyrhizobium* and *Azorhizobium*) secrete lipo-oligosaccharides (Nod factors). These signal molecules are tetra- and pentamers of N-Acetylglucosamine with an acyl group at the nonreducing residue (Lerouge et al., 1990; Roche et al., 1991). These nod factors at very low concentrations (10^{-7} to 10^{-10} M) trigger root hair deformation, nodule primordium formation for establishing specific symbiotic interactions with leguminous plants and helps in fixing atmospheric nitrogen (Truchet et al., 1991) and infection-related processes (Brussel et al., 1992). It has been proposed that plant chitinases might interfere by hydrolyzing these bacterial lipo-oligosaccharides (Roche et al., 1991).

2.4.5. Chitin deacetylases

Chitin deacetylase (CDA; E.C. 3.5.1.41) catalyzes the hydrolysis of acetamido groups of N-Acetylglucosamine in chitin into chitosan, a glucosamine polymer. Extracellular CDA activities from *Colletotrichum lindemuthianum* and *M. anisopliae* were suggested to possess dual function in modification of chitin as well as chito-oligomers for the interaction with host and self-defence (Ghormade et al., 2010).

Chitin deacetylase has been isolated and purified from mycelium extracts of fungi, such as *Absidia coerulea* (Gao et. al., 1995), *A. nidulans* and *C. lindemuthianum* (Kauss et al., 1982). *M. anisophilae* secretes CDA and chitosanase, which facilitate the entry of the fungus into the host insect, *Helicoverpa armigera* (Nahar et al., 2004). A biological role involving the enzyme in plant-pathogen interactions has been suggested for CDA from *C. lindemuthianum*, a plant pathogen, and the enzyme is extracellular and active on chitin oligomers. There is a high similarity with CDAs of fungi and bacterial peptidoglycan deacetylases in the functional domain of conserved polysaccharide deacetylase (Kauss et al., 1982).

Nod-B like protein from *Bacillus subtilis* and acetylxylan esterase A from *Streptomyces lividans* also show same functional domain. *Rhizobia* reside in the roots of their host leguminous plants for symbiotic nitrogen fixation. On the basis of the similarity of Nod-B protein in legumes to chitin deacetylase, John et al., (1993) suggested that the specific deacetylation of the non-reducing end of the precursors provide the necessary free amino group for a subsequent acylation. The more soluble chitooligomers could be more efficiently deacetylated by CDAs.

2.5. CHITIN IN PLANT PROTECTION

Both chitin and chitosan have been explored for plant protection. They have been utilized to control disease or reduce their spread, to chelate nutrient and minerals, preventing pathogens from accessing them, or to enhance plant innate defences (El-Hadrami et al., 2010). This area of application of chitin is important because it suggests alternatives to the use of pesticides in crop production and in post-harvest storage.

Two types of protective roles can be ascribed to these compounds. First, at defined concentrations, it presents antifungal properties as shown by its inhibitory action on the mycelial growth of a number of pathogenic fungi, including root pathogens such as *Fusarium oxysporum* and *Pythium aphanidermatum* (El-Ghaouth et al., 1994), and also by its inhibitory effect on spore germination (Hadwiger and Beckman, 1980).

Secondly, it acts as a potent elicitor, therefore enhancing plant resistance against pathogens (Benhamou, 1996). When used to enhance plant defences, chitin and chitosan induce host defence responses. These responses include the induction of lignification (Barber et al., 1989), ion flux variations, cytoplasmic acidification, membrane depolarization, protein phosphorylation, chitinase and glucanase activation (Kohle et al., 1984), the generation of reactive oxygen species, production of proteinase inhibitors (Walker-Simmons and Ryan, 1984;

Pena-Cortes et al., 1988), the triggering of callose formation (Kohle et al., 1985), biosynthesis of jasmonic acid and the expression of unique early responsive and defence-related genes (Thomma et al., 2002).

2.5.1. Mechanism of action

Chitin derivatives are most commonly used as powerful elicitors of host resistance rather than direct anti-microbial agents. Some of the direct activities include:

1. Formation of a physical barrier around pathogen penetration sites, which is often accompanied by elicitation of a hypersensitive response by accumulation of peroxidases that helps in cell wall fortification (Hirano et al., 1999).

2. Ability to bind to biological membranes and initiate the wound healing process. Activate the synthesis and accumulation of phenylalanine ammonia-lyase and peroxidase. The involvement of these two enzymes in the synthesis and assembly of lignin matrix and in the formation of tylloses, chitin derivatives seems to accelerate the process of wound healing (Hirano et al., 1999).

3. Chelation of nutrients and minerals (ex: Fe, Cu) and thus preventing pathogens from accessing them. These polysaccharide molecules were also reported to bind mycotoxins, which may reduce damage to the host tissues caused by toxins (Bornet and Teissedre, 2007).

4. Chitin and derivatives are known to act as potent inducers, enhancing plant responses both locally around the infection sites and systemically to alert healthy parts of the plant. These include early signaling events as well as the accumulation of defence-related metabolites and proteins such as phytoalexins and pathogenesis related proteins (Ahuja et al., 2012).

5. Modulation of plant responses using chitin compounds has been reported in many

pathosystems involving various plant species and a diverse range of pathogens, including virus and viroids (Vander et al., 1998).

6. Plants are thought to possess mechanisms of trans-membrane pattern recognition receptors (PRRs) that are able to interact with pathogen/microbe-associated molecular patterns - PAMPs/MAMPs (Dangl and Jones, 2001). PAMPs/MAMPs can be any effectors secreted by the pathogens by which plants recognize their intruders. Cell wall polysaccharides such as glucans and chitooligosaccharides (CHOS) have been reported to act as PAMPs/MAMPs in many pathosystems. CHOS and chitosan present the advantage of being recognized by plant PRRs and trigger a panel of defence responses (Zipfel, 2009).

2.5.2. Plant responses and signaling in presence of chitin

Plants are constantly challenged by wide range of pests and pathogens in all ecosystems. A plant's ability to withstand pathogen attack is influenced by numerous factors and involves induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Kue, 1982). Systemic acquired resistance refers to a distinct plant defence response that results in a non-specific and long-lasting systemic resistance to a variety of pathogens. Several pathogenesis related (PR) genes, including those that code for chitinases are induced during SAR and serve as molecular markers of plant defence (Ebrahim et al., 2011).

The onset of systemic acquired resistance is followed by hypersensitive response (cell death), production of reactive oxygen species, anti-microbial compounds (phytoalexins), rapid cross-linking of cell-wall proteins, activation of several defence related genes and ultimately an enhanced resistance to pathogens (Hammond-Kosack and Jones, 1996). Many research studies focus on the genetics and biochemistry of the signaling pathways involved such as salicylic acid

(SA), Jasmonic acid (JA), ethylene (ET) and other defence response compounds secreted in disease resistant phenotypes (Howe, 2004; Grant and Lamb, 2006). In recent years, identification and analysis of several Arabidopsis mutants with altered response to pathogens and SAR-inducing chemicals have helped unravel the molecular basis of defence activation in plants. Mutants in the first-class accumulate high levels of salicylic acid (SA), regulated by *NPR1* gene, constitutively express SAR and are resistant to variety of virulent pathogens (Glazebrook, 2001).

Salicylic acid is synthesized by phenylpropanoid pathway, the first step of which is catalyzed by phenylalanine ammonia lyase (PAL) (Coquoz et al., 1998). Isochorismate synthase (*ICS*) has been shown to be necessary for SA synthesis and SAR. Wildermuth et al., (2001) hypothesized that SA synthesis is mediated by *PAL* in cells undergoing cell death at the site of infection whereas it is regulated by *ICS* at adjacent and distal parts of the plant. Arabidopsis mutants unable to accumulate SA show reduced expression levels of pathogenesis related *PR1* gene (Gaffney et al., 1993; Delaney et al., 1994).

Jasmonic acid mediated responses involved pathogenesis related genes, defensins, thionins (sulfur-rich proteins) and proteinase inhibitors (Zhou, 1999; Thomma et al., 2002). SA independent pathogenesis related *PR3* gene encodes for endochitinase and is regulated by JA dependent pathway.

The resistance to some pathogens such as *Alternaria brassicicola* and *Botrytis cinerea* is independent of salicylic acid (Penninckx et al., 1996). These mutants independent of SA/*NPR1* pathway are characterized by the induction of *PDF1*.2 and thionin genes that encode anti-microbial peptides, and require functional ethylene (ET) and jasmonic acid (JA) signaling pathways. Although SA- and ET/JA-mediated signaling appears to regulate distinct defence pathways, several studies indicate cross-interference between these pathways (Maleck and

Dietrich, 1999; Glazebrook, 2001). Expression of some chitinase genes is attenuated in the presence of SA or JA suggesting an alternate signaling pathway in the defence response (Shinya et al., 2007).

Ramonell et al. (2002) used a microarray consisting of 2,375 EST clones representing putative defence-related and regulatory genes to characterize changes in the gene expression patterns of *A. thaliana* in response to treatment with chitin. Interestingly, the levels of transcription of numerous genes were altered as early as 10 min after exposure to chitin, hence translating the earliest changes that may occur in chitin-treated plants. These genes included commonly elicited immune response related genes and produced defence proteins (i.e., phenylalanine amonia-lyase, chitinase, peroxidase) as well as other genes with a function not yet identified. A decrease in transcript abundance of a number of genes encoding cell wall strengthening and wall deposit proteins were all found to downstream the chalcone synthase promoter, suggesting their potential suppression during plant-pathogen interactions (Ramonell et al., 2002).

Chitinases secreted in the apoplasm play a role in the early stage of pathogenesis. They degrade fungal cell walls and release elicitor molecules, which are involved in signal transfer and inform the plant of the attack from hyphae that penetrate the intercellular space. Subsequently, these elicitors bind to particular receptors to switch on the active defence mechanisms. This results in increased apoplastic chitinase secretion, as well as synthesis of vacuolar chitinases. During pathogenesis, when fungal hyphae penetrate and destroy the cell, leading to protoplast burst, the vacuolar chitinases come into action. They degrade newly synthesized chitin chains of hyphae and suppress fungal growth (Collinge et al., 1992).

2.5.3. Application of chitosan as a protective agent

Substratum amendment with chitosan was reported to enhance plant growth and suppress some devastating soil-borne diseases. For example, in tomato, root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* was suppressed using chitosan amendments (Lafontaine and Benhamou, 1996). Various methods of application of chitooligomers (CHOS), chitosan and chitin are practiced to control or prevent the development of plant diseases or elicitation of plant innate defences against pathogens.

Part of the effect observed by chitosan on the disease incidence of pathogens comes from the fact that it acts as an elicitor and enhances plant defence responses. The other part is linked to the fact that this biopolymer is composed of polysaccharides that stimulate the activity of beneficial microorganisms in the soil, such as *Bacillus*, *Pseudomonas*, Actinomycetes, mycorrhiza and other rhizobacteria (Murphy et al., 2000). This alters the microbial equilibrium in the rhizosphere disadvantaging plant pathogens. Beneficial microorganisms, on the other hand, exhibit antagonistic properties through mechanisms such as parasitism, antibiosis, and induced resistance (Daayf et al., 2003).

Applied as seed coating agents

Guan et al., (2009) examined the use of chitosan in the development of maize seeds. Chitosan induced a decline in malonyldialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars, proline, peroxidase and catalase activities. In other studies, seed priming with chitosan improved the vigor of maize seedlings (Shao et al., 2005).

Reddy et al., (1999) reported that chitosan increases wheat seed resistance to certain

diseases and improves their quality and/or their ability to germinate. In carrot, seed coating helps restrain further development of Sclerotinia rot (Cheah et al., 1997). Ruan and Xue (2000) showed that rice seed coating with chitosan might accelerate their germination and improve their tolerance to stress conditions.

Applied as foliar treatment

Foliar application of chitosan has been reported in many systems and for several purposes. Foliar application of a chitosan pentamer affected the net photosynthetic rate of maize and soybean one day after application (Khan et al., 2002). This correlated with increases in stomatal conductance and transpiration rate.

Iriti et al., (2009) unveiled some of the aspects through which chitosan was able to reduce transpiration rate in bean plants after being used as a foliar spray. The authors showed that this activity was likely occurring due to the increase in abscisic acid (ABA) content in the treated leaves. Chitosan has also been extensively utilized as a foliar treatment to control the growth, spread and development of many diseases caused by viruses, bacteria, fungi and pests (Rabea et al., 2003). Faoro et al. (2008) showed that the foliar application of chitosan on barley reduced locally and systemically the infection by powdery mildew pathogen *Blumeria graminis* f. sp. *hordei*.

Application as soil amendment

Chitosan used as a soil amendment was shown to control Fusarium wilts in many plant species (Rabea et al., 2003). Applied at an optimal concentration, chitosan was able to induce a delay in disease development, in forest nurseries suffering from *F. acuminatum* and *Cylindrocladium floridanum* infections. These infections were dramatically reduced upon the use

of chitosan as a soil amendment (Laflamme et al., 1999).

2.6. SUMMARY

The microbial break down of lobster shells provides an environmentally safe and economically viable alternative to conventional chemical process to synthesize chitin derivatives. The decomposition activities of various microorganisms investigated, suggests their potential application in sustainable approaches to extract chitin from crustacean shell wastes. Many factors, such as initial pH value, temperature, external carbon source and carbon to nitrogen ratio have been reported to influence the degradation process and consequently effect the degradation efficiency. The review also capitulates the application of chitin and chitosan as elicitors in plant-pathogen interactions.

2.7. OBJECTIVES

The project focused on microbial degradation of lobster shells to extract bioactive compounds for use in plant protection. The objectives of the present study were,

1. To isolate and characterize microorganisms that degrade lobster shells, study their optimum cultural conditions for shell digestion, extraction of chitin derivatives and purification of chitinase from the digested extracts.

2. To investigate the efficacy of bioactive compounds present in the extracts on enhancing plant protection in *Arabidopsis thaliana* by inducing disease resistance against a bacterial pathogen *Pseudomonas syringae pv. tomato* DC3000 and a fungal pathogen *Botyrtis cinerea*.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF SOIL MICROBES TO EXTRACT CHITIN DERIVATIVES FROM LOBSTER SHELLS

3.1. INTRODUCTION

The lobster industry in Nova Scotia contributes to the economy through local markets and exports. The demand for Atlantic lobster around the world has made it the most profitable fishery resource, generating $1 billion CAD in exports (Fisheries and Oceans Canada, 2012). More than 150 million pounds of lobster meat (half of the landed volume) is processed every year. The industry provides employment in the Atlantic region spanning across traditional fishermen families, processing industries, domestic and international markets. Annual lobster landings average 50,000 to 55,000 metric tonnes. Conservation measures to protect lobster populations are regulated by the sustainability framework of Atlantic lobster (Maritime Lobster Panel, 2013).

The processing of lobster meat involves removal of the exoskeleton and the shells are usually dumped on the shoreline, in the sea or landfills. However, with an increase in production (both fresh catch and processing) discarding huge volume of shells threatens the environmental sustainability of coastal areas due to the foul smell and release of biogenic amines (Xu et al., 2008). The present waste management practices challenge the processing industries.

Lobster shell is composed of chitin, calcium, protein, lipids and carotenoids. Chitin fibers are associated with proteins and calcium carbonate molecules, forming the tough exoskeleton (Stirn, 2012). Chitin is the second most abundant polysaccharide in nature, next to cellulose and is made up of (β-1,4) N-Acetylglucosamine units. Chitin and its derived compounds are used

extensively in chemical, agriculture and pharmaceutical industries (Kandra et al., 2012). Commercial production of chitin presently involves alkali and acid treatment of crustacean exoskeleton to remove protein and calcium carbonate constituents.

Previous studies have been carried out to extract chitin through a combination of biochemical methods. Proteolytic enzymes like alcalse, trypsin (Guerard et al., 2007) and lactic acid have been used to remove protein and calcium counterparts of the shells, respectively. Microorganisms from crustacean gut microbiota and probiotic curd were used to deproteinise and demineralise the shells (Rao et al., 2000). Nevertheless, the major production of chitin is from sea sources with approximately 10 gigatons recycled every year (Arcidiacono and Kaplan, 1992). Microorganisms found in chitin rich niches can enzymatically breakdown the shells by chitinases, proteases and organic acids.

In terrestial and aquatic ecosystems chitin is recycled by free-living bacteria, fungi and those in association with animal guts (Gooday, 1990a). The microbial population may use chitin as the sole source of carbon and nitrogen. Organisms that degrade chitin, using chitinases are labelled as chitinolytic. The chitinolytic system cleaves glycosidic bonds of chitin by hydrolysis and chitinases belong to families 18 and 19 of glycosyl hydrolases. Organisms that are not composed of chitin can also synthesis chitinase to breakdown chitin in the environment (Davis and Eveleigh, 1984). The chitinolytic mechanism provides an environmentally safe alternative to extract chitin and other derived compounds from lobster shells.

In the following research, microorganisms were isolated from soil samples taken from fields where lobster shells were applied as a soil amendment over a period of one to three years. This study aims to extract chitin derivatives from lobster shells using microorganisms and to optimize cultural conditions and understand the process of microbial degradation.

3.2. MATERIALS AND METHODS

3.2.1. Preparation of lobster shell powder

Lobster shells (fresh and cooked) were obtained from Aquashell Holdings Inc., Wallace, NS, Canada. The shells were washed in rainwater and sun-dried for three consecutive days, then crushed in a feed – mill grinder and stored at 4 °C. They were then finely grounded in a home coffee grinder, refined through a 1 mm sieve (Appendix II, Figure A.1) and used as the lobster shell powder (LSP).

3.2.2. Isolation and screening of microbes for degrading lobster shells

Soil samples were collected at Jost vineyards, Malagash, NS, Canada. Serial dilutions were made from 1 g of homogenized soil samples and spread-plated on lobster shell agar plates containing 0.5% (w/v) lobster shell powder (carbon source) in M9 minimal buffer (Appendix I) with 2% (w/v) agar (pH 7.0). The Petri plates were incubated at 25 °C for three to five days. Colonies growing well or showing distinct clearing zones were selected from nearly 20 different microorganisms (data not shown) for subsequent sub-culturing on LSP agar plates. They were given arbitrary numbers (such as S113, S224, S223, S2231, S232, S232) for convenience. The isolated cultures were maintained in LSP agar plates and stored in 60% (v/v) glycerol at -80 °C (Chang *et al.,* 2003).

Four microorganisms which were previously studied (or belong to same genus) for chitinolysis and bioconversion of crustacean shells (see Chapter 2), *Bacillus subtilis* (Sini et al., 2007), *Pseudomonas fluorescens* (Oh et al., 2007), *Trichoderma harzianum* (Woo et al., 2001) and *Lactobacillus acidophilus* (Adour et al., 2008) were investigated (cultures obtained from laborotary stocks) along with the soil isolates on lobster shell degradation activity. Liquid

cultures of the microorgnisms were grown (by inoculating a single colony picked from agar plates) in M9 buffer containing lobster shell powder (carbon source) for 48 h at 25 °C. spot inoculated (30 µL) on agar plates containing 0.5% (w/v) chitin (powder chitin from shrim shells, Sigma®), fresh or cooked lobster shell powder in M9 buffer and incubated at 25 °C. Colony diameter was measured from average of three plates (biological replicates) of each microorganism and clear zones were observed under a light microscope (Olympus®) on 7th day after incubation.

3.2.3. Screening for deproteinisation, demineralisation and chitinolytic activity

Liquid cultures of microorganisms (1 mL) were inoculated in 50 mL tubes containing 1% (w/v) of fresh or cooked lobster shell powder and 20 mL M9 buffer. The tubes were incubated at 25 °C on an orbital shaker (New Brunswick Scientific®) at 250 rpm and samples were collected every day and centrifuged at 14,000 × *g* (Thermo scientific® Sorvall). The supernatants were used for determining protease (deproteinisation), calcium (demineralisation), N-Acetylglucosamine and chitosan (chitinolytic activity) in the culture extracts. The assays were performed in triplicates.

3.2.3.1. Protease assay

The protocol is adapted from the phenol quantification method described by Folin and Ciocalteau (1927). The reaction mixtures of sample (100 µL) and 0.65% (w/v) casein (500 µL, in 50 mM potassium phosphate buffer, pH 7.5) were incubated in 1.7 mL Eppendorf tubes at 37 °C for 10 min. The reaction was stopped by adding 110 mM trichloroacetic acid (500 µL) for 30 min and centrifuged at 14,000 × g for 2 min (Beckman Coulter™ Microfuge®). The supernatants (200 µL) were transferred to clean tubes containing Folin and Ciocalteau's reagent

(100 μL) and 0.5 M Na_2CO_3 (500 μL). The reaction was incubated again for another 30 min and the tubes were centrifuged.

Protease activity was detected by the development of blue colour due to the binding of tyrosine units with the Folin's reagent. The supernatants (200 μL) were transferred to a 96-well plate and read at absorbance 660nm (Biotek® Epoch microplate spectrophotometer). Protease units were quantified with tyrosine as the standard and expressed in units / mg of protein (Regression equation: $y = 0.0266x+0.1576$, $R^2 = 0.99768$). Protein concentration of the samples was measured by the Bradford (1976) method with bovine serum albumin (Sigma®) as the standard and read at absorbance 590nm (Regression equation: $y = 0.6863x+ 0.2416$, $R^2 = 0.942$).

3.2.3.2. Calcium quantification

Approximately 5 mL of samples collected on 7th day after incubation was fed into a Varian® atomic absorption spectrometer and the concentration of calcium (in ppm) in the culture extracts was measured with known calcium standards (laborotary stock).

3.2.3.3. N- Acetylglucosamine and chitosan assays

The experimental setup was the same as described above but chitin powder (from shrimp shells, Sigma) was used as the carbon source in place of the lobster shells. The reaction mixture containing samples (1.5 mL) and 3,5 Dinitrosalicylic acid reagent (750 μL) in glass vials were heated in a boiling water bath for 5 min. Presence of N-Acetylglucosamine (GlcNAc) was detected by a change of colour from yellow to red after the reaction with 3,5- Dintirosalicylic acid. The vials were cooled down to room temperature and absorbance was measured at 545nm (Biotek®) with N- Acetylglucosamine (Sigma®) as the standard (Monreal and Reese, 1969). (Regression equation: $y = 0.0144x-0.0282$, $R^2 = 0.9908$).

Colorimetric assay of chitosan was performed according to the method of Badawy (2012). Microbial culture extracts (1 mL) and standard solutions of chitosan (Sigma®) were pipetted into Eppendorf tubes and 0.5 M $NaNO_2$ (100 µL) was added. The mixture was shaken briefly and the tubes were incubated in a water bath at 80 °C for 30 min. The pH of the reaction was raised to 8.0 by adding 100 mM NaOH (200 µL) and then 40 mM thiobarbituric acid solution (500 µL) was added. The tubes were again incubated in a water bath for 10 min. The solutions were cooled and the purple color that developed was read at absorbance 555nm (Biotek®). (Regression equation: $y = 0.0077x-0.0356$, $R^2 = 0.94526$).

Two microorganisms isolated from soil samples, S223 and S224, exhibiting higher degradation activities than others were selected and studied in further experiments on the biological breakdown of lobster shells.

3.2.4. Optimization of culture conditions

To find the optimum growing conditions for S223 and S224, various factors affecting degradation process were taken into account (Table 3.1). Liquid cultures of microorganisms were inoculated in 50 mL tubes and agar plates containing lobster shell powder in M9 buffer. The standard conditions of experimental setup in tubes were 0.1 g of shells in 10 mL media incubated at 25 °C, pH 7.0 for 14 days and modified accordingly for each factor tested. Treatments were run in triplicate and the study analyzed one factor at a time. Supernatants were collected by centrifugation at 6,000 × *g* (Thermo scientific® Sorvall) for 20 min. Deproteinisation and decalcification activities were measured as described above.

N-Acetylglucosamine (GlcNAc) was quantified by the method described by Reissig et al., (1955). The reaction mixture containing culture extracts (1 mL) and 80 mM potassium tetraborate, pH

8.9 (500 µL) was boiled vigorously in a water bath for 3 min. The glass vials were cooled and 6 mL of diluted p-Dimethylaminobenzaldehyde reagent was added. The vials were then incubated at 37 °C for 30 min. The samples were transferred to 1 mL cuvettes and read without delay at absorbance 545nm in a spectrophotometer with N-Acetylglucosamine (Sigma®) as the standard. (Regression equation: $y=0.021x+0.0013$, $R^2=0.99996$). Temperature and pH experiments were carried out on agar plates by spot inoculation as described earlier in Section 3.2.2.

Table 3.1. Factors investigated for optimizing culture conditions of microorganisms

	Factors	Levels
1.	Shells	Cooked, Fresh
2.	Fermentation	Solid, Liquid state
3.	Concentration of shells	1, 2, 5% (w/v)
4.	Inoculum	1, 5, 10% (v/v)
5.	Time point	7, 14, 21, 28 days
6.	Temperature	20°, 25°, 30°, 37 °C
7.	pH	5, 7, 9

3.2.5. Extraction of chitinase from culture filtrates

The microbes were grown under optimal conditions in 100 mL M9 buffer (pH 7.0) containing 1 g of fresh lobster shells at 25 °C, 150 rpm for 14 days in 250 mL flasks closed with cotton plugs. Following centrifugation at 6,000 × *g* for 20 min (Thermo scientific® Sorvall), the supernatants were collected and filter sterilized using a 0.2 µm filter for use as crude enzyme extract. They were stored frozen at -20 °C for long term use.

3.2.5.1. Detection of chitinase activity by gel diffusion assay

Gel plates were prepared as explained in the method of Velasquez and Hammerschmidt (2004). Glycol chitin (1 mL) solution was added to 100 mL of 1% (w/v) agarose solution in 10 mM sodium phosphate buffer (pH 7.0). The resulting suspension was stirred to ensure homogeneity of the substrate and 30 mL aliquots were poured into Petri plates (150 × 15 mm). After solidification small wells (2mm diameter) were carved in the agarose gels at a distance of 2 cm from each other to form a square grid. 10 µL of the crude extracts were loaded to each well and the plates were incubated at 37 °C for 4 h. After incubation gels were stained with 0.01% (w/v) fluorescent brightener 28 in 500 mM Tris- HCl (pH 8.9) and incubated again for 10 min at room temperature.

The plates were rinsed and flooded with distilled water followed by overnight color development in the dark. Dilutions of *Streptomyces griseus* chitinase (Sigma®) were prepared to serve as the standard. After the assay the gel was photographed under long wave UV transillumination (BioRad GelDoc™). Glycol chitin binds to the fluorescent brightener and contrast develops between the fluorescent background and dark circular zones, which indicate chitin hydrolysis by chitinase.

3.2.5.2. Purification of chitinase enzyme

The protein from crude samples was precipitated with ammonium sulfate (85% saturation). The precipitate was collected by centrifugation at 14,000 × *g* for 30 min (Thermo scientific® Sorvall) and suspended in 50 mM sodium phosphate buffer (pH 7.0). It was dialyzed against the same buffer overnight. Chitin affinity chromatography was carried out according to the method described by Escott et al., (1998).

Equal volume of 1% (w/v) colloidal chitin was added to the enzyme solutions and incubated for 2 h at 4 °C. The suspensions were transferred to a glass column (1 × 40 cm) with sufficient glass beads (1 mm diameter) to occupy a bed volume of 25 mL and a plug of cotton was placed over the upper surface of the column matrix. The column was eluted at a flow rate of 1 mL/min against gravity in sodium phosphate buffer to remove unbound proteins. After that, the bound chitinase was eluted in 50 mM sodium acetate buffer (25 mL, pH 4.0). The obtained preparations were dialyzed overnight against sodium phosphate buffer (pH 7.0). The desalted samples were concentrated using Amicon® ultrafiltration (PM30) centrifugal tubes and stored at -20 ºC (Brzezinska et al., 2013).

3.2.5.3. Chitinase quantification

At each purification step chitinase activity was measured using a chitinase assay kit (Sigma®) containing substrates 4-nitrophenyl-β-D-N,N′,N′-triacetyl- chitotriose [4-NP-$(GlcNAc)_3$], 4-nitrophenyl-β-D-N,N′-diacetylchitobioside [4-NP-$(GlcNAc)_2$] and 4-nitrophenyl-N-acetyl-β-D-glucosaminide [4-NP-GlcNAc] to detect endochitinases, exochitinases and N-acetylglucosaminidases, respectively. The released 4-nitrophenol (NP) was measured colorimetrically at a 405nm absorbance (Biotek® Cytation 3 Imaging reader). One unit of enzyme activity is defined as the amount of enzyme releasing 1µM NP.min^{-1} at pH 4.8 at 37 °C. The protein content was measured by the Bradford method (1976).

3.2.5.4. SDS-PAGE

Molecular mass of the purified chitinase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel in Tris-glycine buffer, pH 8.3, adopting the method of Laemmli (1970). The protein molecular marker ladder ranged from

10kDa to 175kDa and chitinase from *Streptomyces griseus* was used as a reference. The gel was stained in 0.15% (w/v) Coomassie brilliant blue R-250 in ethanol-acetic acid-water (20: 10: 70, by volume) for 2 h and destained overnight in the same solvent without the dye. The gel was photographed with a white tray underneath to visualize proteins in bright field illumination.

3.2.6. Antifungal activity

Hyphal inhibition assay of the filtrates was performed according to the method of Roberts and Selitrennikoff (1988). A 2mm mycelial plug of the test fungi was placed at the center of one-half strength potato dextrose agar (Difco®) plates. Wells of 6mm diameter were carved along the perimeter in the agar plates and 50 μL of microbial culture extracts were added to the wells. The inhibition zones of hypahe were observed in comparison to its growth around the control well after three to six days of incubation from four replication plates.

3.2.7. Identification of soil isolates

Identification of the microorganisms (S223 and S224) isolated from soil samples involved morphological observations, biochemical tests and gene sequencing.

3.2.7.1. Morphology and physiological characterisations

The Gram – positive, filamentous and spore bearing nature of the soil isolates suggested that they might belong to Actinomycetes. They were observed for their morphology and physiological characteristics according to the procedures as described in the International Streptomyces project (Shirling and Gottlieb, 1966). The isolates were grown on Tryptone–malt extract broth and streaked onto Petri plates (three replications) containing yeast extract malt extract agar (YEME), oats infusion agar, starch – inorganic salts agar, glycerol – asparagine agar or lobster shell powder agar. The colony growth and characteristics were recorded on the 7th, 14th

and 21st days of incubation. Slants of peptone–iron agar and tyrosine agar were used to detect any melanin pigments produced by the microorganisms.

3.2.7.2. Nutrient utilization tests

Nutrient metabolism of the microbes were tested by adding different carbohyrdrates, amino acids and other organic compounds to M9 buffer with universal pH indicator at a final concentration of 1% (w/v) and incubated in 24-well plates at 25 °C. The growth of microorganisms was observed visually after 10 to 15 days.

3.2.7.3. 16S rDNA amplification, sequencing and alignment

Gene coding for 16s rRNA of the actinomycetes was amplified with universal eubacterial primers and purified DNA preparations were sequenced at Marine Station Biologique de Roscoff, France. PCR amplification with Taq polymerase (Promega®), and two universal eubacterial specific primers: 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) was performed according to the procedure of Salun et al., (2010). The PCR products were purified using Montage™PCR96 Cleanup kit (Millipore). The full-length sequences of 16S rDNA were sequenced in ABI 3130 x 1 capillary sequencer (Applied Biosystems®) and compared to public nucleotide databases using the advanced Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST).

3.2.8. Data analysis

Experiments were setup in a completely randomized design and the quantification assays were measured in three technical replicates of individual biological sample. Linear regression analysis for standard curves used in quantification assays was done using a Microsoft Excel (Microsoft®, 2013) scatter plot. An analysis of variance on data was performed after satisfying

the assumptions of normality and constant variance using the SAS v. 9.3 statistical software package with General Linear Model (proc glm) or Mixed procedure (proc mixed) at a 95% confidence interval. Multiple means comparison was done using a Tukey's HSD (honest significant difference) test at $\alpha = 0.05$ (α – level of significance). In the graphs presented in the results section, data points sharing the same letter are not significantly different and SAS macro pdmix.sas was used to generate letter groupings.

3.3. RESULTS

3.3.1. Isolation and screening of microbes for degrading lobster shells

Six microorganisms were subsequently isolated from soil samples on lobster shell agar plates, based on colony growth and formation of a clearing zone. They were arbitrarily named as S113, S224, S223, S2231, S23, and S232. Preliminary identifications were done by Gram staining and morphological observations of colonies formed on lobster shell agar plates (data not shown). The first four (S113, S224, S223, S2231) were tentatively identified as actinomycetes (filamentous bacteria) and last two (S23, S232) as bacteria.

The soil isolates in addition to four other microorganisms (*Bacillus subtilis*, *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Lactobacillus acidophilus*) were screened for their lobster shell degrading activity. All ten microorganisms grew on lobster shell agar plates and the colony diameter (Figure 3.1) was measured on the seventh day of incubation. *Trichoderma* (not listed in the figure) had covered the entire plate in three days. Bacteria formed a slimy growth at the point of inoculation whereas actinomycetes grew in patterned colonies (examples are shown in Figure 3.2). Clearing zones were observed under a light microscope and circular halo zones were visible around and beneath the colonies (Appendix II, Figure A.2).

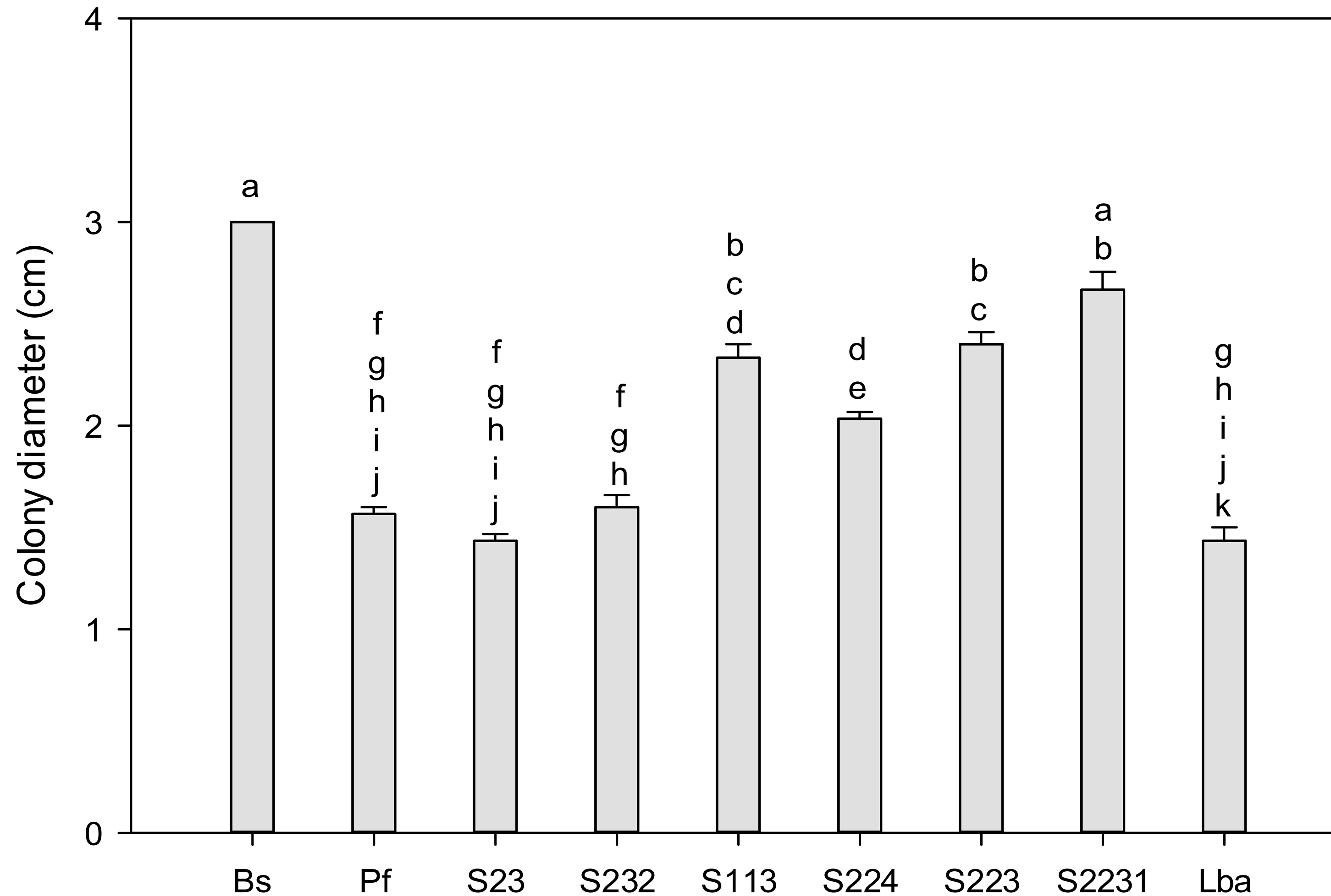

Figure 3.1. Colony diameter measured on 7th day after inoculation of microbial cultures on agar plates containing 0.5% (w/v) fresh lobster shells [*SEM* ± 0.052, P < 0.0001]. Values represent mean ± SE of 3 replications per treatment and values sharing the same letter(s) are not significantly different (Tukey's HSD at α = 0.05). In order to simplify the graph, data for only fresh lobster shells are shown and extended data are presented in Appendix II, Table A.1. (Bs – *B. subtilis*, Pf – *P. fluorescens*, Lba – *L. acidophilus*).

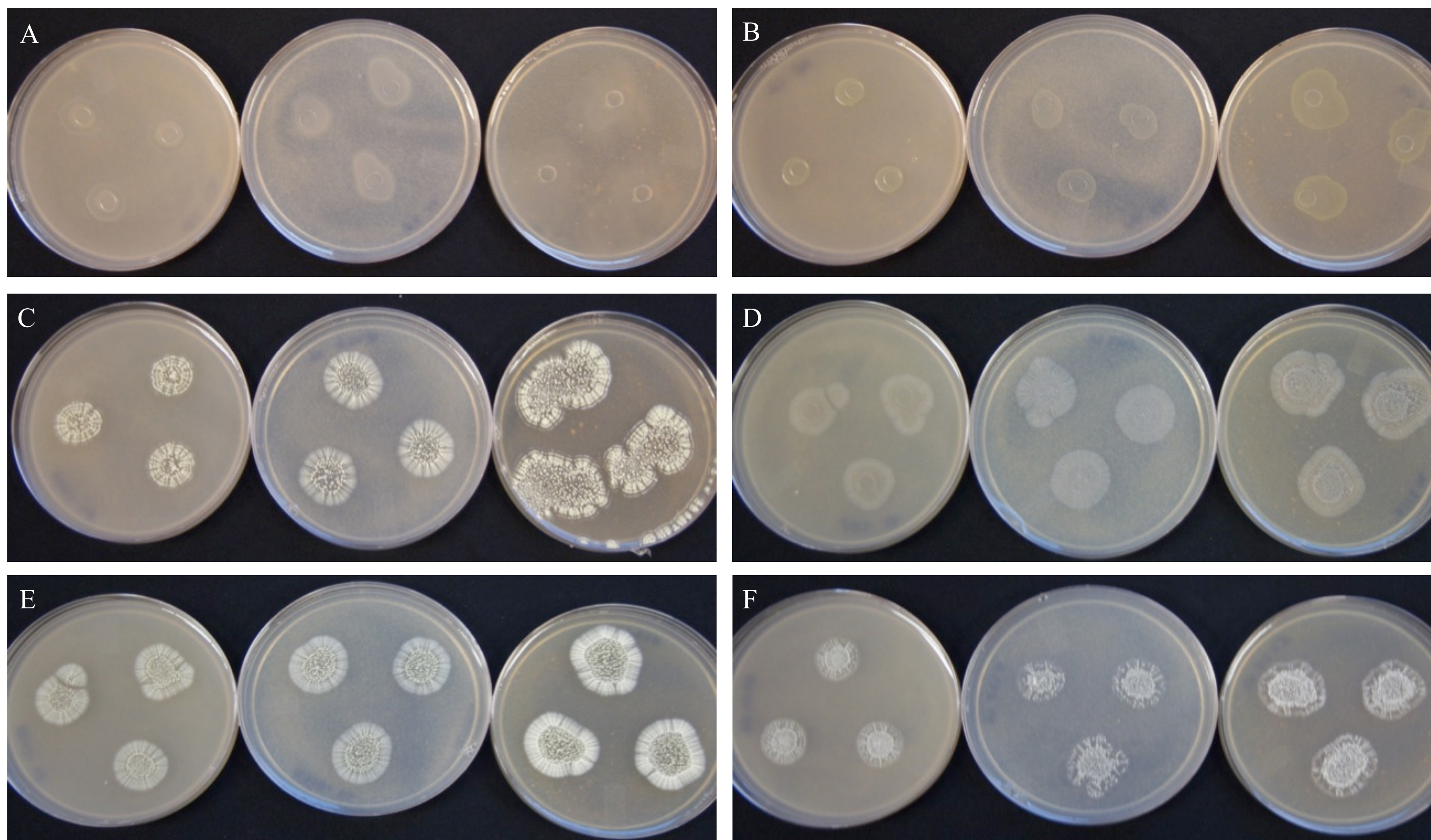

Figure 3.2. Microbial colonies growing on agar plates containing 0.5% (w/v) chitin, cooked or fresh lobster shells, respectively (left to right). Plates were incubated at 25 °C for seven days. (A) *B. subtilis* (B) S23 (C) S223 (D) S224 (E) S2231 (F) S113

3.3.2. Screening for deproteinisation, demineralisation and chitinolytic activity

Four microbes, *B. subtilis*, *T. harzianum*, S223 and S224 exhibited deproteinisation activity in fresh lobster shells (Figure 3.3), whereas only *B. subtilis* showed detectable protease units in digestion with cooked lobster shells among the ten microorganisms screened. *B. subtilis* exhibited significantly greater (P < 0.0001) deproteinisation activity in fresh shells than other microorganisms. However, two actinomycetes (S223 and S224) showed a gradual increase in protease units in their samples over the incubation period.

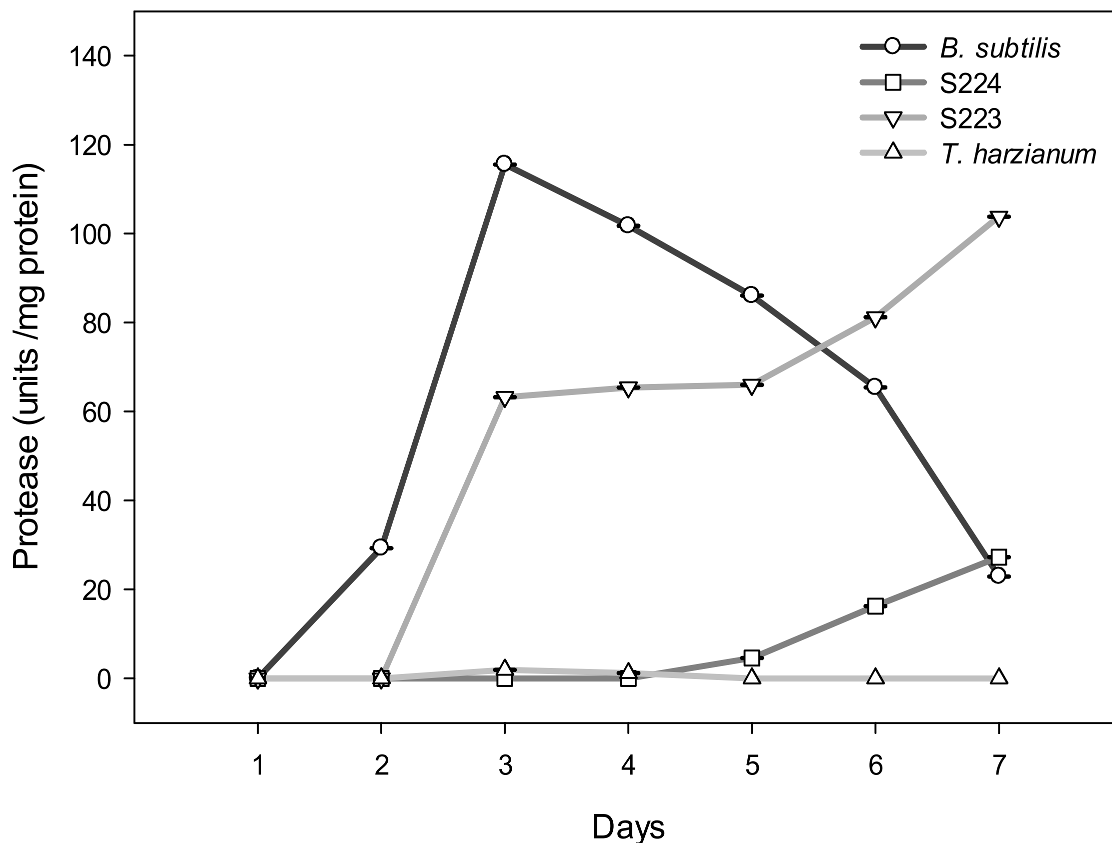

Figure 3.3. Protease activity measured in the culture supernatants of microbes grown in fresh lobster shells from one to seven days after incubation, using Folin-Ciocalteau's phenol reagent [*SEM* ± 7.34E-03, P < 0.0001]. Four microorganisms (*B. subtilis*, *T. harzianum*, S223 and S224) exhibited deproteinisation activity among ten screened. Values represent mean ± SE of 3 replications per treatment. To minimize complexity of the graph, letter groupings are presented in Appendix II, Table A.2.

The samples were continuously monitored for their pH changes every 24 h of incubation and the pH moderately shifted towards alkaline. The lobster shells themselves contributed to an increase in pH and samples incubated with microorganisms had relatively lower pH than the shells (Appendix II, Tables A.3 and A.4).

Calcium content of cooked shells was higher than that of fresh shells (Appendix II, Figure A.3) and so, the release of Ca^{2+} to the medium was expected to be higher in incubation with cooked shells. S224 and *B. subtilis* had higher calcium content in their samples on incubation with cooked and fresh lobster shell powder respectively (Figure 3.4). The microorganisms exhibited significant differences (P < 0.0001) in demineralisation activity.

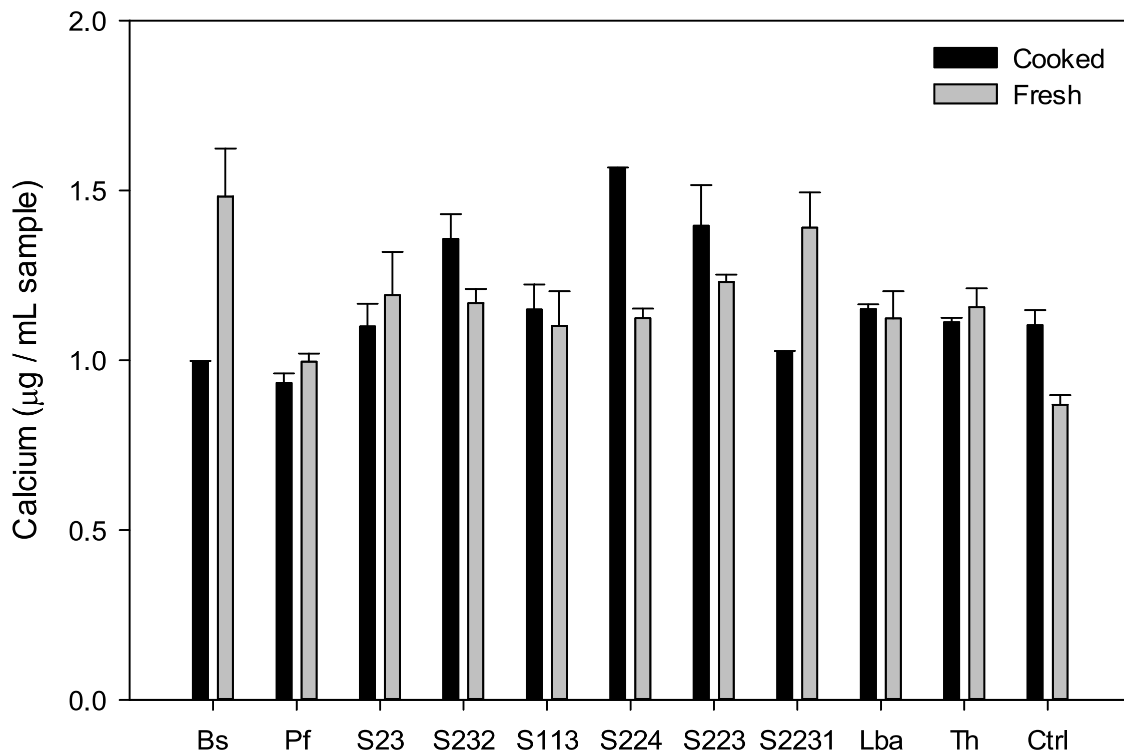

Figure 3.4. Calcium content measured in the culture supernatants of microbes grown in cooked and fresh lobster shells on seven days after incubation by AAS [*SEM* ± 5.62E-02, P < 0.0001]. Values represent mean ± SE of 3 replications per treatment. To minimize complexity of the graph, letter groupings are presented in Appendix II, Table A.5. (Bs – *B. subtilis*, Pf – *P. fluorescens*, Lba – *L. acidophilus*, Th – *T. harzianum*, Ctrl – control-no inoculation).

Endo and exochitinases hydrolyze chitin to produce N-Acetylglucosamine units, the monomer of chitin. Chitin is digested by another enzymatic pathway, where chitin deacetylase cleaves it to form chitosan and acetyl group. Pure chitin was used as the carbon source to screen chitinolytic microorganisms because preliminary colorimetric assays using 3,5- Dinitrosalicylic acid reagent was not able to recognize N-Acetylglucosamine in the samples collected from incubation with lobster shells because of the pigments present in the shells (data not shown).

Chitinolytic activity of the microorganisms was detected by quantifying N-Acetylglucosamine (Figure 3.5 A) and chitosan (Figure 3.5 B) in the samples collected from days 1 to 7 after incubation. *P. fluroscens*, S23, S224 and S223 exhibited significantly ($P < 0.0001$) higher N-Acetylglucosamine content. Isolates S224 and S113 produced pink colour pigments in the medium that interfered with the chitosan assay, where the compound is detected by development of purple colour. Their sample blanks (without reagent) were read to subtract from the original value to quantify chitin deacetylase activity. S223 generated significantly ($P < 0.0001$) high quantities of chitosan.

Screening for chitinolytic activities lead to select S223 and S224 for further experiments on lobster shell degradation. Research studies have been published (see Chapter 2, section 2.4.) on *Bacillus subtilis, Pseudomonas fluorescens, Trichoderma harzianum* and *Lactobacillus acidophilus* degrading other crustacean shells (shrimp, crab). These soil isolates (S223 and S224) are unknown of their chitinolytic metabolism and exhibited degradation activities in all three pathways (deproteinisation, decalcification and chitinolysis) and were comparatively higher than the other microorganisms screened.

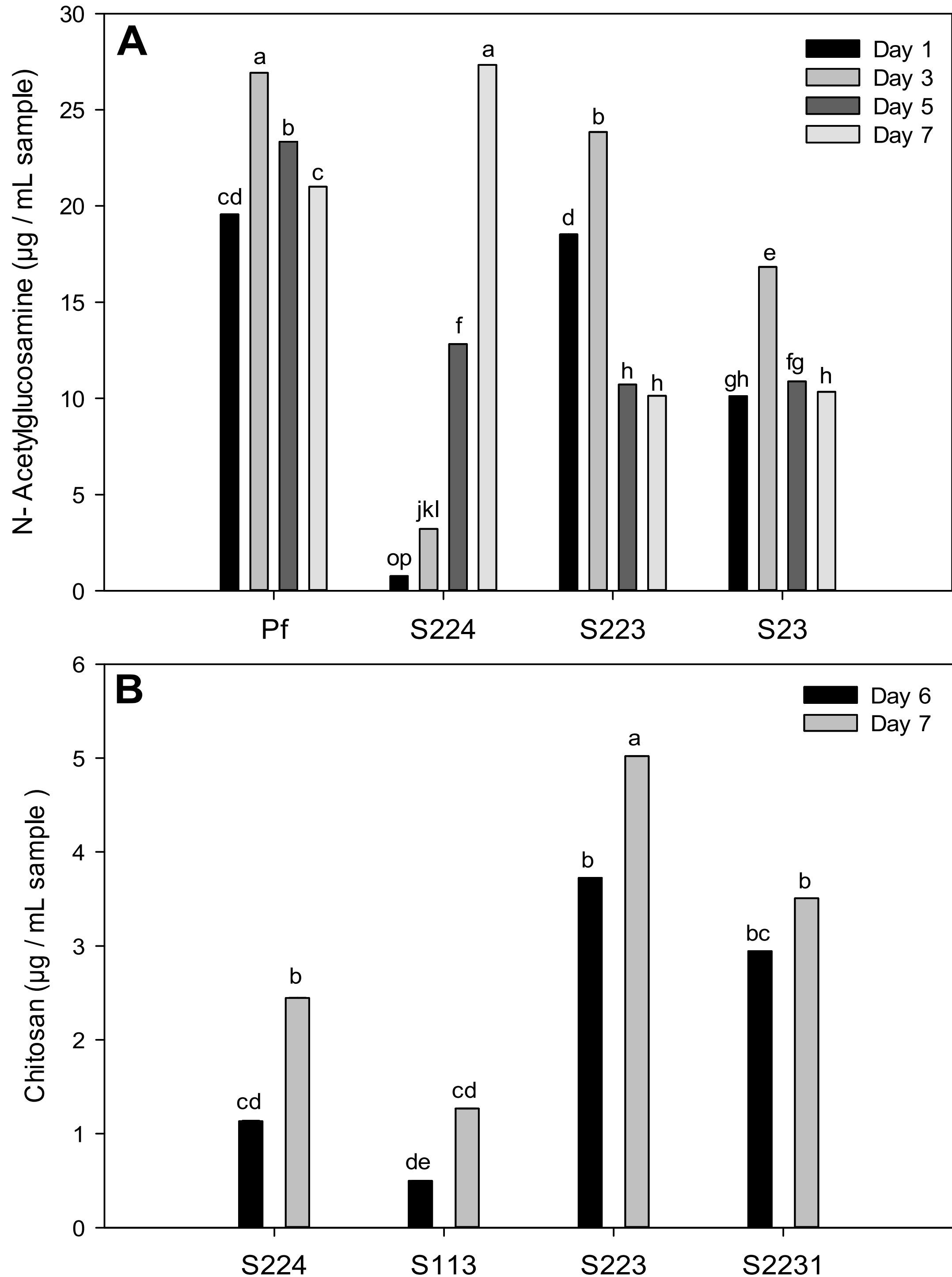

Figure 3.5. (A) N-Acetylglucosamine and (B) chitosan quantified in the culture supernatants where pure chitin was used as the substrate medium for microorganisms. Result of four microorganisms with significantly high N-Acetylglucosamine [*SEM* ± 2.70E-03, P < 0.0001] and chitosan [*SEM* ± 1.24E-03, P = 0.0002] content are shown. Values represent mean ± SE of 3 replications per treatment and values sharing the same letter(s) are not significantly different (Tukey's HSD at $\alpha = 0.05$). To minimize complexity of the graphs, extended data are presented in Appendix II, Tables A.6 and A.7. (Pf – *P. fluorescens*).

3.3.3. Optimization of culture conditions

Several factors were taken in account for optimizing the culture conditions of the microorganisms in lobster shell digestion (Table 3.1). The factors were analysed in a one-at-a-time manner to reduce complexity. The first factor to study was the type of shells and since the microbial digestion of cooked shells produced lesser N-Acetylglucosamine units than fresh shells, cooked shells were excluded from other experiments. The concentration of shells and microbial inoculum factors were expected to increase the degradation parameters quantified at increased levels yet constrained by the incubation time. The optimization of time period for degradation of lobster shells is of particular interest because preliminary studies were not able to detect N-Acetylglucosamine in the samples collected seven days after incubation with lobster shells (data not shown). The state of digestion (solid vs. liquid) was studied to observe the degradation process under the presence of very little moisture in the media, the water content in solid digestion was one hundred times lesser than in liquid digestion. Temperature and pH ranges were studied to observe the adaptability of the microorganisms at different temperatures and pH. The optimum temperature for growth is 25 – 30 °C and pH is 7 and results of that experiment are shown in Appendix II, Figures A.4.

Deproteinisation: Protease quantity was not detected in cooked shells because of low protein content in the shells (Figure 3.6). 2% exhibited significantly ($P < 0.0001$) higher deproteinisation than 5% (w/v) concentration of shells. Solid and liquid digestion had no significant ($P = 0.96$) difference on deproteinisation whereas microbial inoculum significantly ($P < 0.0001$) increased deproteinisation. Samples from 14 days of incubation had protease units but the activity is not significant ($P = 0.06$) over the incubation period. S224 exhibited higher protease activity when compared to S223.

Demineralisation: Cooked shells filtrates obtained from S224 digestion had significantly ($P = 0.001$) higher calcium content, similar to the trend observed in screening experiments (Figure 3.7). Increasing concentration of shells showed a significant ($P < 0.0001$) increase in calcium quantity and the volume of microbial inoculum had no significant ($P = 0.72$) effect on demineralisation. Liquid digestion released ($P = 0.069$) more calcium into the media because of increased solubility. There was significantly ($P < 0.0001$) higher calcium content in the samples obtained on seventh day after incubation than at later time points of the experiment.

Chitinolytic activity: Preliminary experiments (data not shown) were not able to detect the presence N-Acetylglucosamine in the samples collected from lobster shells on days 3, 7, 10 and 14 after incubation using 3,5-Dinitrosalicylicacid due to sensitivity of the assay affected by lobster shell pigments. Hence, the protocol to quantify N-Acetylglucosamine was changed in the optimization experiments using p-Dimethylamino benzaldehyde. Cooked shells had significantly less ($P < 0.0001$) N-Acetylglucosamine (GlcNAc) units in the extracts than fresh shells (Figure 3.8). Concentration of shells and microbial inoculum significantly ($P < 0.0001$) increased the release of N-Acetylglucosamine into the medium. Liquid digestion had significantly ($P < 0.0001$) high N-Acetylglucosamine units in the samples than the solid-state digestion. N-Acetylglucosamine quantified from 14 days of incubation were significantly ($P = 0.0009$) higher than other incubation periods. The shells show N-Acetylglucosamine in their samples because p-Dimethylaminobenzaldehyde reagent binds to the N-Acetylglucosamine units at the terminal ends of broken chitin chains that resulted from mechanical grinding. The shells themselves did not have any chitnolytic activity and this was confirmed in the next experiment.

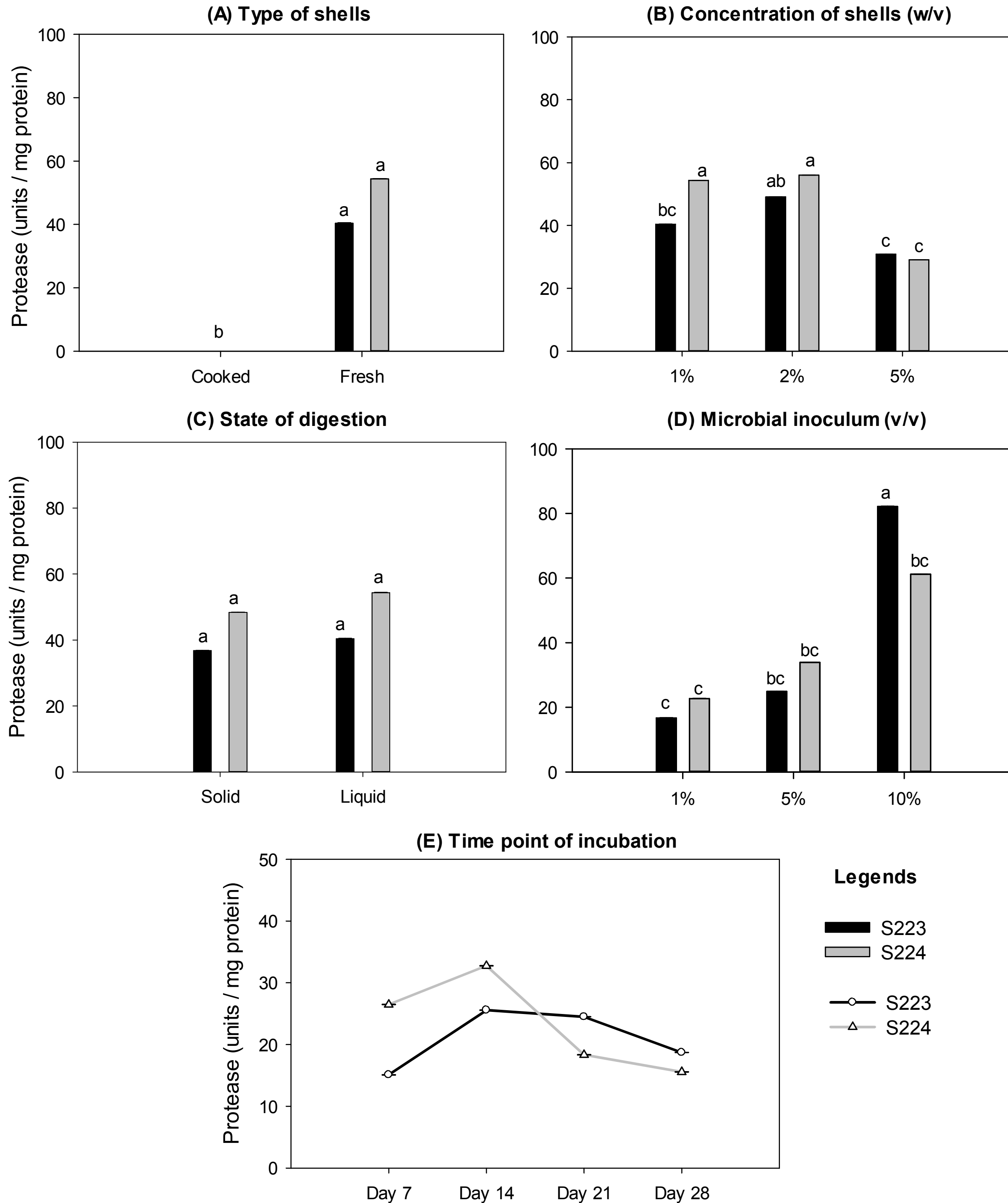

Figure 3.6. Microbial deproteinisation of lobster shells optimized for factors: (A) type of shells [*SEM* ± 1.40E-02, P < 0.0001], (B) concentration of shells [*SEM* ± 2.37E-02, P < 0.0001], (C) state of digestion [*SEM* ± 1.78E-02, P =0.96], (D) microbial inoculum [*SEM* ± 2.49E-02, P < 0.0001] and (E) time period of incubation [*SEM* ± 1.58E-02, P = 0.06]. Deproteinisation activity was measured by protease units present in the culture supernatants, quantified using Folin's reagent. Values represent mean ± SE of 3 replications per treatment and values sharing the same letter(s) are not significantly different (Tukey's HSD at $\alpha = 0.05$).

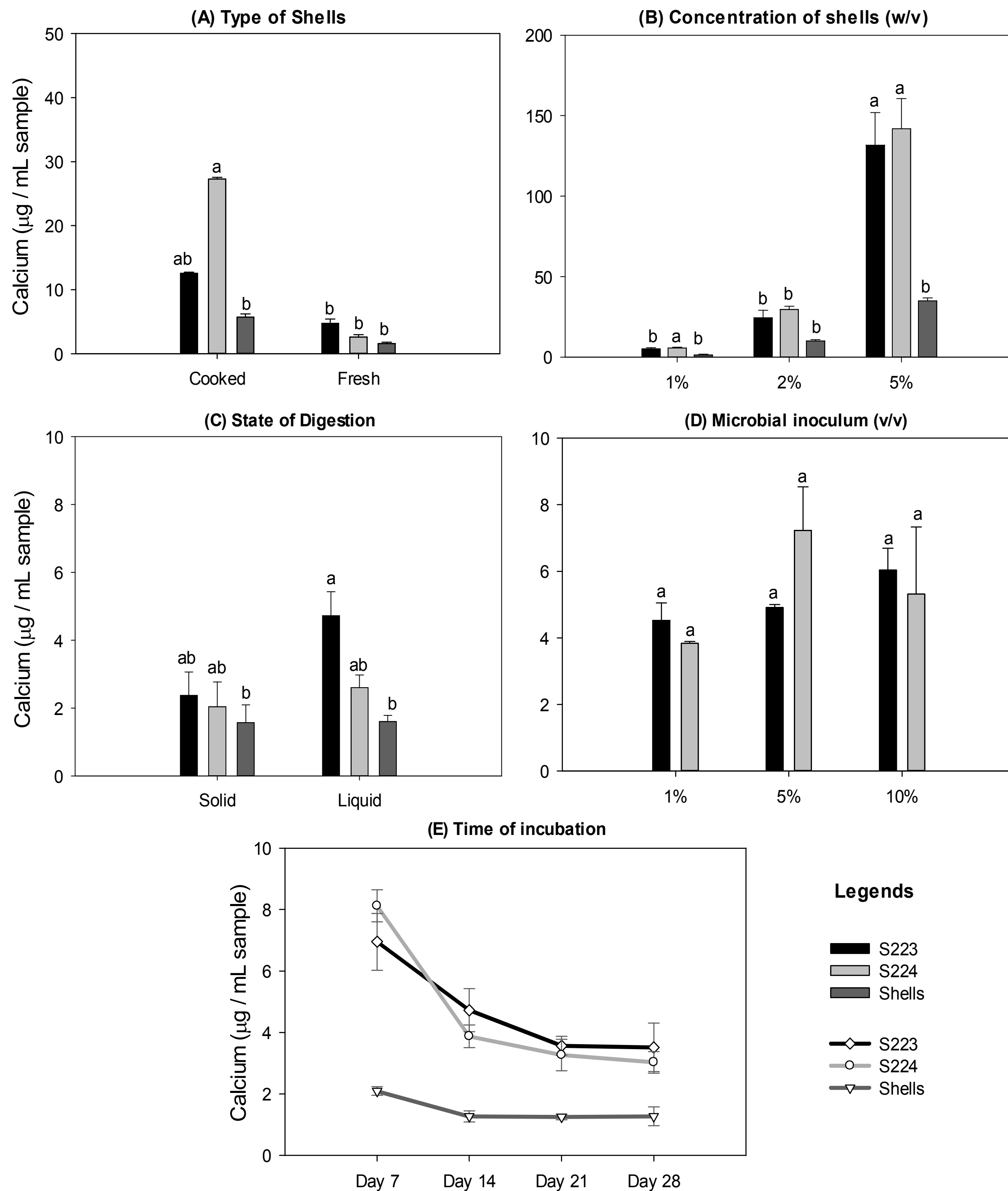

Figure 3.7. Microbial demineralisation of lobster shells optimized for factors: (A) type of shells [*SEM* ± 2.35E-01, P = 0.001], (B) concentration of shells [*SEM* ± 3.19, P < 0.0001], (C) state of digestion [*SEM* ± 2.64, P = 0.069], (D) microbial inoculum [*SEM* ± 4.81E-01, P = 0.72] and (E) time period of incubation [*SEM* ± 4.33E-01, P < 0.0001]. Demineralisation activity was measured by calcium content present in the culture supernatants, quantified using AAS. Values represent mean ± SE of 9 replications per treatment and values sharing the same letter(s) are not significantly different (Tukey's HSD at α = 0.05).

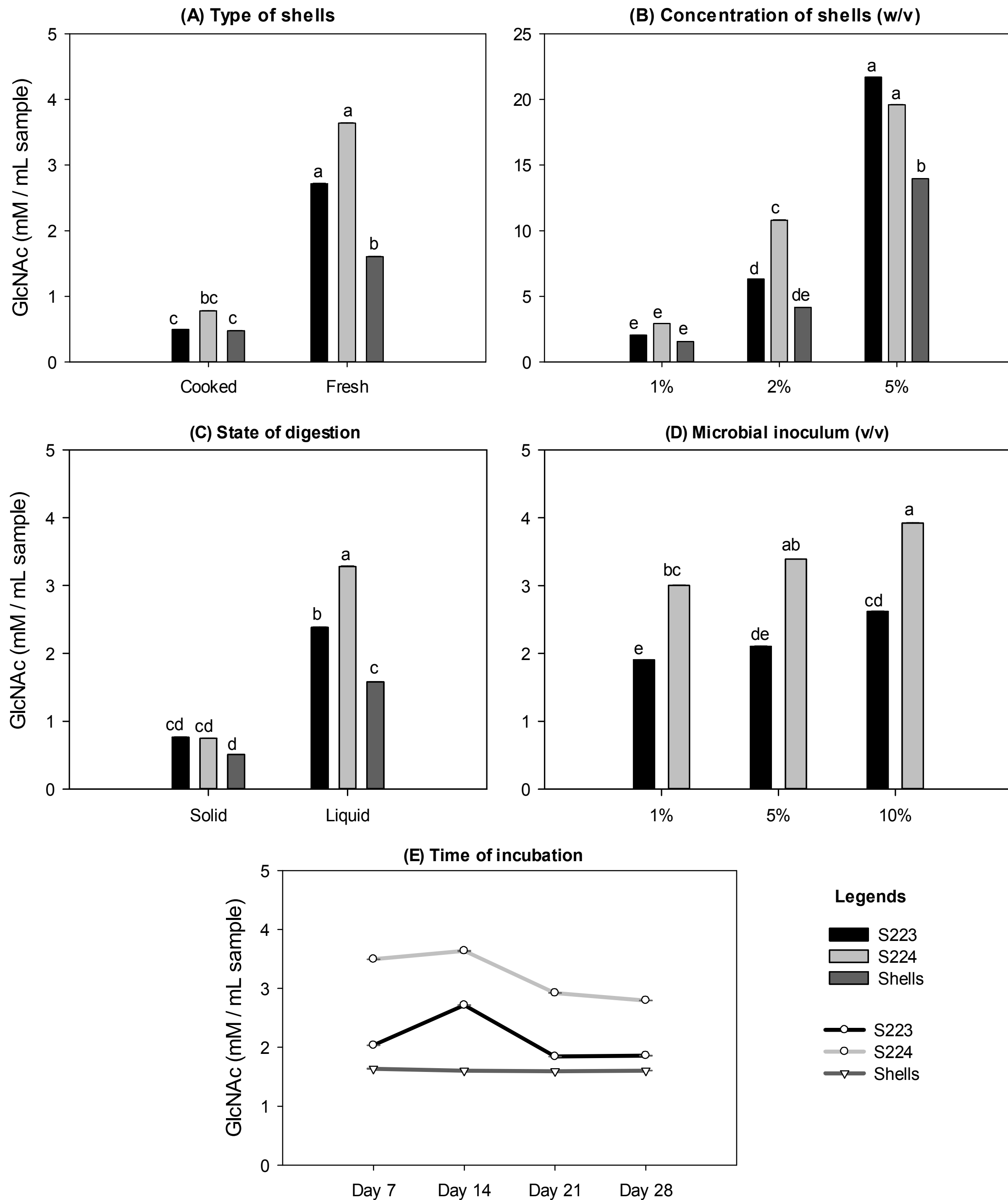

Figure 3.8. Chitinolysis of lobster shells optimized for factors (A) type of shells [*SEM* ± 3.5E-03, P < 0.0001], (B) concentration of shells [*SEM* ± 7.82E-03, P < 0.0001], (C) state of digestion [*SEM* ± 3.66E-03, P < 0.0001], (D) microbial inoculum [*SEM* ± 1.92E-03, P < 0.0001] and (E) time period of incubation [*SEM* ± 3.58E-03, P = 0.0009]. Chitnolysis activity was measured by N-Acetylglucosamine (GlcNAc) present in the culture supernatants, quantified using p-Dimethyaminobenzaldehyde Values represent mean ± SE of 3 replications per treatment and values sharing the same letter(s) are not significantly different (Tukey's HSD at α = 0.05).

Based upon these experiments, the optimal conditions for culturing S223 and S224 were decided to be 1 g of fresh lobster shells with 5 mL inoculum in 100 mL M9 buffer (pH 7.0) incubated at 25 °C, 150 rpm for 14 days. The microbes were grown under these conditions to prepare crude extracts.

3.3.4. Detection of chitinase activity by gel diffusion assay

Chitinase activity was detected on agarose gel containing glycol chitin (Figure 3.9). Culture extracts from S223 and S224 grown on lobster shell powder exhibited chitinase activity whereas extracts from YEME did not exhibit any. This suggested that chitinase synthesis of microorganisms is strongly dependent on the nature of the substrate present (Saito et al., 2000).

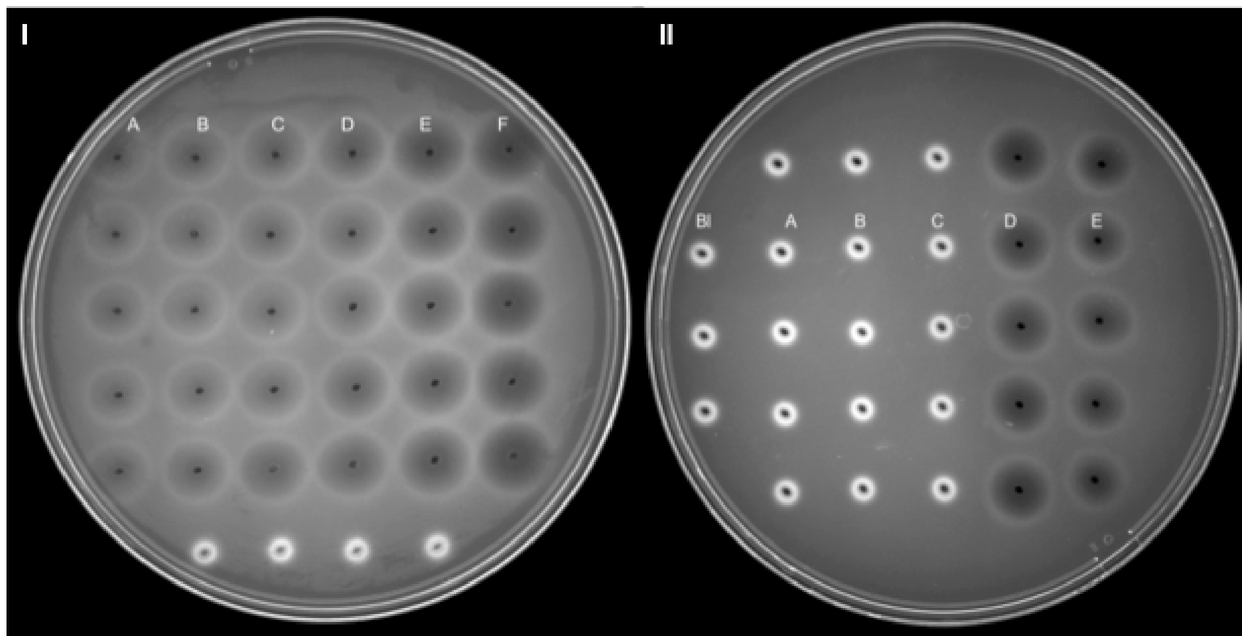

Figure 3.9. Dark circular zones indicate chitinase activity on glycol chitin agarose plates, visualized under UV transillumination. (I) Serial dilutions of chitinase standards from *Streptomyces griseus* (Sigma®) (II) Chitinase activity of microbe digested lobster shell extracts, Bl is water, A and B are culture filtrates of S223 and S224 grown on YEME, respectively, C is extract from lobster shells, D and E are extracts of S223 and S224 grown on lobster shell media, respectively.

3.3.5. Purification of chitinase enzyme

Chitinase was purified by affinity chromatography with colloidal chitin as the substrate. Table 3.2. shows the purification process of chitinase obtained from culture filtrate S223 grown in lobster shell powder. Chitinase activity increased with each step resulting in 2-fold purification at the final stage. Molecular weight of the purified enzyme preparation was checked on SDS-PAGE with protein markers and its size was found to be ~30 kDa (Figure 3.10).

Table 3.2. Chitinase activity of S223 culture filtrate using different substrates

Purification Step	Specific activity (U/mg of protein)				Purification factor
	Endochitinase	Chitobiosidase	N-Acetyl β–glucosaminidase	Total	
Culture supernatant	23.05	8.18	27.88	59.11	1
NH_4SO_4 (85%) dialysis	24.43	10.05	36.47	70.96	1.2
Affinity adsorption	49.84	38.31	58.81	146.96	2.4
Amicon PM30	66.68	52.93	73.1	192.71	3.2

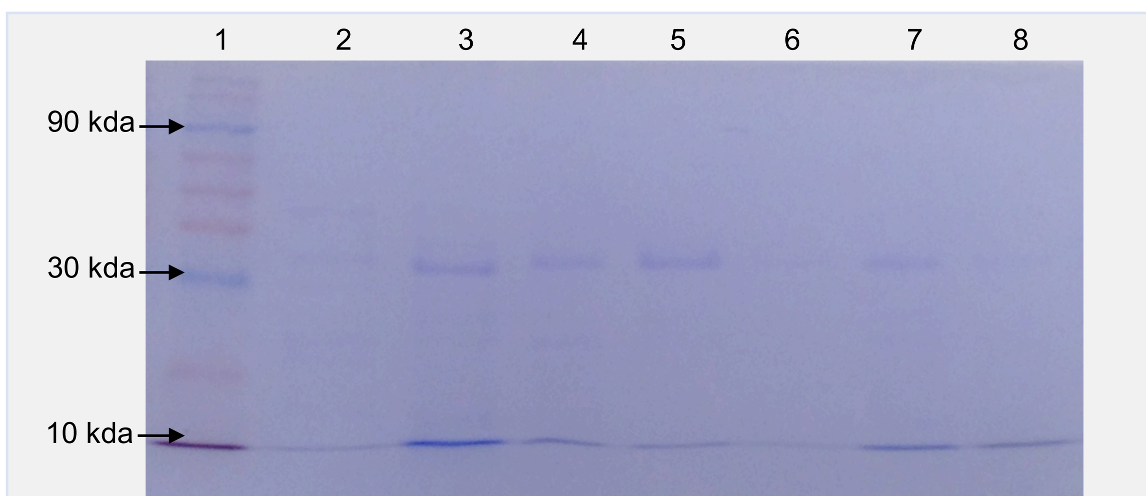

Figure 3.10. SDS-PAGE analysis of purified chitinase from S223. Lanes (1) Molecular weight markers 10-175 kDa (2) Chitinase from *Trichoderma* (3) Chitinase from *Streptomyces griseus* (4) Purified chitinase from S223 after affinity chromatography (5) S223 chitinase concentrated by Amicon PM30 filter unit (6) S223 unbound proteins (7) S223 crude extract after dialysis (8) S223 crude enzyme extract.

3.3.6. Antifungal activity

Antifungal activity of the extracts collected from microbial digestion of lobster shells was tested on potato dextrose agar plates. S224 crude extract had inhibited hyphal extension of test fungi when compared to the zones around wells containing water (control) and other extracts, which can be seen from Figure 3.11.

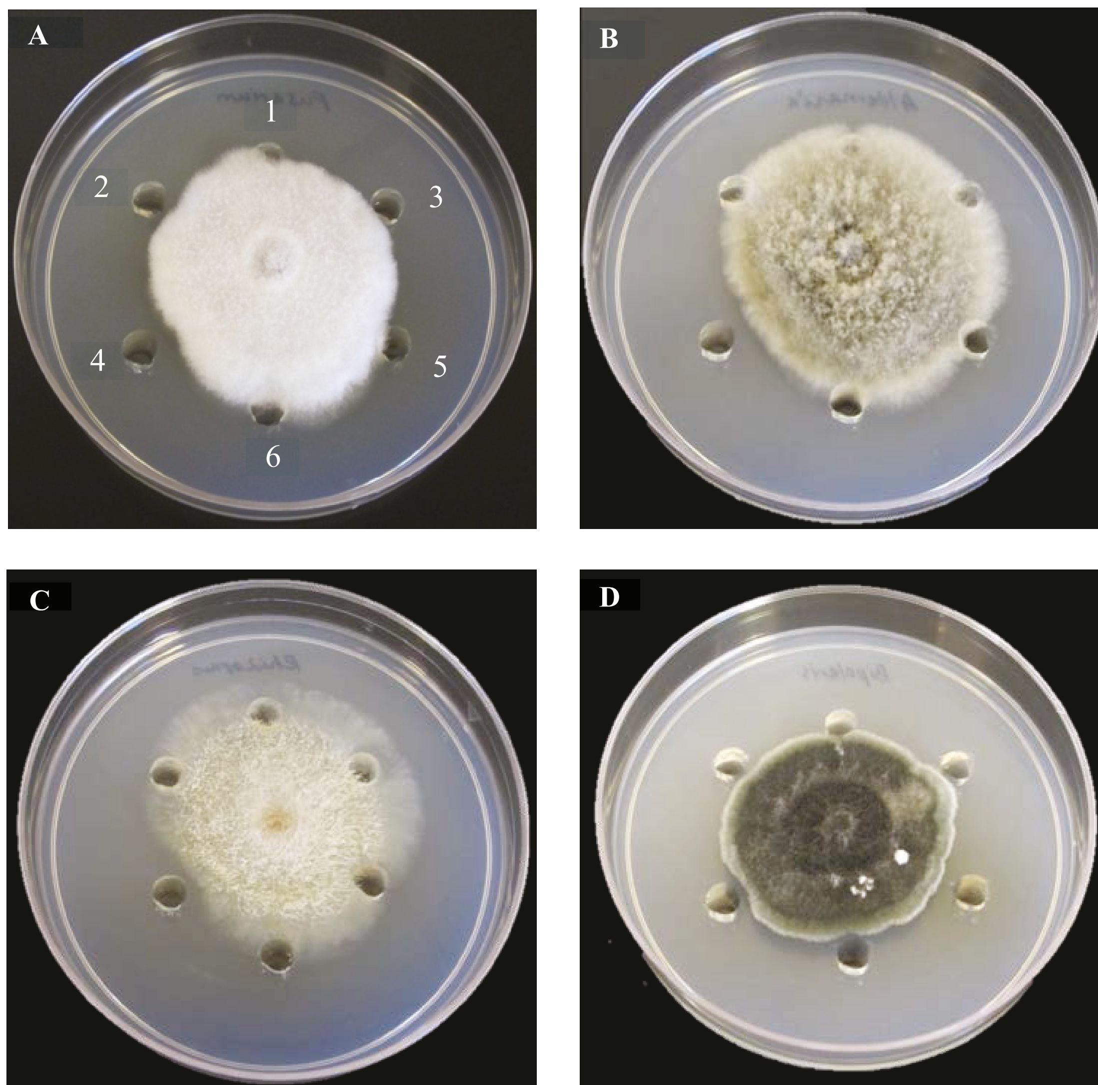

Figure 3.11. Antifungal activity of digested lobster shell extracts tested on potato dextrose agar plates, (A) *Fusarium* (B) *Alternaria* (C) *Rhizopus* (D) *Bipolaris*. 50µL of extracts are added to the wells (1) Water (2) and (3) are culture filtrates from S223 and S224 grown on YEME, respectively (4) and (5) are extracts from S224 and S223 grown on lobster shell powder (6) is Control, extract from undigested lobster shell powder.

3.3.7. Identification of the soil isolates

Morphological observations were made on 14 and 21 days after incubation (Appendix II, Figure A.5). Spore bearing hypha is flexible, slender and branched (Figure 3.12). Dense network of filaments bear conidia like spores on aerial hypha. Spores are round shaped, endogenous in origin and form smooth chain. Other characters observed are powdery mass on the surface, raised colonies with crates and distinct earthy odour (Appendix II, Table A.3). In starch agar clearing zones were observed. The isolates produced melanin pigments in peptone iron and tyrosine agar. They are aerobes and this was determined by thioglycollate tests (Appendix II, Table A.4). Morphological characteristics placed the soil isolates close to *Streptomyces* genus.

Nutrient utilization tests with carbon and nitrogen sources were observed visually by microbial cell growth (white mass on the media surface) and pH colour changes (Appendix II, Figure A.6). Cell growth was more dense in monosaccharaides like glucose, fructose, galactose and similar to what was known in previous experiments, S223 and S224 have strong chitin utilization. pH was changed from neutral to acidic in some test sources due to oxidation of carbonyl groups in the nutrient compounds to carboxylic/carbonic acids. However, pH changes were not observed or changed to alkaline in some test sources and the utilization metabolism appeared to be different (Appendix II, Table A.5). The full-length nucleotide sequences of 16s rDNA was compared with the sequences deposited in NCBI database using BLAST (Figure 3.13). Based on the alignment, the soil isolates S223 and S224 share 99% and 96% identity respectively to *Streptomyces sp*. and differ by one base pair between themselves.

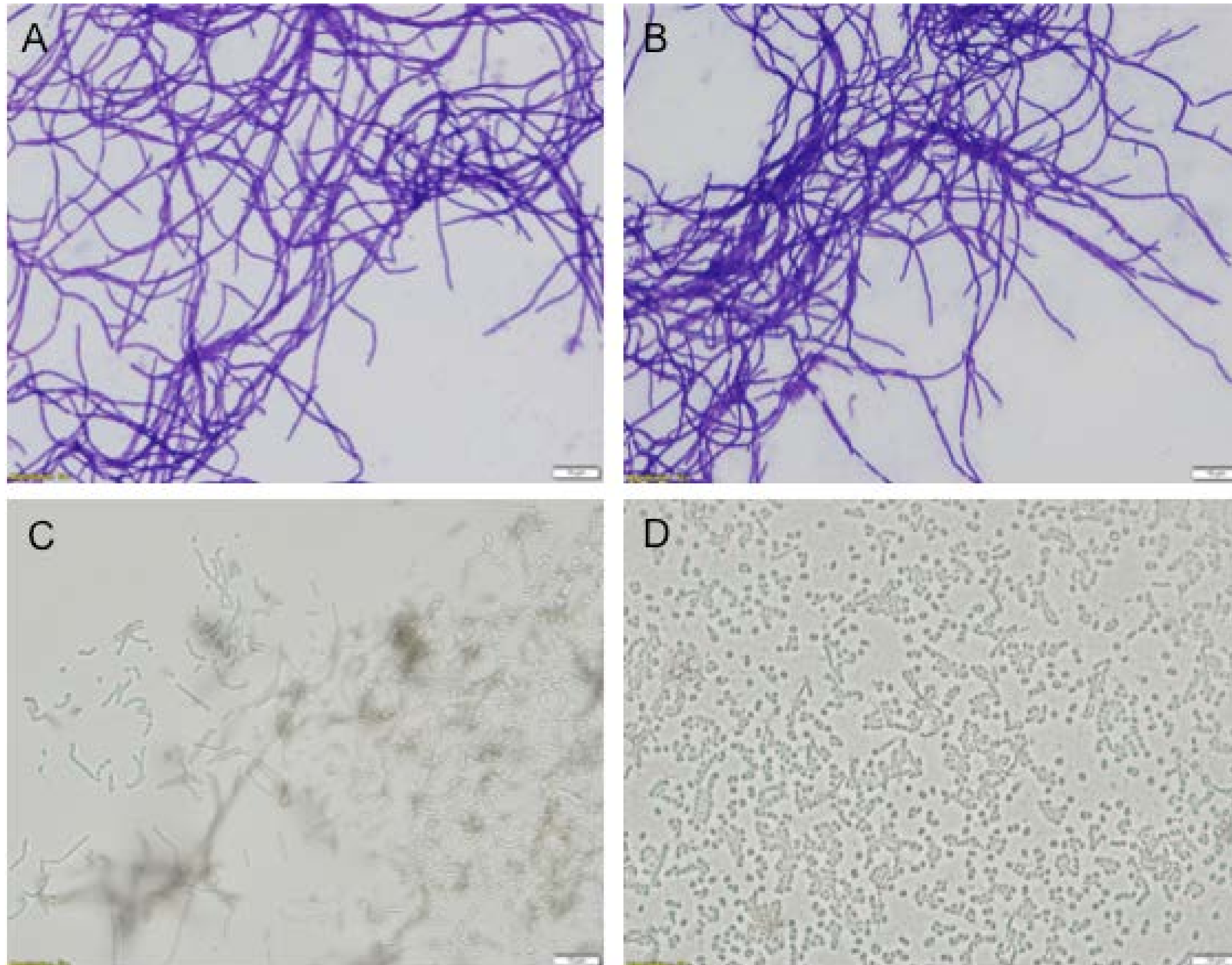

Figure 3.12. (A) and (B) Gram staining (C) and (D) Spore chains of S223 and S224, respectively observed under light microscope (bright field, 1000X magnification).

BLAST hits of query sequence of S223 16S rRNA gene						
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces coelicolor strain DSM 40233 16S ribosomal RNA gene, partial sequence	2571	2571	99%	0.0	99%	**NR_116633.1**
Streptomyces exfoliatus strain NBRC 13475 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	**NR_041229.1**
Streptomyces sampsonii strain NBRC 13083 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	**NR_112362.1**
Streptomyces coelicolor strain NBRC 12854 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	**NR_112305.1**
Streptomyces limosus strain NBRC 12790 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	**NR_112279.1**
Streptomyces felleus strain NBRC 12766 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	**NR_112266.1**
Streptomyces globisporus subsp. caucasicus strain NBRC 100770 16S ribosomal RNA gene, partial sequence	2564	2564	99%	0.0	99%	**NR_112593.1**
Streptomyces odorifer strain NBRC 13365 16S ribosomal RNA gene, partial sequence	2564	2564	99%	0.0	99%	**NR_112382.1**
Streptomyces albidoflavus strain NBRC 13010 16S ribosomal RNA gene, partial sequence	2564	2564	99%	0.0	99%	**NR_041095.1**
Streptomyces resistomycificus strain ISP 5133 16S ribosomal RNA gene, partial sequence	2564	2564	99%	0.0	99%	**NR_042100.1**
Streptomyces albidoflavus strain DSM 40455 16S ribosomal RNA gene, complete sequence	2564	2564	99%	0.0	99%	**NR_119341.1**
Streptomyces odorifer strain DSM 40347 16S ribosomal RNA gene, partial sequence	2564	2564	99%	0.0	99%	**NR_026535.1**
Streptomyces albus J1074 strain J1074 16S ribosomal RNA, complete sequence	2562	2562	99%	0.0	99%	**NR_102949.1**
Streptomyces champavatii strain NRRL B-5682 16S ribosomal RNA gene, partial sequence	2562	2562	99%	0.0	99%	**NR_115669.1**

BLAST hits of query sequence of S224 16S rRNA gene						
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. PGPA39 16S ribosomal RNA gene, partial sequence	2311	2311	100%	0.0	96%	**KJ854443.1**

Table 3.3. BLAST of full-length 16S rDNA sequences of S223 and S224 in NCBI database.

3.4. DISCUSSION

Natural sources such as soils and sediments have been useful in the isolation of bacteria on chitinous substrates that can produce chitinases and antifungal compounds (Chen and Li, 1993). In this study, chitinolytic microorgansisms were isolated from rhizospheric soils of a vineyard where lobster shells were applied as an amendment. More than half of isolated microorganisms on lobster shell media were actinomycetes with few bacteria and two fungi (data not shown). Further quantification experiments on the degradation activity substantiate that the microorganisms can degrade lobster shells by deproteinisation, demineralisation and chitinolysis processes.

The absence of detectable protease activity in cooked shells might be due to the relatively low protein content of the shells, because the shells lose protein during the boiling process (Waterman, 1991). Deproteinisation of 90-94% of shrimp shells was achieved by digesting the shells with *Serratia marcescens* (Jung et al., 2007). *Bacillus licheniformis*, capable of deproteinising shrimp shells exhibited a protease activity of 60units/ml (Waldeck et al., 2006).

Digestion of $CaCO_3$ involves organic acids and dissociates Ca^{2+} ions in to the solution, which in turn would form insoluble calcium salts of oxalate, formate or lactate (Oh et al., 2007). Ensilage of shrimp shells with lactic acid bacteria had resulted in >99% demineralisation of the shells in 2 to 3 days of incubation, by precipitation of calcium to calcium lactate, which lowered the pH and induced activation of proteases (Xu et al., 2008). Hence, quantification of calcium in the samples, as described in the method, reflects the quantity of calcium ions present in the solution at any given time point. Analysis of the digested sediments would reveal the amount of calcium precipitated by organic acids.

In this study, *B. subtilis* secreted >300 units/mg of protease on day 3 after incubation with fresh lobster shells and the bacteria is known for deproteinisation activity in crustacean shells (Sini et al., 2007). Deprotenisation of lobster shells can be improved by initial digestion with *B. subtilis* to remove the entire protein fraction of the shells. *B. subtilis* also exhibited high calcium content in the extracts followed by a soil actinomycete S2231. These microorganisms can be employed for efficient lobster shell degradation, to remove the protein and calcium constituents and yield a partially purified chitin.

In the optimization experiments, the quantity of protease units decreased after two weeks over the incubation time. A suggested explanation could be the increased microbial proteins in the culture medium because the total protease units remained high (data not shown) but decreased when measured in terms of units /mg of protein. The higher calcium content in the samples obtained on seven days after incubation than at later stages of the experiment might be due to the precipitation of calcium with organic acids.

Shrimp shells and chitin powder were reported to be excellent sources of carbon and nitrogen and used by microorganisms for chitinase production (Chang et al., 2003; Wang et al., 2005). The similar chemical composition in lobster shells suggested that they could strongly induce microbial chitinase synthesis. The microorganisms must produce both endochitinases and exochitinases to yield N-Acetylglucosamine (GlcNAc) as end product. With lobster shells being used as the substrate carbon source, the rate of GlcNAc production increased up to 2 weeks and then decreased over the incubation period. This suggested that the high concentration of the substrate and accessibility of all available sites of chitin particles for the digestion by enzyme hydrolysis are the main reasons for the production of GlcNAc during the initial stages.

α-Chitin has antiparallel microfibril orientation with strong hydrogen bonding and it is the major form of chitin present in crustacean shells (see Chapter 2, Section 2.2.2.). There could be a limited accessibility to the β-glycosidic linkages in the interior chitin chains for the enzyme attack and the rate of chitinolysis was slowed down. Other researchers suggested the same reasons for the absence of chitin hydrolysis after 24 h incubation (Klaikherd et al., 2004; Chen et al., 2010). The difference in the yield of GlcNAc at later stages of incubation could be attributed to end product feedback inhibition and/or enzyme denaturation during the reaction. Berger and Reynolds (1958) reported that intra or extracellular proteolytic enzymes could destroy chitinases of *Streptomyces griseus*.

Chitosan was detected in the samples six days after incubation, which suggested that the chitin deacetylase pathway begins later than the chitinase pathway. Chitosan could not be detected in the samples collected from lobster shell digestion because the carotenoid pigments of the shell interfered with the colorimetric assay.

Gel diffusion assay for chitinase using glycol chitin embedded in agarose gel, developed by Zou et al., (2002) was showed to be sensitive to detect chitinase activity in muskmelon seeds. The same procedure has been adopted by Velasquez and Hammerschmidt (2004) and to quantify chitinase activity in cucumber leaf samples. The method proved to be effective to detect chitinase in the microbial culture extracts, prior to the purification process.

Interestingly, chitinase activity was not detected in the culture filtrates of S223 and S224 grown in YEME media. This suggested that chitinase production is dependent on the susbtrate present. Several *Streptomyces* chitinase genes, *chiA*, *chiB*, *chiC*, *chiD*, and *chiF* of *S. coelicolor* A3 (Saito et al., 2000), *chiA* and *chiC* of *S. lividans* (Fujii and Miyashita, 1993; Miyashita and Fujii, 1993), exo-*chiO1* of *S. olivaceoviridis* (Blaak and Schrempf, 1995), *chb1* of *S.*

olivaceoviridis (Schnellmann et al., 1994) and *chi63* of *S. plicatus* (Delic et al., 1992), were found to be transcribed in the presence of chitin but downregulated in the presence of glucose.

The acetylation of chitosan resulted in a uniformly dispersed gel and it provided suitable column packing for the purification of chitinase by affinity chromatography (Molano et al., 1977). Microbes and cell-free microbial enzyme preparations were reported to digest chitin (Reynolds, 1954). The chitin substrates used for quantifying individual chitinases of *Streptomyces griseus* were described by Berger and Reynolds (1958). They found that the predominant enzyme secreted by *Streptomyces grisues* is chitobiase (now known as chitobiosidase), whereas in S223 endochitinase and β-N-Acetylglucosaminidase were abundant.

Brezezinska et al., (2013) purified chitinase up to 3.9 fold from *Streptomyces albidoflavus* and Han et al., (2008) reported up to 6.15 fold purification of chitinase from *Streptomyces sp*. Da11. Mukerjee and Sen (2006) obtained a 3.19 fold purified chitinase from *S. venezulae* P10. Many researches have reported the molecular weight of purified chitinase from *Streptomyces* species was between 20 and 71 kDa (Joo, 2005; Kim et al., 2007; Han et al., 2008). In this study, chitinase of 3.2 fold purification and 30 kDA molecular weight was obtained.

Antifungal activity of *Streptomyces* chitinase has been described in numerous research studies. El-Abyad et al., (1993) reported that the growth of *Fusarium oxysporum, Verticillium alboaturn* and *Alternaria solani* were inhibited by culture filtrates of *Streptomyces sp*. Mukerjee and Sen, (2006) observed that the *Streptomyces venezulae* chitinase inhibited growth of *Aspergillus niger, Alternaria alternata* and *Helminthosporium sativum*. Brezinska et al., (2013) revealed the antifungal activity of crude extracts from *Streptomyces albidofavus* grown in shrimp/crab shell as an additional carbon source, inhibit the growth of *F. culmorum, A. alternate*

and *Botrytis cinerea*. The extracts of S224 had restricted the hyphal extension of all the four plant-pathogenic fungi tested.

Waksmann and Henrici (1943) proposed the name *Streptomyces* and described it as, "… generic name for aerobic, saprophytic actinomycetes, which form catenulate spores, indicates the essential character of the group." They further described, "…forms spores in chains on aerial hyphae. Spores are apparently endogenous in origin formed by the segregation of protoplasm with in the hyphae into a series of round, oval or cylindrical bodies. Sporophores may be simple or branched."

Based on the same description that has been incorporated to Bergey's Manual of Systematic Bacteriology: The Actinobacteria (2012), the morphological characters of S223 and S224 observed revealed that the isolates belong to the *Streptomyces* genus. The sequencing of 16s rRNA gene further confirmed the identification by close identity of the microbes to *S. coelicolor*.

The study investigated the degradation of lobster shells by two *Streptomyces sp*. isolated from soil samples. The research shows the potential of these microorganisms to produce chitinases and proteases in lobster shell media. In this way they can contribute to alternative methods of recycling lobster shell wastes. The bioactive compounds found in the culture extracts can potentially be used in plant protection, which is discussed in the next chapter of this thesis.

CHAPTER 4

BIODEGRADED LOBSTER SHELL EXTRACTS INDUCE DISEASE RESPONSES IN ARABIDOPSIS

4.1. INTRODUCTION

Chitin is a natural polymer of N-acetylglucosamine with β -1, 4 linkages. It is the second most abundant renewable polysaccharide in nature next only to cellulose (Muzzarelli, 1997). Chitin and chitosan have been applied to control disease in plants or reduce their spread, to chelate nutrients and minerals, preventing pathogens from accessing them, or to enhance plant innate defenses (El-Hadrami et al., 2009).

Chitin acts as a potent elicitor and induces host defense responses, therefore enhancing plant resistance against pathogens (Benhamou, 1996). Plants respond to the presence of chitin in the environment by triggering a panel of defense responses. Chitin and its derived compounds are utilized as structural components in many species, including plant pathogenic bacteria, fungi and insects. Cell wall polysaccharides, such as chitooligosaccharides (CHOS), hexoseamines and glucans have been reported to act as pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) in many pathosystems (Zipfel, 2009). CHOS and chitosan present the advantage of being recognized by plant mechanisms of trans-membrane pattern recognition receptors (PRRs) (Ahuja et al., 2012).

The defense responses begin from early signaling events as well as the accumulation of defense-related metabolites and pathogenesis related proteins such as phytoalexins (Vander et al., 1998). CHOS lead to the induction of lignification (Barber et al., 1989), ion flux variations, cytoplasmic acidification, membrane depolarization, protein phosphorylation, chitinase and

glucanase activation (Kohle et al., 1984), generation of reactive oxygen species, biosynthesis of jasmonic acid, production of proteinase inhibitors (Walker-Simmons and Ryan, 1984; Pena-Cortes et al., 1988) and triggers callose formation (Kohle et al., 1985).

Plants respond to attack by pathogenic microorganisms by the induction of expression of a large number of genes encoding diverse proteins (Collinge et al., 1992). Many of these genes encode for chitinases, but there are no suitable substrates for these in plants, whereas chitin is the principle component of fungi and insects, which led to the proposal that the major natural role for chitinase is primarily defense against fungal pathogens (Mauch et al., 1988). Heterologous chitinase gene expression is observed in various plants that enhance their defense mechanisms against fungal pathogens (Schickler and Chet, 1997).

Plant chitinases have been focused on their role as pathogenesis related proteins and are synthesized in response to assorted environmental stimuli, such as fungal (Busam et al., 1997) or insect attack, osmotic pressure or developmental stage, such as fruit ripening (Clendennen and May, 1997). Endochitinases are the most extensive type of chitinase studied in plants (Molano et al., 1977) generating soluble low molecular mass oligomers of N-Acetylglucosamine. In addition, synthesis of exochitinase has been reported in many plants. Purified plant endochitinases also show some degree of lysozyme activity, they can hydrolyze β-1,4-linkages between N-Acetylmuramic acid and N-Acetylglucosamine residues in peptidoglycan (Boller, 1983; Majeau et al., 1990).

In the following study, extracts collected from microorganism digested lobster shells containing chitin derived compounds were tested for induced disease resistance in *Arabidopsis thaliana* against a bacterial pathogen *Pseudomonas syringae pv. tomato* DC3000 and a fungal pathogen *Botrytis cinerea*.

Arabidopsis thaliana is a member of Brassicaceae family and model organism used in plant biology studies. It offers advantage for conducting laboratory experiments with a short life cycle (6 weeks), easy to maintain in growth chamber and greenhouse conditions with less input requirements (soil, light, water and space), prolific seed production, whole genome sequenced and methods developed to research genetic, biochemical, physiological and developmental traits, which much is already known (The National Science Foundation, 2013).

Pseudomonas syringae pv. *tomato* DC3000 is a easily culturable gram-negative bacteria and pathogenic on Arabidopsis, causing water-soaked spreading lesions with chlorosis (Preston, 2000). *Botrytis cinerea* is considered as one of the most destructive plant pathogens infecting at pre- and post-harvest stages. It is a necrotrophic fungus with a broad host range and results in water-soaked lesion turning necrotic (Dean et al., 2012). The host-pathogen interaction studies of these pathogens in Arabidopsis had greatly increased the understanding of pathogenesis, virulence factors and plant responses. Therefore, the pathogens were chosen for pathogenicity studies in Arabidopsis to study the elicitation of defense reactions in plants triggered by the application of chitin derivatives present in the lobster shell extracts.

Chitosan, the deacetylated derivative of chitin was used as a positive control in the study based on its known function as an antimicrobial and potent elicitor of defense responses in plants. Chitosan has been applied as a protective agent against soil-borne diseases in seeds, foliar spray in plants and used as a soil amendment (Lafontaine and Benhamou, 1996).

4.2. MATERIALS AND METHODS

4.2.1. Preparation of lobster shells extracts

Fresh lobster shells were ground and sieved as described in the Chapter 3, Section 3.2.1. Two soil-isolates, S223 and S224 grown on 0.5% (w/v) lobster shell powder in M9 buffer for two days were used as the seed culture. The cultures were added to autoclaved fresh lobster shell powder (0.5 and 1 g) in glass jars containing 100 mL M9 buffer. They were incubated along with non-inoculated (undigested) lobster shell powder for 14 days at 25 °C and occasionally stirred with sterile needles every four days. The digested lobster shell extracts (LSE) 1% and 0.5% (w/v), were collected by centrifugation (Thermo scientific® Sorvall) at 18,000 × *g* for 20 min. The extracts were filter sterilized using 0.2 μm filters through vacuum and stored at -20 °C until use.

4.2.2. Antimicrobial activity

4.2.2.1. Effect of lobster shell extracts on the growth of Pseudomonas syringae pv. tomato

Pseudomonas syringae pv. tomato (*Pst*) DC3000 (laboratory stock) was grown in King's B broth overnight. Optical density was adjusted with blank to 0.01 at λ 600nm in a spectrophotometer. Bacterial culture (100 μL) was added to digested lobster shell extracts (100 μL), and water (negative control) and chitosan (positive control) were pipetted into a 96-well plate with blanks and incubated at 28 °C. The plates were read at absorbance 600nm (Biotek) after inoculation for 28 h and 48 h (Subramanian et al., 2011). Inhibition ratio was calculated by the absorbance of control (water) minus the average absorbance of tested extracts from six replicates, divided by the absorbance of the control.

4.2.2.2. Effect of lobster shell extracts on the growth of Botrytis cinerea

Botrytis cinerea (laboratory stock) was grown in one-half strength Potato dextrose agar (PDA) plates. Fungal spores collected by adding potato dextrose broth to the agar surface were filtered through sterile cheesecloth. A spore suspension of (100 µL, 10^4/mL) of *Botrytis cinerea* in potato dextrose broth and digested extracts and controls (100 µL) were added to a 96-well plate and incubated at 25 °C. Fungal spore germination was observed microscopically and optical density was measured at absorbance 595nm (Biotek®) after inoculation for every 12 h up to 3 days (Troskie et al., 2012). Inhibition ratio was calculated similarly as described for *Pst* DC3000

4.2.3. Pathogenicity studies of Arabidopsis in growth chamber conditions

The crude lobster shell extracts were applied as a foliar spray on Arabidopsis grown in growth chamber conditions. Stratified Arabidopsis seeds were planted on Jiffy peat pellets. They were covered with transparent plastic trays for 4-5 days to build humidity and encourage uniform seed germination. The trays were transferred to growth chambers set at 22 °C under light intensity of 150-200 $E.m^{-2}.sec^{-1}$ in 16:8 h day-night cycle. Three to four weeks old plants were sprayed with lobster shell extracts (flow-rate 6 mL/min) and inoculated with pathogens after 48 h of treatment application.

4.2.3.1. Disease intensity in Arabidopsis against Pst DC3000 infection

Pst DC3000 was grown for 48 h in King's B broth. Optical density was adjusted to 0.05 at λ 600nm and mixed with surfactant Silwett (0.02% v/v) in distilled water. The plants were dipped in *Pst* DC3000 inoculum and kept under a 100% relative humidity.

After 5 days post inoculation, six plants per treatment were scored on a numerical scale of 0 to 5 (0 = no infection, 1 = 25%, 2 = 50%, 3 = 75%, 4 = 100%, 5 = plant death) assigned to percentage of disease incidence on the plants. Disease intensity (DI) was calculated according to the formula below (Singh and Prithiviraj, 1997).

$$\textbf{Disease intensity} = ((sum\ of\ ratings\ (0-5))\ /\ (Maximum\ possible\ score)) \times 100$$

Growth of bacteria in leaf tissue

Arabidopsis leaf samples were collected at 24, 48, 72 and 96 h after inoculation and were surface sterilized with ethanol immediately. Nine randomly selected leaf samples were pooled into three replications, weighed and macerated independently using micropestles. The suspensions were serial diluted and plated on King's B agar containing rifampcin (25 µg/ mL). The plates were incubated at 28 °C for 48 h and number of colony forming units (cfu/g fresh weight) were counted.

4.2.3.2. Disease intensity in Arabidopsis against Botrytis infection

Botrytis was grown on one-half strength potato dextrose agar plates and spores were collected by adding potato dextrose broth to the agar surface. Six to eight leaves per plant (six plants per treatment) were inoculated with 20 µL spore suspension (1×10^6/mL) of *B.cinerea*, placed on the surface of leaves. The plants were kept under a 100% relative humidity for encouraging spore germination. The size of lesions formed was measured on days 3 and 5 post inoculation.

4.2.4. Estimation of enzyme activity

Arabidopsis plants were grown in similar conditions and subjected to infection as described above expect that the pathogen inoculums were foliar sprayed rather than dip or spot

inoculation. A control group of plants were treated with lobster shell extracts to observe induced defense responses without any pathogen inoculation and they were noted as mock (no infection). Samples from nine plants per treatment were pooled to form three biological replications and 200 mg of leaf tissues from treated and control plants were excised at 24 and 48 h post treatment (mock) and inoculation (*Pst DC3000* or *Botrytis*), immediately weighed and snap frozen in liquid nitrogen and stored at -80 °C. Frozen leaf tissues were ground using mortar and pestle in liquid nitrogen. The material was collected in 1.5 mL microcentrifuge tubes using an extraction buffer (1mL) containing 0.3 g/L polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 16,000 × *g* (Beckman Coulter™ Microfuge®) for 30 min at 4 °C and the supernatant was decanted into a clean tube (Subramanian et al., 2011). This was used as the crude enzyme extract. Protein content of the extracts was measured using the Bradford method (1976) with bovine serum albumin (BSA) as standard (Regression equation: $y = 5.6644x+0.0496$, $R^2 = 0.9887$).

4.2.4.1. Chitinase assay

Plant chitinases digest a pathogen's cell wall and play a key function in defending the host against the pathogens. The chitinase in the leaf samples were quantified, based on the method of Imato and Yagishita (1971). Grounded leaf tissues were collected in 25 mM Sodium acetate buffer (pH 5.0). The reaction preparation contained crude enzyme (100 µL) and glycol chitin (0.1%) in 100 mM Na-acetate buffer (500 µL). The reaction was incubated for 30 min at 37 °C and potassium ferricyanide (1 mL) was added to the reaction tubes, which results in development of yellow color. After boiling in a water bath for 30 min, the samples (200 µL) were transferred to a microplate and measured at absorbance 420nm in a Biotek® spectrophotometer (Dietrich et al., 2004). Chitinase from *Streptomyces griseus* (Sigma) was used

as the standard (Regression equation: $y = -2.72x+0.3792$, $R^2 = 0.99805$).

4.2.4.2. Phenylalanine ammonia lyase estimation

Anti-microbial phenol compounds and phytoalexins are synthesized via the Phenyl propanoid pathway where phenylalanine ammonia lyase is a primary catalase. Leaf tissues were ground as described above and homogenized in 25 mM ice-cold borate buffer (pH 8.8). The reaction mixture containing extract (200 μL) and 15 mM L-phenylalanine in 25 mM borate buffer (800 μL) was incubated for 1 h at 37 °C. The cinnamic acid produced during the reaction was read at λ 290nm in a Cary® UV 100 visible spectrophotometer and the quantity present was calculated using cinnamic acid standards (Subramanian et al., 2011). The activity of PAL is expressed as μmol cinnamic acid/min/mg protein with reference to the standard curve (Regression equation: $y = 9.5581x+0.0189$, $R^2 = 0.99287$).

4.2.5. Gene expression studies

The expression of pathogenesis related genes was studied (Table 4.1) to understand the activity of lobster shell extracts on induced disease resistance in plants. Leaf samples were harvested and ground in the same method for enzyme assays.

4.2.5.1. RNA extraction

RNA from the leaf tissues was extracted by Trizol method (Chomcsynzki and Mackey, 1995) using TRIzol® reagent (Life technologies) and chloroform. RNA content in the samples were quantified on a NanoDrop spectrophotometer (ThermoScientific®) and analyzed by electrophoresis on 1% (w/v) agarose gel, visualized under UV transilliumination (BioRad GelDoc™).

4.2.5.2. cDNA synthesis

The RNA samples (2 µg) were treated with RNase free DNase for 30 min in a thermal cycler at 60 °C and stop solution was added. Reverse transcription of RNA to cDNA was performed using High capacity cDNA Reverse transcription kit (Applied Biosystems®). cDNA synthesis were confirmed by PCR amplification with *Actin* primers and electerophoresis of the samples on agarose gel.

4.2.5.3. Real-time PCR

The cDNA synthesized was loaded on to qPCR reaction plates (Applied Biosystems®) with gene specific primers (Invitrogen™) and SYBR® Green master mix (Promega). The reactions were performed in StepOne™ platform (Applied Biosystems®). Expression levels of four genes of interest, *PR1, PR3, PDF1.2* and *ICS1* (Table 4.1) were normalized with *Actin* with reference to control (water) treatment.

Table 4.1. Gene specific primers used to study induced disease resistance in Arabidopsis

Gene	Gene locus	Gene specific primers
PR1	AT2G14610.1	F 5'-ACATGTGGGTTAGCGAGAAG-3' R 5'-ACTTTGGCACATCCGAGTCT-3'
PR3	AT3G12500.1	F 5'-ACGAAGGATCTTTGGTTGTA-3' R 5'-ACATCATTAACGGTGGATTG-3'
PDF1.2	AT5G44420.1	F 5'-TGCTGGGAAGACATAGTTGC-3' R 5'-TGGTGGAAGCACAGAAGTTG-3'
ICS1	AT1G74710.1	F 5'-TTCTTCCGTGACCTTGATGG-3' R 5'-CCAAAAGGTTCCCATTCAAC-3'

4.2.6. Data analysis

Experiments were setup in a completely randomized design and the quantification assays were measured in three technical replicates of individual biological sample. Linear regression analysis for standard curves used in quantification assays was done using a Microsoft Excel (Microsoft®, 2013) scatter plot. An analysis of variance on data was performed after satisfying the assumptions of normality and constant variance using the SAS v. 9.3 statistical software package with General Linear Model (proc glm) or Mixed procedure (proc mixed) at a 95% confidence interval. Multiple means comparison was done using a Tukey's HSD (honest significant difference) test at $\alpha = 0.05$ (α – level of significance). In the graphs presented in the results section, data points sharing the same letter are not significantly different and SAS macro pdmix.sas was used to generate letter groupings.

4.3. RESULTS

4.3.1. Antimicrobial activity

Growth of *Pseudomonas syrinage pv. tomato* DC3000 was significantly ($P < 0.0001$) inhibited by the addition of extracts from microbe digested of lobster shell powder. Culture filtrates from S223 and S224 grown on yeast extract and malt extract (YEME) broth were included as controls and they inhibited pathogen growth yet not as high as the lobster shells digested extracts (Figure 4.1). This suggested that chitinase and other antimicrobial compounds in the extracts are specific to the substrate media. S224 (1%) extracts exhibited the highest inhibition on the growth of *Pst* DC3000 and S223 (1%) showed the significantly ($P = 0.0028$) highest inhibition on the spore germination and hyphal growth of *Botrytis* (Figure 4.2).

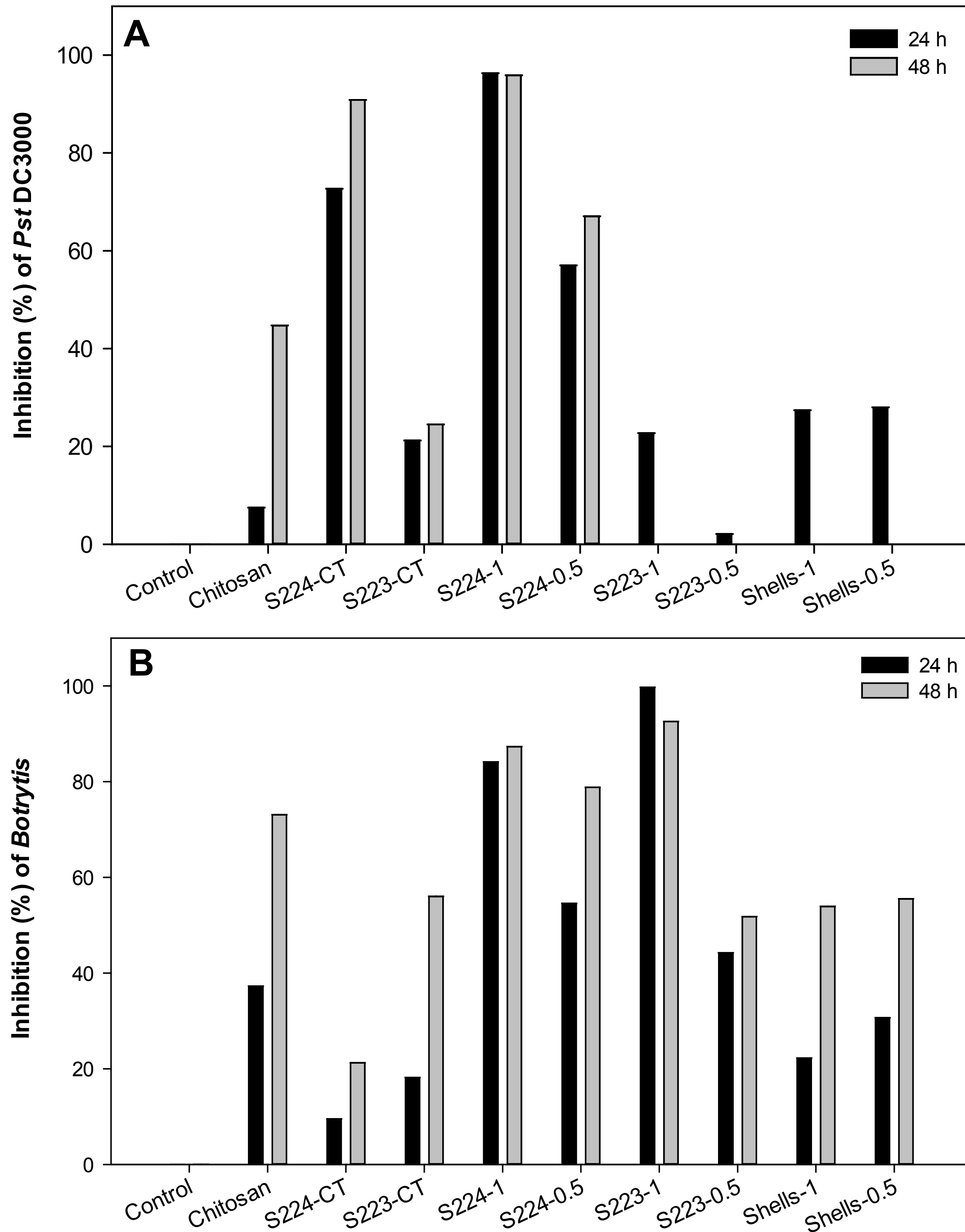

Figure 4.1. Antimicrobial activity of lobster shell digested extracts on pathogen growth. Inhibition percentage of (A) *Pst* DC3000 [*SEM* ± 9.89E-03, P < 0.0001] and (B) *Botrytis cinerea* [*SEM* ± 1.81E-02, P = 0.0028] was calculated respective to control treatment. Control – water, Chitosan – positive control, S224-CT, S223-CT – control extracts of S224 and S223 grown in YEME, S224-0.5, S224-1 – extract of 0.5% and 1% of S224 digested lobster shells, S223-0.5, S223-1 – extract of 0.5% and 1% of S223 digested lobster shells, Shells-0.5, Shells-1 – extract of 0.5% and 1% of undigested lobster shells. Values represent mean ± SE of 6 replications per treatment. Letter groupings are presented in Appendix II, Tables A.11 and A.12.

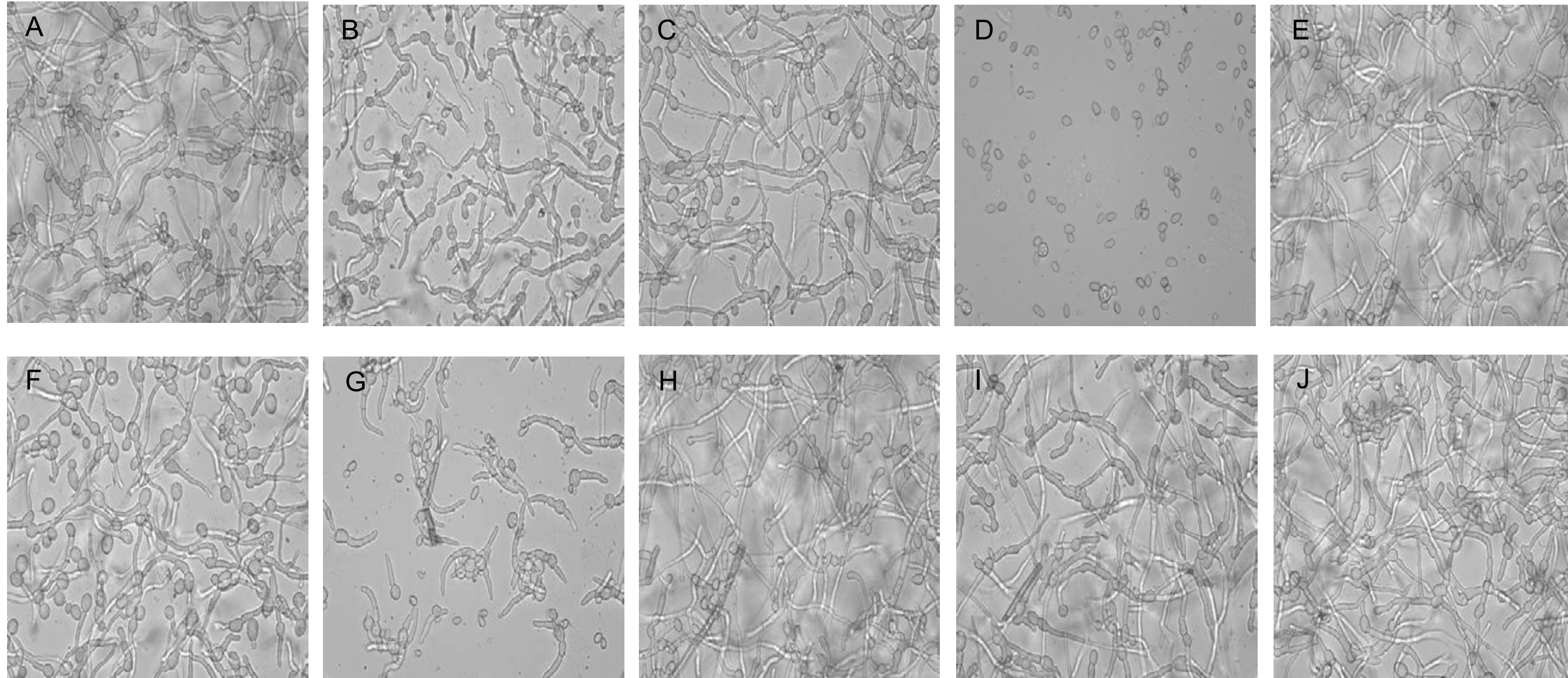

Figure 4.2. Spore germination of *Botrytis* observed 12 h after incubation under light microscope (20X magnification). (A) Control – water, (B)-Chitosan – positive control, (C) S223-0.5 – extract of 0.5% of lobster shells digested by S223, (D) S223-1 – extract of 1% of lobster shells digested by S223, (E) S223 grown on YEME (F) S224-0.5 – extract of 0.5% of lobster shells digested by S224, (G) S224-1 – extract of 1% of lobster shells digested by S224, (H) S224 grown on YEME, (I) Shells-0.5 – extract of 0.5% of undigested lobster shells, (J) Shells-1 – extract of 1% of undigested lobster shells).

4.3.2. Pathogenicity studies of Arabidopsis in growth chamber conditions

4.3.2.1. Disease intensity in Arabidopsis against Pst DC3000 infection

Arabidopsis leaves infected with *Pst* DC3000 were scored on a numeric scale and the percentage of disease intensity was calculated according to the formula mentioned in Section 4.2.3.1. Plants treated with S224 digested extracts of lobster shells (1%) exhibited significantly (P < 0.0001) higher restriction on disease spread compared to other treatments (Figure 4.3 A). Yellowing of leaves appeared three days after inoculation (Figure 4.3 B).

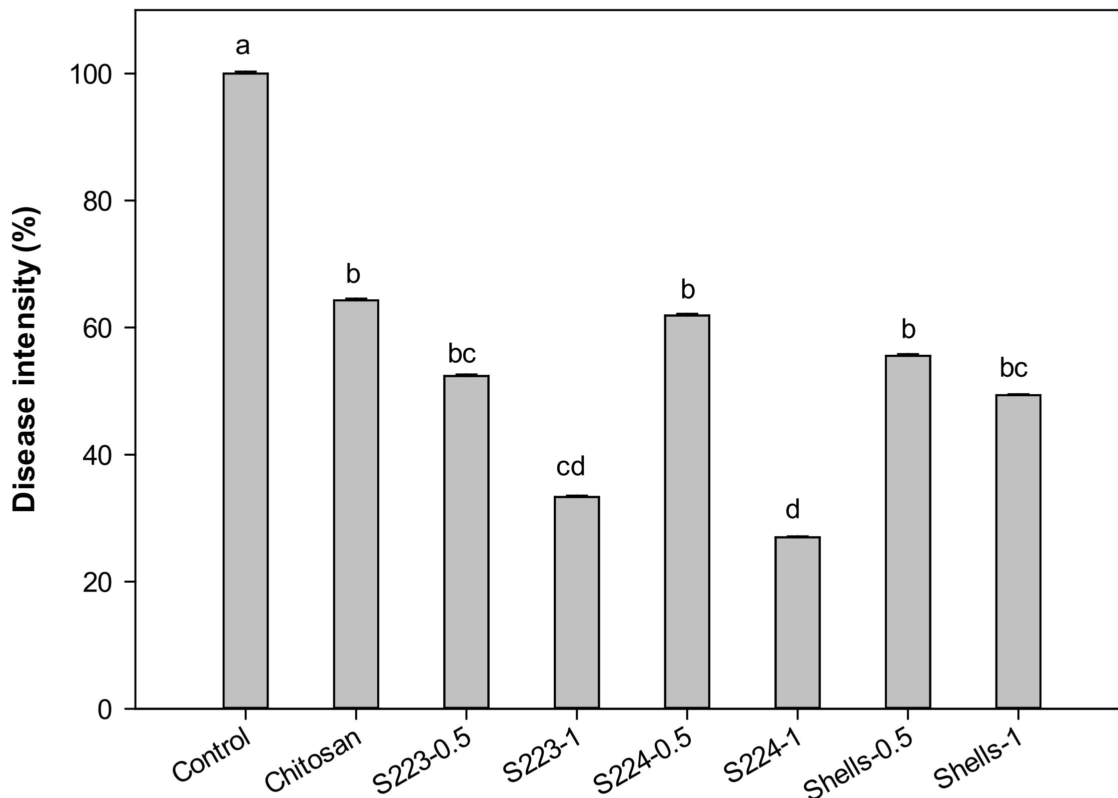

Figure 4.3. (A) Disease intensity of *Pst* DC3000 infection in Arabidopsis treated with lobster shell extracts measured by visual observation [*SEM* ± 2.39E-01, P < 0.0001]. Control – water, Chitosan – positive control, S223-0.5 – extract of 0.5% of lobster shells digested by S223, S223-1 – extract of 1% of lobster shells digested by S223, S224-0.5 – extract of 0.5% of lobster shells digested by S224, S224-1 – extract of 1% of lobster shells digested by S224, Shells-0.5 – extract of 0.5% of undigested lobster shells, Shells-1 – extract of 1% of undigested lobster shells. Values represent mean ± SE of 6 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at α = 0.05). (continued on next page)

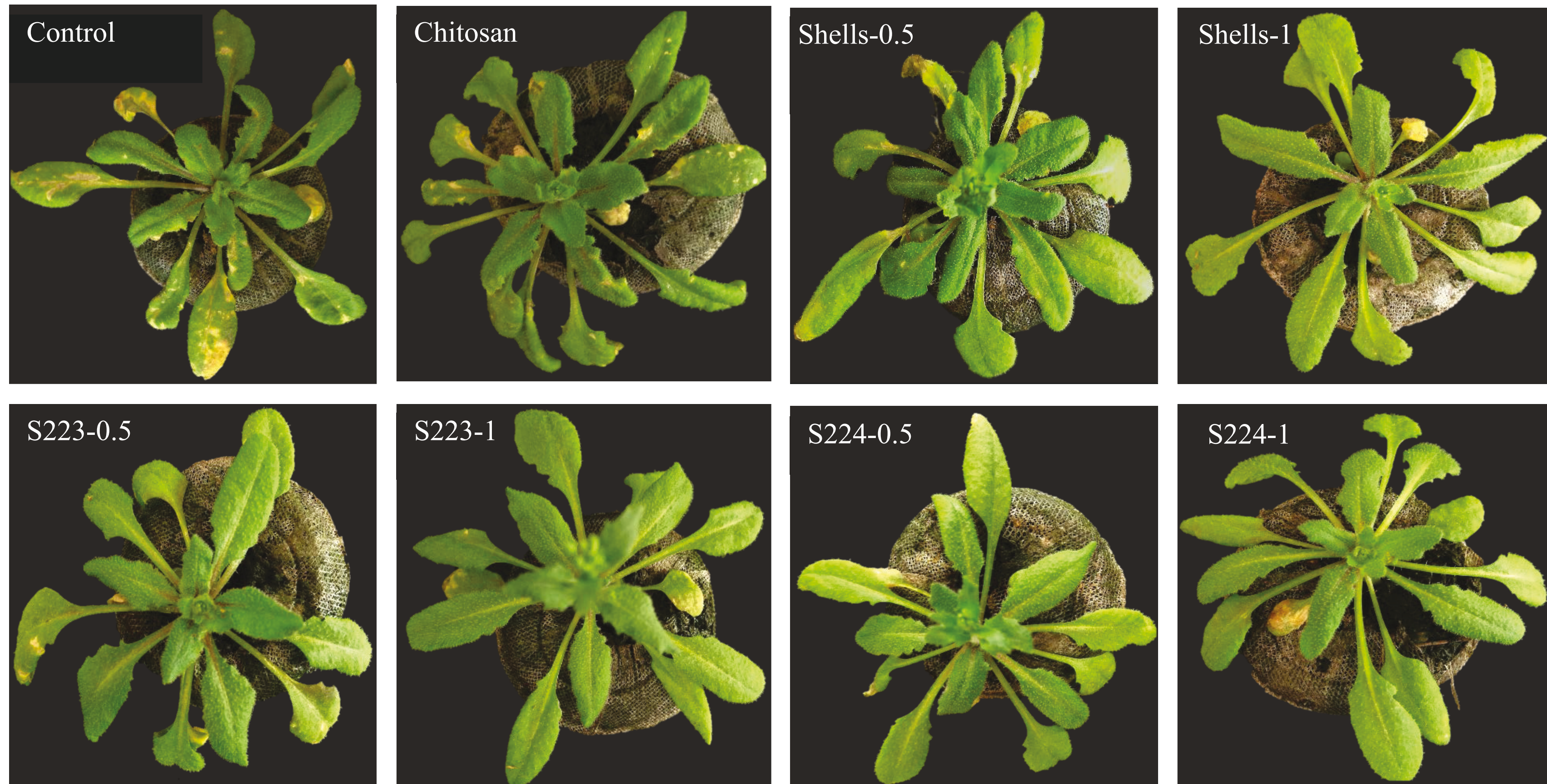

Figure 4.3. (B) Disease intensity of *Pst* DC3000 infection in Arabidopsis treated with lobster shell extracts measured by visual observation. (Control – water, Chitosan – positive control, S223-0.5 – extract of 0.5% of lobster shells digested by S223, S223-1 – extract of 1% of lobster shells digested by S223, S224-0.5 – extract of 0.5% of lobster shells digested by S224, S224-1 – extract of 1% of lobster shells digested by S224, Shells-0.5 – extract of 0.5% of undigested lobster shells, Shells-1 – extract of 1% of undigested lobster shells).

Growth of bacteria in Arabidopsis leaves

Pst DC3000 colonizing Arabidopsis leaves were enumerated on King's B agar plates since visual observations might not imply the actual infection on bacteria in leaf tissue. S223 and S224 digested lobster shell extracts (1%) showed a significant (P < 0.0001) reduction of number of bacteria in the leaves, and revealed a similar trend of the disease incidence experiment (Figure 4.4).

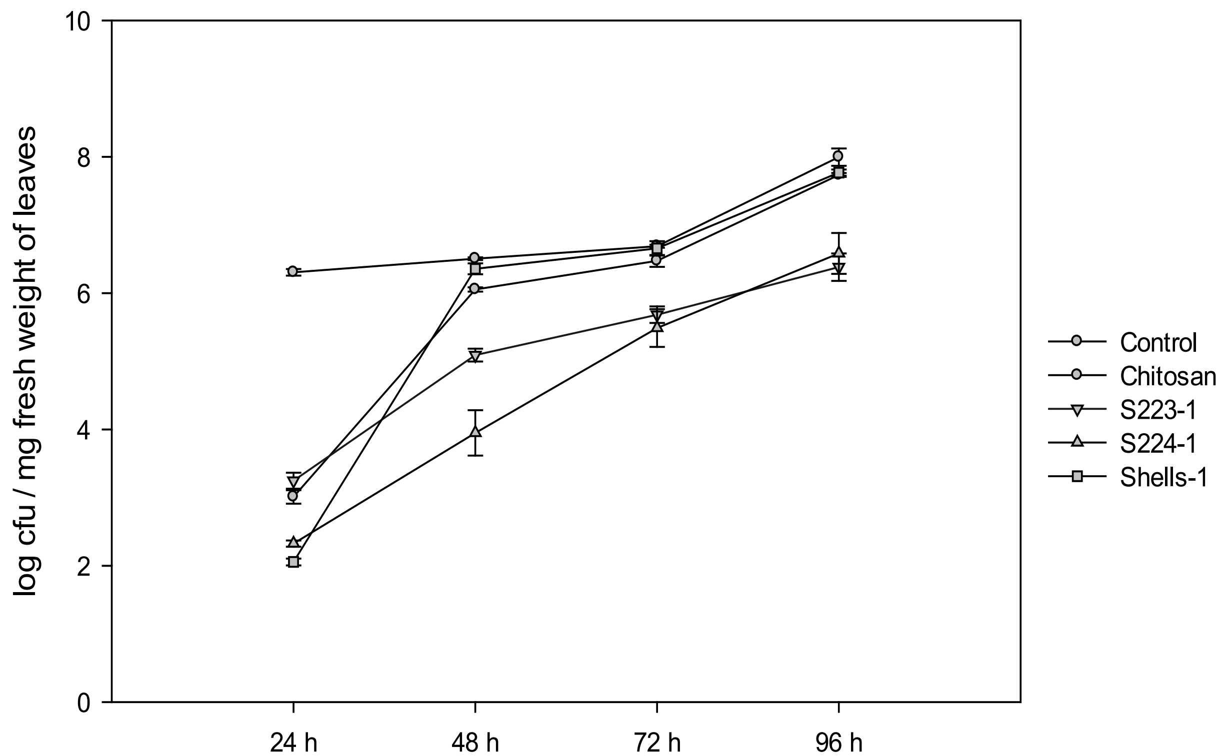

Figure 4.4. Proliferation of *Pst* DC3000 in Arabidopsis enumerated by cfu counted on King's B agar plates [*SEM* ± 1.24E-01, P < 0.0001]. Control – water, Chitosan – positive control, S223-1 – extract of 1% of lobster shells digested by S223, S224-1 – extract of 1% of lobster shells digested by S224, Shells-1 – extract of 1% of undigested lobster shells. Values represent mean ± SE of 3 replications per treatment. To minimize complexity of the graph, extended data and letter groupings of Figure 4.4 are presented in Appendix II, Table A.13.

4.3.2.2. Disease intensity in Arabidopsis against Botrytis infection

Arabidopsis leaves were spot inoculated with *Botrytis* spores. The infection resulted in rapidly expanding, water-soaked lesions (examples are shown in Figure 4.5). Leaf lesions formed at the infection spots were measured on days 3 and 5 after inoculation (Figure 4.6). Lobster shell digested extracts showed significant ($P < 0.0001$) reduction on the spread of leaf lesion (Figure 4.6). Microbe digested extracts from 1% lobster shells exhibited higher restriction on disease spread than 0.5% extracts. The infected spots become localized necrosis resulting from increased disease resistance.

Figure 4.5. Formation of *Botrytis* leaf lesions on treated Arabidopsis plants. (A- Control – water, B- S223-1 – extract of 1% of lobster shells digested by S223, C- S224-1 – extract of 1% of lobster shells digested by S224, D- Shells-1 – extract of 1% of undigested lobster shells).

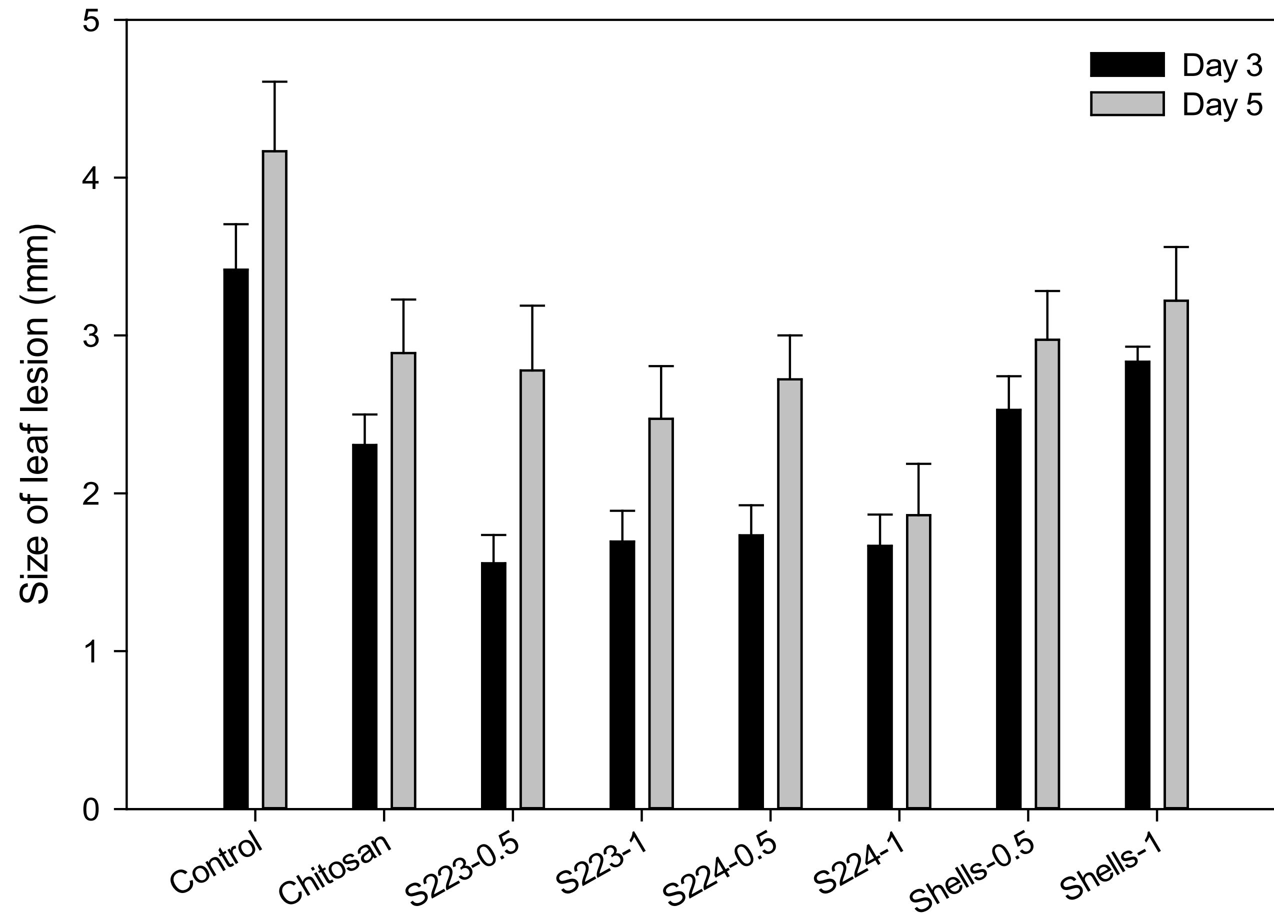

Figure 4.6. Size of leaf lesions was measured on *Botrytis* infected Arabidopsis leaves on days 3 and 5 after inoculation [*SEM* ± 2.71E-01, P < 0.0001]. (Control – water, Chitosan – positive control, S223-0.5 – extract of 0.5% of lobster shells digested by S223, S223-1 – extract of 1% of lobster shells digested by S223, S224-0.5 – extract of 0.5% of lobster shells digested by S224, S224-1 – extract of 1% of lobster shells digested by S224, Shells-0.5 – extract of 0.5% of undigested lobster shells, Shells-1 – extract of 1% of undigested lobster shells). Values represent mean ± SE of 6 replications per treatment. Letter groupings of Figure 4.5 are presented in Appendix II, Table A.14.

4.3.3. Estimation of enzyme activity

The resulted disease resistance is due to the elicitation of defense responses upon application of the extracts and also their antibiotic properties. The effect of microbial digested lobster shell extracts on induced disease resistance in Arabidopsis were investigated through biochemical analysis of two enzymes, which are involved in metabolic pathways secreting antimicrobial compounds (see Chapter 2, Section 2.5).

Chitinase activity was not detected in measurable quantity in the Arabidopsis leaf tissues after 24 h of treatment or inoculation with pathogens. Chitosan treated plants exhibited significantly ($P < 0.0001$) higher chitinase activity in the leaf tissue after 48 h upon infection with *Botrytis*. S223 digested lobster shell extracts had induced significant chitinase activity in place of both *Pst* DC3000 and *Botrytis* infection. The reduced chitinase activity in S224 treated plants suggested that it acted as a protective agent because of antibiotic properties of the extracts and the plants were least affected by the pathogen (Figure 4.7. A).

There was significant ($P = 0.0044$) difference in phenylalanine ammonia lyase (PAL) activity at 48 h after treatment (Figure 4.7. B). When compared to mock and *Botrytis* infection, PAL activity was significantly ($P < 0.0001$) higher in plants with *Pst* DC3000 at 24 and 48 h post infection (Appendix II, Table A.16). The lobster shell extracts, both digested and undigested, had elicited chitinase and PAL activity in non-infected (mock) plants, which suggested their possible functions as PAMPs (Pathogen associated molecular patterns).

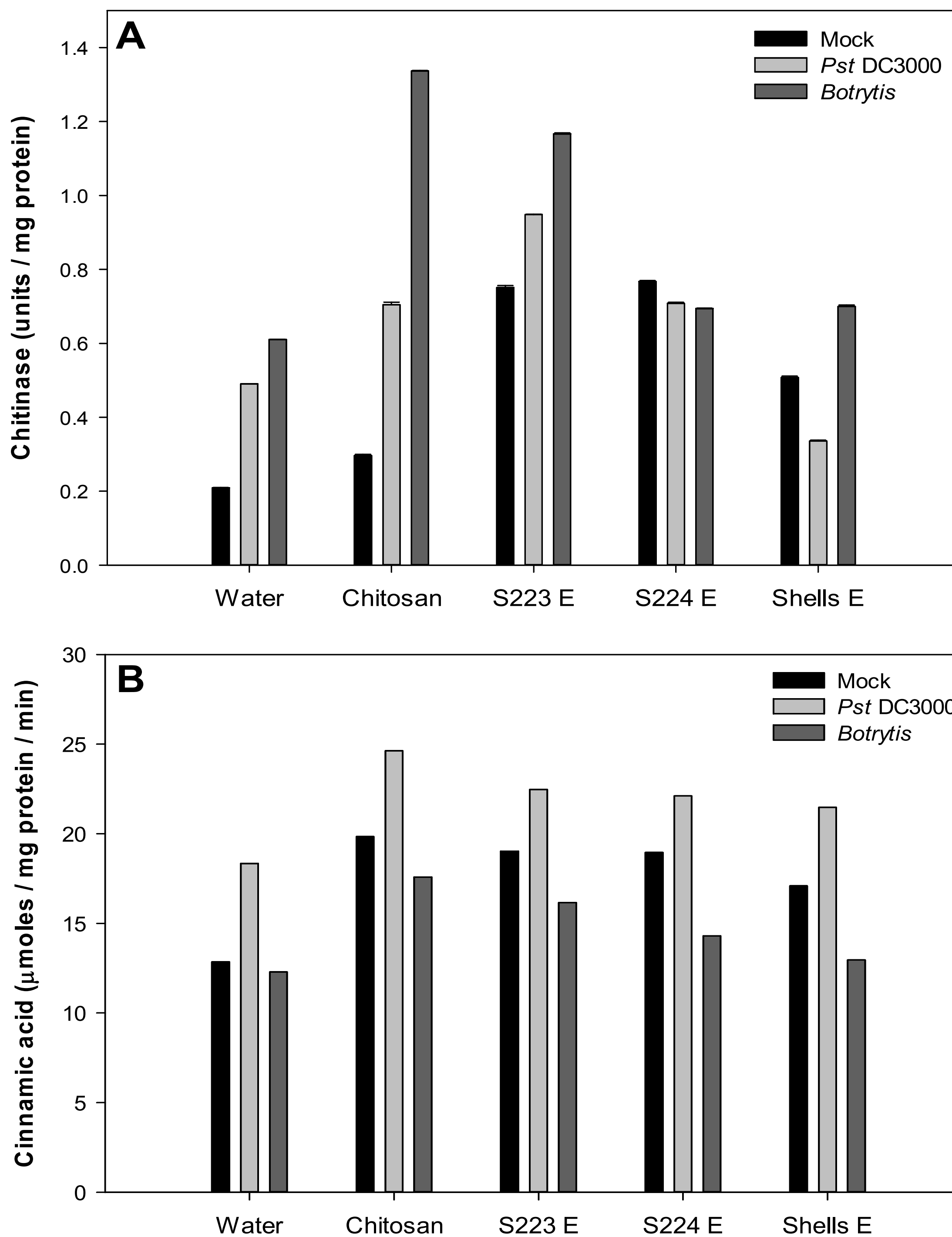

Figure 4.7. (A) Chitinase [*SEM* ± 2.47-03, P < 0.0001] and (B) Phenylalanine ammonia lyase activity [*SEM* ± 4.65E-04, P = 0.0044] quantified at 48 h (post treatment and inoculation) in Arabidopsis leaf samples. (Water – control, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells). Values represent mean ± SE of 3 replications per treatment. Letter groupings are presented in Appendix II, Tables A.15 and A.16

4.3.4. Gene expression studies

The genes most commonly studied to examine the roles of salicylic acid, jasmonic acid phytoalexins, mediated defense responses include *PR1*, *PR3*, *PDF1*.2 and *ICS1*. The effect of foliar treatments (Control –water, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells) on the transcription of these genes in Arabidopsis leaf tissues upon mock (Figure 4.8) *Pst* DC3000 (Figure 4.9) and *Botrytis* (Figure 4.10) infection were investigated. The gene expression levels were higher in infected than non-infected (mock) plants and different at 24 and 48 h after treatment. The interaction between the treatments and time had a significant ($P < 0.0001$) effect on the transcript abundance of the genes studied. The expression of *PR1* (salicylic acid) and *PDF1*.2 (jasmonic acid) were greater in *Pst* DC3000 and *Botrytis* infected plants.

S224 extract treatment resulted in the highest transcription of *PR1* (500 fold) in plants infected with *Pst* DC3000 at 24 h (Figure 4.9 A). Jasmonic acid dependent *PDF1*.2 expression was higher in plants treated with S224 extracts (40 fold) and chitosan under *Pst* DC3000 infection at 24 h (Figure 4.9 C) whereas in *Botrytis* infection, the expression was higher at 48 h (Figure 4.10 C). An increase in the transcript of *PR3* (endochitinase) was observed in S224 extracts (8 fold) in *Pst* DC3000 infection (Figure 4.9 B). ICS1 transcription, which induces salicylic acid, was increased with S224 and shell extracts in *Botrytis* infection at 48 h after treatment (Figure 4.10 D).

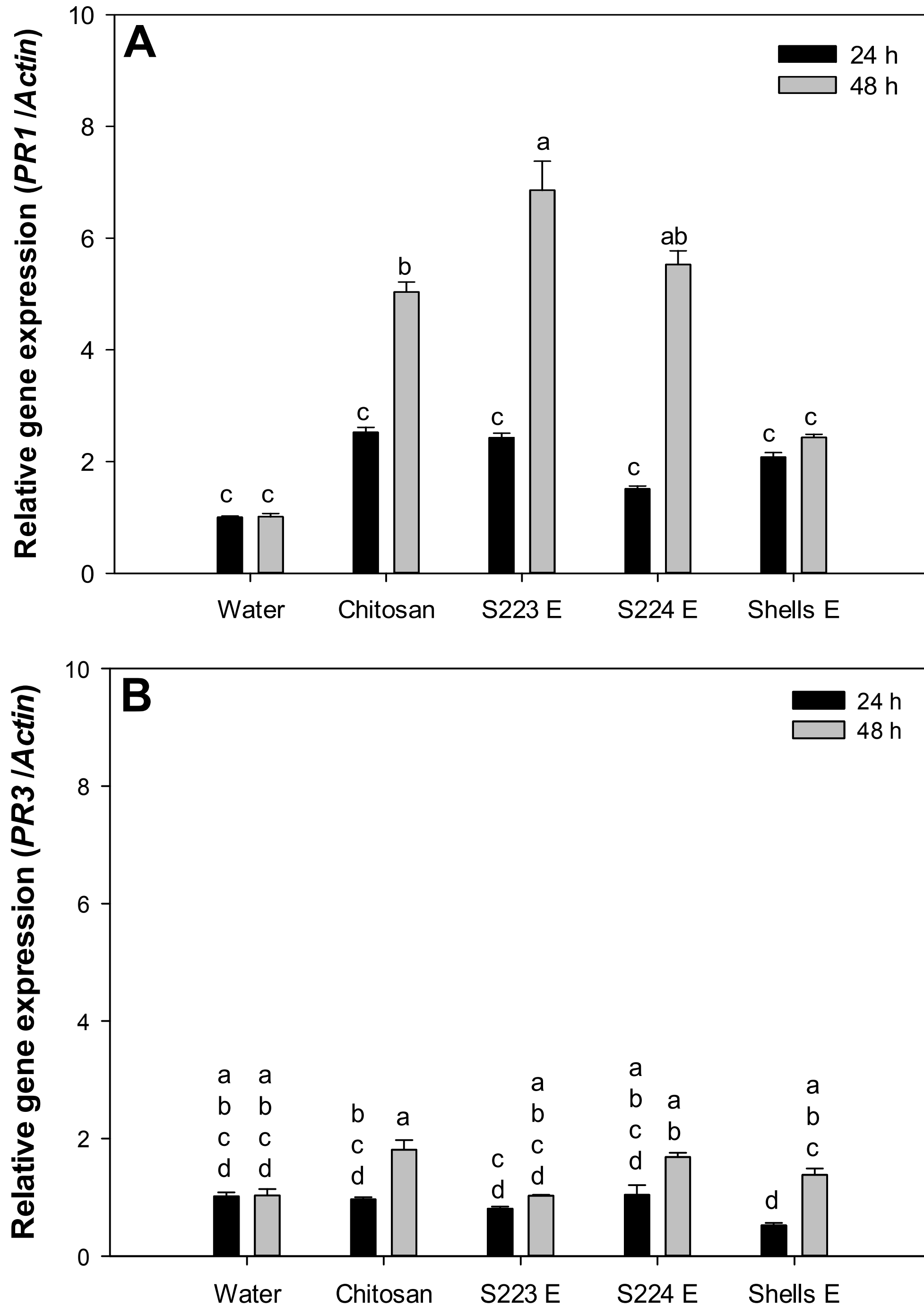

Figure 4.8. Relative expression levels of (A) *PR1* [P < 0.0001] and (B) *PR3* [P = 0.058] in Mock (Non-infected) leaf tissues of Arabidopsis treated with lobster shell extracts. Control –water, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells. Values represent mean ± SE of 2 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at α = 0.05). (continued on next page)

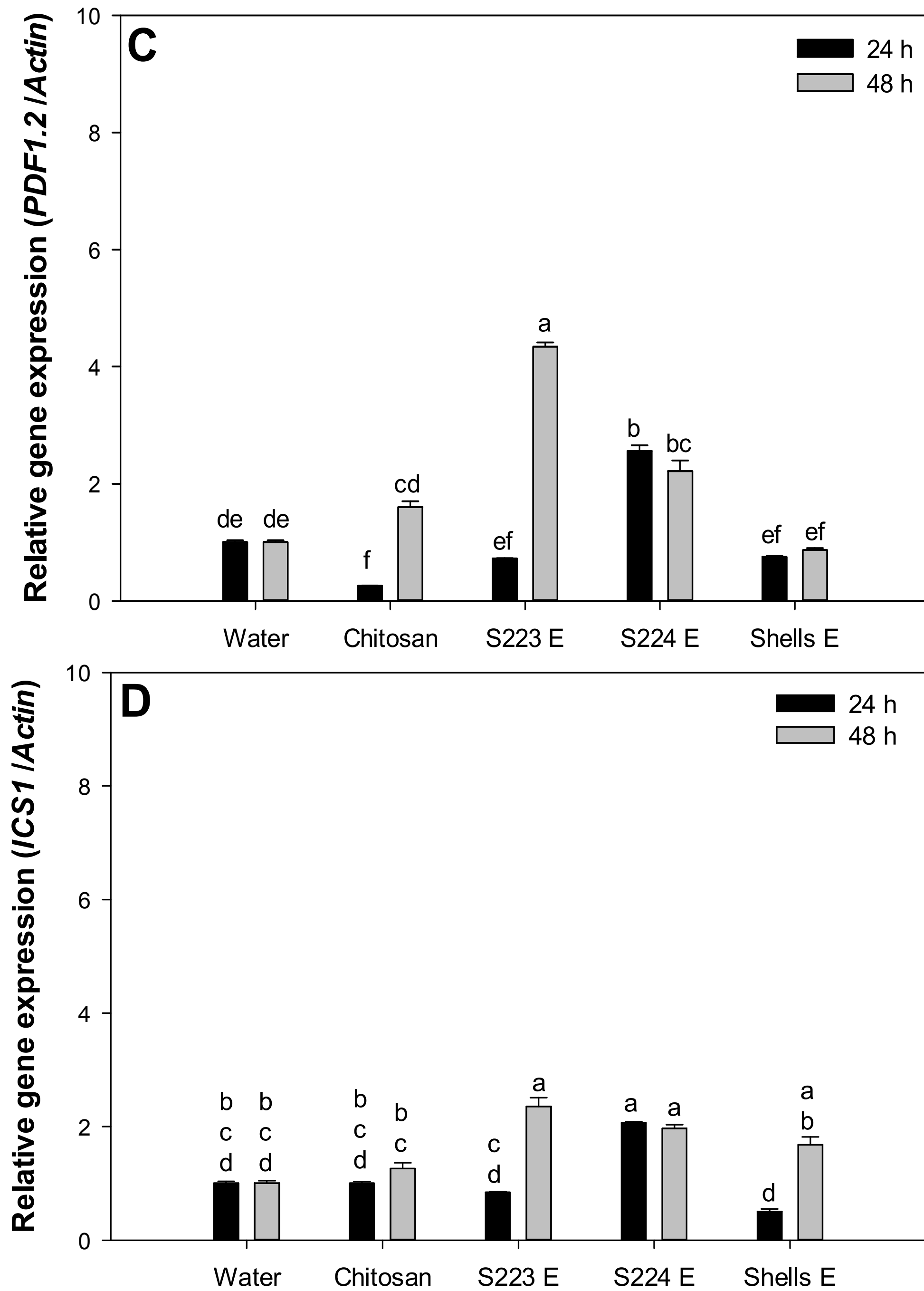

Figure 4.8. Relative expression levels of (C) *PDF1.2* [P < 0.0001] and (D) *ICS1* [P < 0.0001] in Mock (Non-infected) leaf tissues of Arabidopsis treated with lobster shell extracts. Control – water, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells. Values represent mean ± SE of 2 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at α = 0.05).

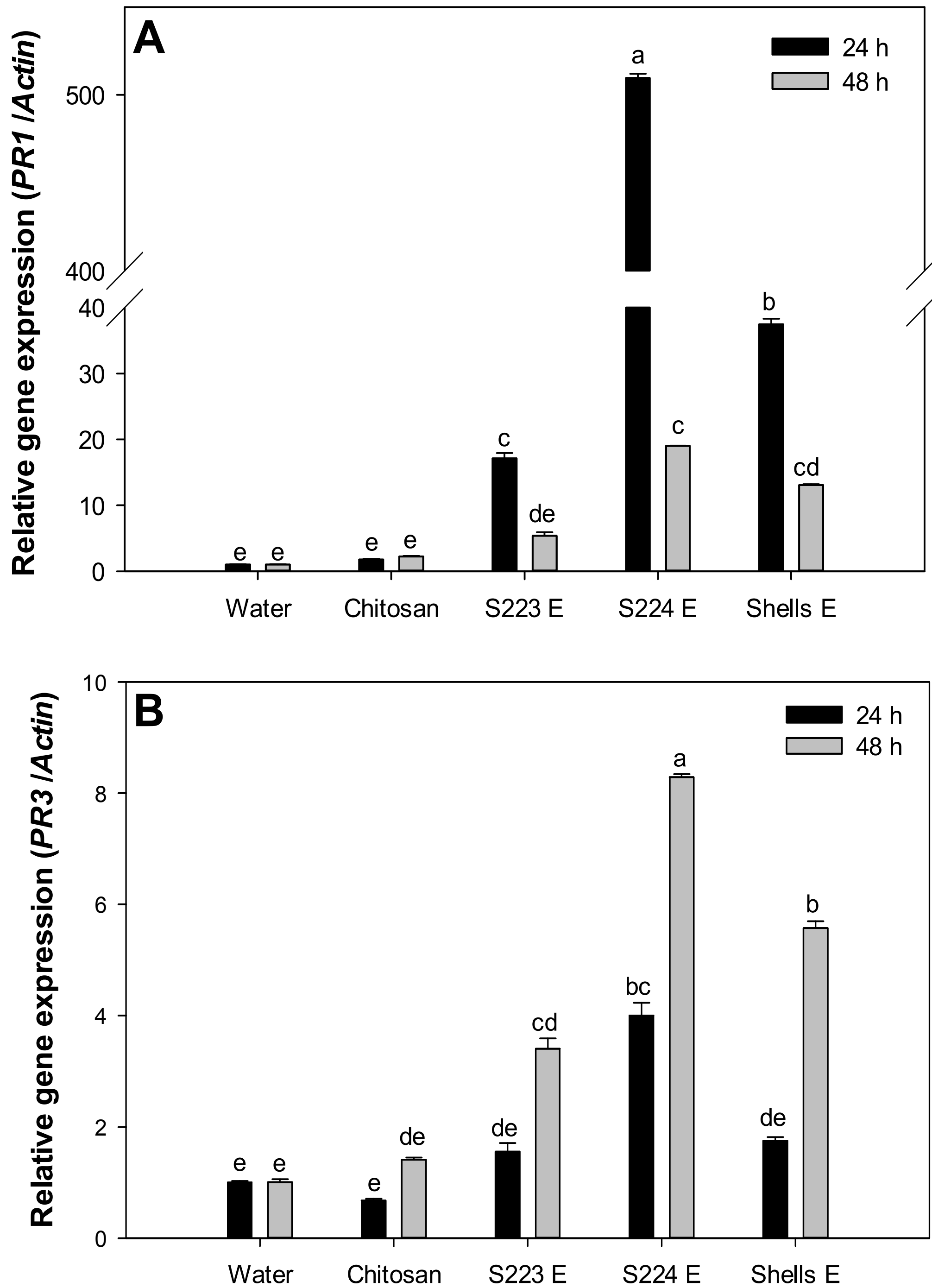

Figure 4.9. Relative expression levels of (A) *PR1* [P < 0.0001] and (B) *PR3* [P = 0.0001] in *Pst* DC3000 infected leaf tissues of Arabidopsis treated with lobster shell extracts. Control –water, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells. Values represent mean ± SE of 2 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at α = 0.05). (continued on next page)

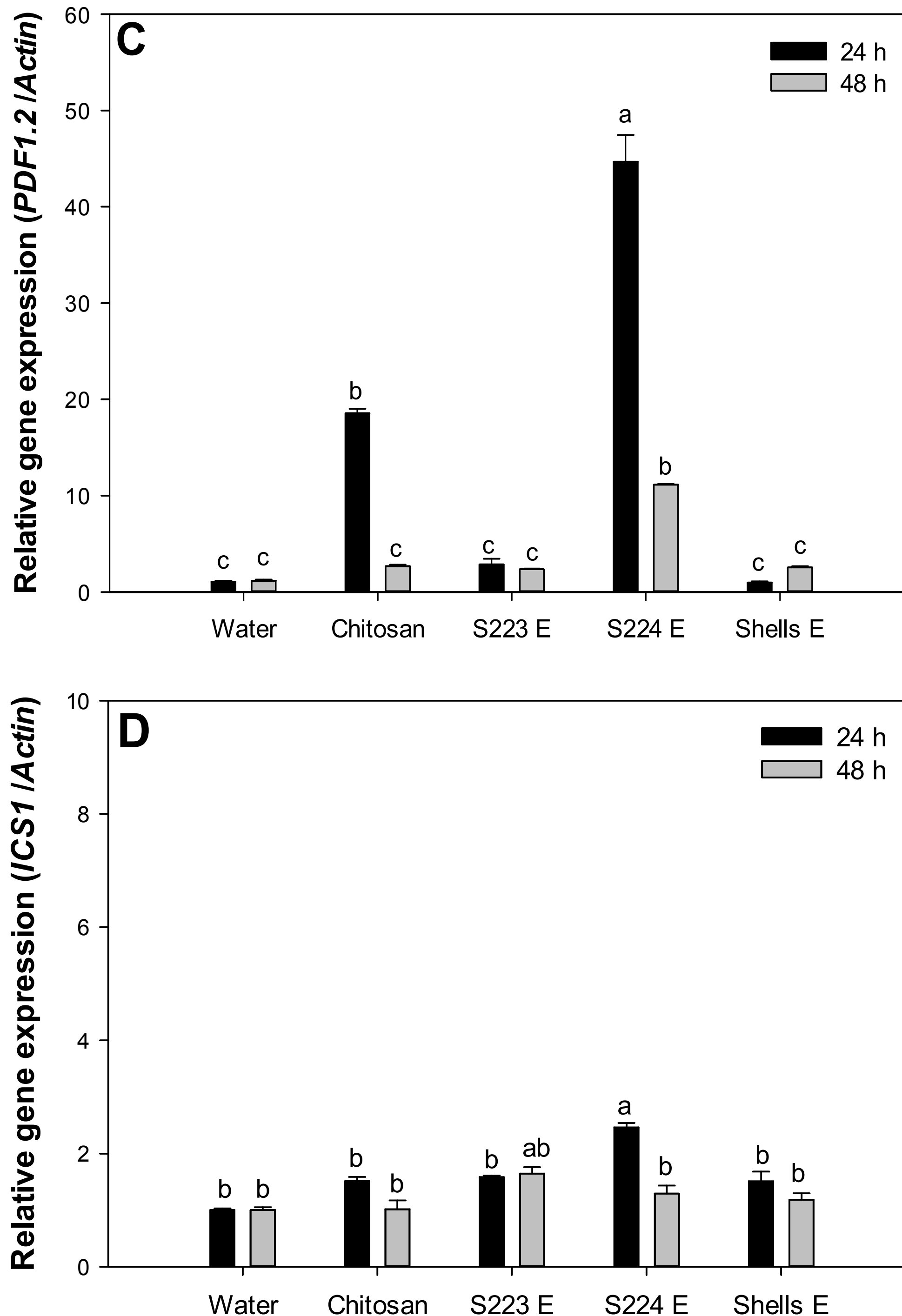

Figure 4.9. Relative expression levels of (C) *PDF1.2* [P < 0.0001] and (D) *ICS1* [P = 0.01] in *Pst* DC3000 infected leaf tissues of Arabidopsis treated with lobster shell extracts. Control – water, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells. Values represent mean ± SE of 2 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at α = 0.05).

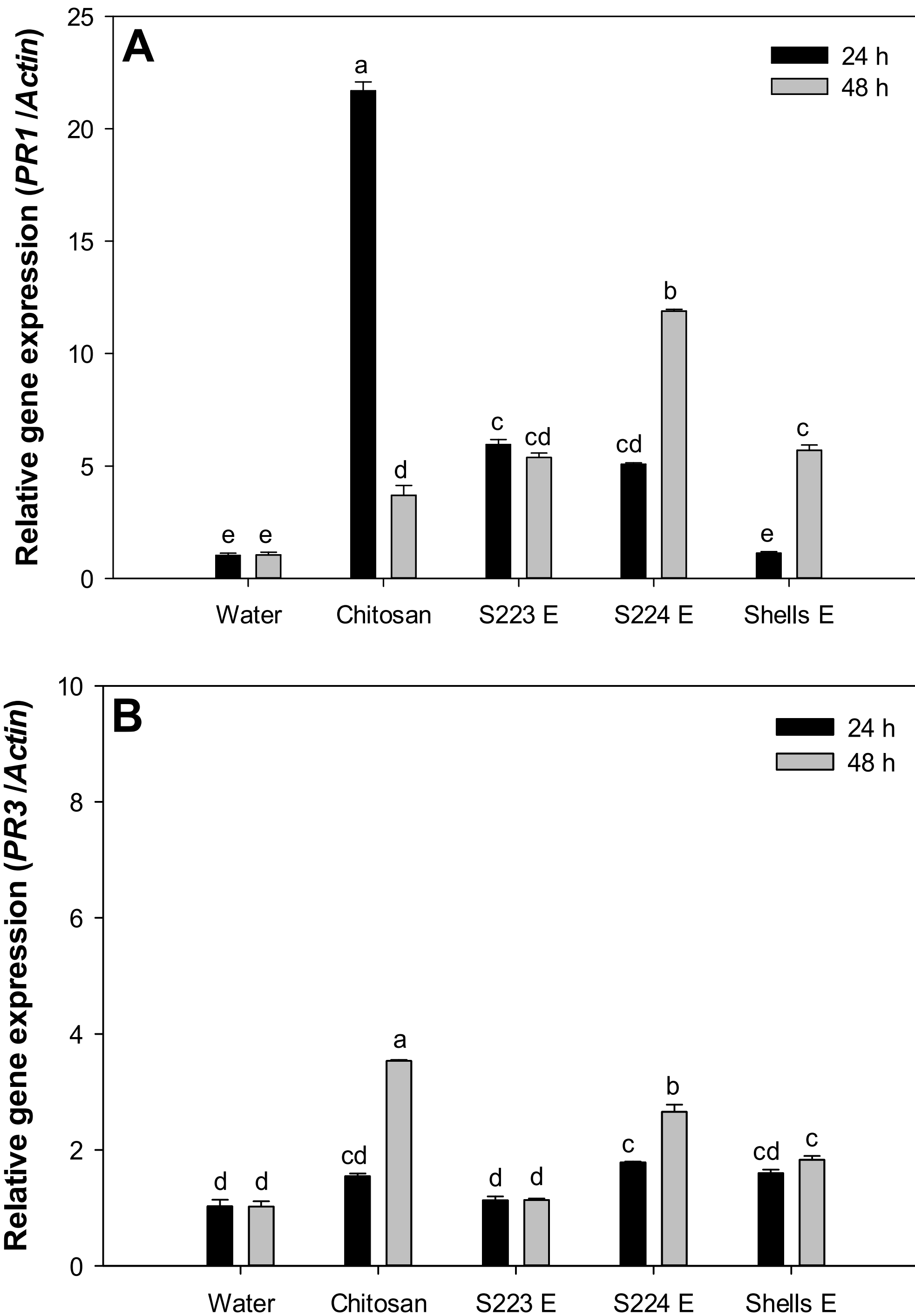

Figure 4.10. Relative expression levels of (A) *PR1* [P < 0.0001] and (B) *PR3* [P < 0.0001] in *Botrytis* infected leaf tissues of Arabidopsis treated with lobster shell extracts. Control –water, Chitosan – positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells. Values represent mean ± SE of 2 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at $\alpha = 0.05$). (continued on next page)

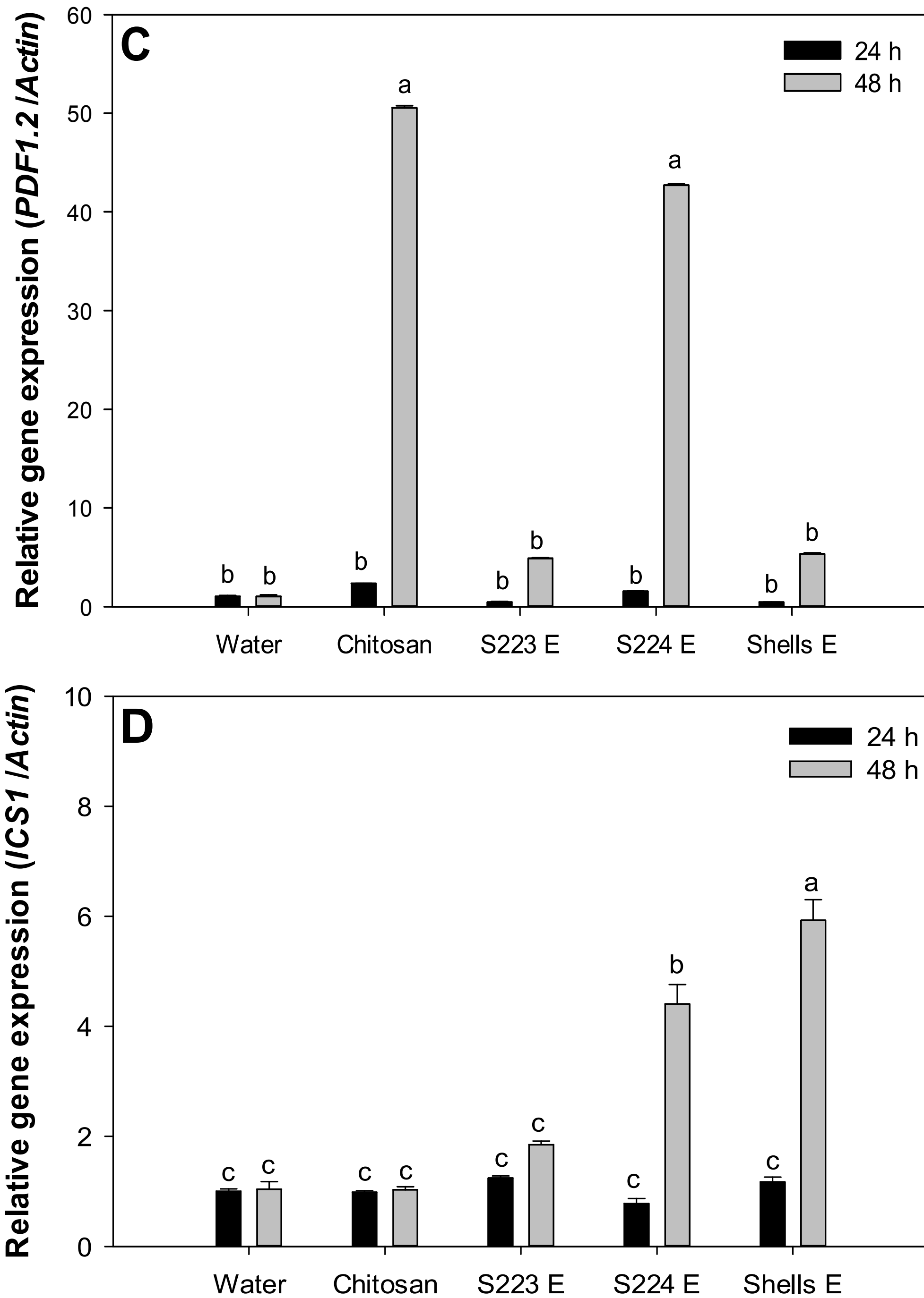

Figure 4.10. Relative expression levels of (C) *PDF1.2* [$P < 0.0001$] and (D) *ICS1* [$P < 0.0001$] in *Botrytis* infected leaf tissues of Arabidopsis treated with lobster shell extracts. Control –water, Chitosan –positive control, S223-E – extract of 1% of lobster shells digested by S223, S224-E – extract of 1% of lobster shells digested by S224, Shells-E – extract of 1% of undigested lobster shells. Values represent mean ± SE of 2 replications per treatment and values sharing the same letter are not significantly different (Tukey's HSD at $\alpha = 0.05$).

4.4. DISCUSSION

In this study, the microbe digested lobster shell extracts elicited disease resistance in Arabidopsis against *Pst* DC3000 and *Botrytis cinerea* infection. From the biochemical and gene expression studies, it can be understood that both induced systemic resistance (ISR) and systemic acquired resistance (SAR) regulated the induced disease resistance. In addition, the extracts also exhibited significant antimicrobial properties and affected the growth of the pathogens. Chater (2006) reported the production of secondary metabolites by *Streptomyces*, which had antibiotic properties and the secretion being regulated by extracellular signaling molecules.

The microbial culture filtrates obtained from lobster shell digestion might also contain secondary metabolites possessing antibiotic properties and along with chitinases had detrimental effect on the growth of plant pathogens. Chitinases also possess lysozyme properties and they can digest peptidoglycan of the bacterial cell walls, which is made of alternating β-1,4 linked residues of N-Acetylglucosamine and N-Acetylmuramic acid resembling the structure of chitin (Roberts and Selitrennikoff, 1988). When chitin in fungal cell wall is digested by chitinases, the associated β-glucan is solubilized as well causing the disruption of the cell wall (Schlumbaum et al., 1986).

The activities of chitinase and phenylalanine ammonia lyase are used in the present study as induced resistance markers after biotic stress and they correlate with the disease intensity and time course of infection. The increased enzyme activity in the infected plants is positively linked with increased disease resistance (Summermatter et al., 1995). The lobster shell extracts induced defense responses in non-infected plants (mock), which exhibited enzyme activities and expression of genes related to pathogen attack.

Arabidopsis plants pre-treated with S224 and S223 extracts had resulted in significant reduction in disease severity and number of *Pst* DC3000 colony forming units in the leaf tissue. While results of S224 extracts correspond to the inhibition experiments where significant control on the pathogen growth was observed, resistance of S223 extracts treated plants can be attributed to the induced defense mechanisms against *Pst* DC3000. The S223 extracts induced chitinase and Phenylalanine ammonia lyase (PAL) activities in infected plants followed by S224 extract and chitosan treatments.

However, S224 extracts induced disease resistance in addition to the biocontrol activity and this can be understood from gene expression studies. The expression level of *PR1* (500 fold) at 24 h in S224 extracts treated plants implied the induction of SAR mediated by salicylic acid pathway. The expression of *ICS1* did not change at 24 h except in S224 treated plants. Induction of *PDF1.2* at 24 h after *Pst* DC3000 suggested the up-regulation of Jasmonic acid (JA) mediated defense pathways, because among the plant defensins only *PDF1.2* is induced upon pathogen challenge (Thomma et al., 2002). Subramaniam et al., (2011) showed that *PDF1.2* was induced in Arabidopsis infected with *Pst* DC3000. Jasmonic acid dependent *PR3* expression of S224 extract treated plants indicated endochitinase activities being induced in the plants at 48 h after infection.

The results of *Botrytis* infection studies show that S223 extracts predominantly acted as a biocontrol against the fungi. S224 and S223 extracts had significantly controlled the lesion spread. *B.cinerea*, a necrotrophic pathogen, induced SA mediated systemic acquired resistance (Murphy et al., 2000; Audenaert et al., 2002). S224 extracts induced disease resistance was on par with the effects observed in chitosan treatment. The increased PAL activity in chitosan

treated plants resulted in the induction of *PR1* at 24 h after infection but PR1 expression decreased at 48 h when PAL activity still remained high. The expression of *ICS1* was higher at 48 h in S224 extracts treated plants, which correlates to the *PR1* induction by *ICS1* mediated salicylic acid pathway. Interestingly, the levels of ICS1 were higher than mock and *Pst* DC3000 infected plants. *Botrytis* also caused the induction of JA mediated PDF1.2, which regulates an antifungal defensing like peptide (Penninckx et al., 1996; Zimmerli et al., 2001). Ferrari et al., (2003) showed that the rate of lesion formation by *Botrytis* at the infection site depends on ethylene, JA and SA signaling pathways.

Chitin oligosaccharides are known to have activities as antimicrobial agents (Wang et al., 2008) and elicitors of plant defense responses (Xia et al., 2011). Extracts from non-inoculated lobster shells exhibited inhibition of *Pst* DC3000 at 24 h after incubation but showed 50-55% inhibition of Botrytis at 48 h after incubation. This suggested that chitin itself has a certain extent of antibiotic properties. The undigested lobster shell extract treated plants also exhibited an increase in enzyme activity and gene expression levels and the induced disease resistance conferred was due to the pathogen associated molecular patterns (PAMPs) of the chitinous extracts.

The results indicate that different signaling pathways activate the defense mechanisms. Disease resistance mediated by SA, JA and ET pathways can be induced by application of elicitors, which are often recognized by the plants as pathogen associated molecular patterns (Zipfel, 2009). The study showed that lobster shell extracts elicited defense responses and the plants exhibited increased disease resistance when infected by pathogenic microbes. The elicitors can be used as an alternative to chemical controls in plant protection (Benhamou, 1996).

CHAPTER 5

CONCLUSION

Lobster is an integral part of the seafood industry in Canada and contributes significantly to the economy of Maritime Provinces, generating $50million CAD in exports each year. Lobster shells are a waste material from the lobster meat processing facilities that is discarded in landfills and on shores causing a foul smell and other environmental issues. Any recycling process in land would take much longer than in a marine ecosystem because the chitinolytic microbiota would have to be established in order to degrade the huge mass of shells.

The exoskeleton of lobster is a source of chitin, proteins and calcium. Chitin is the second most abundant natural polysaccharide and is commercially produced by chemical treatment of crustacean shells, which leads to effluent residues. Chitin has wide applications in many fields including agriculture and pharmaceutical industries. If proper chitinous waste disposal can be carried out effectively, it would solve the lobster shell environmental problems and has potential economic value (Wang et al., 2002). Detailed information on this problem is summarized in Chapter 2 of this thesis.

Based on previous research on the biological breakdown of other crustacean shells, such as shrimps and crabs, but not lobster, this project attempted to utilize lobster shells to investigate the process of microbial degradation and potential applications of the digested extracts. The objectives of this project were to (1) isolate microorganisms able to degrade lobster shells, optimize their culture conditions and extract chitin derivatives and (2) to apply the extracts as plant protection compounds by inducing disease resistance in *Arabidopsis thaliana* against pathogens *Pseudomonas syringae* pv. tomato DC3000 3and *Botyrtis cinerea*.

The first objective is addressed in Chapter 3 and was met by isolating chitinolytic microorganisms from soil samples that had been amended with lobster shells as a source of nutrients. The ecosystem niche would naturally host communities of microorganisms that are actively degrading the organic matter present, including lobster shells. A growth media was formulated with lobster shells as the sole carbon source to isolate and screen microorganisms that can degrade the shells. Two soil isolates, S223 and S224 exhibited higher deproteinisation, demineralisation and chitinolysis in lobster shell media than other microbes studied.

The cultural conditions for the isolated microbes were optimized for various factors and each factor studied holds a key role in achieving efficient degradation. Specifically, there were two types of lobster shells, cooked and fresh left after processing and they differ in calcium, protein and chitin content. Both were studied. The state of digestion, solid vs. liquid, was used to study whether the microorganisms can carry out degradation at low moisture content in the media. The concentration of shells and load of microbial inoculum in the media were varied to investigate competent levels of both the factors for degradation in a particular time period.

The chitinases presumably involved in chitin breakdown were detected in a gel diffusion assay and their substrate specificity confirmed. The enzyme from a crude extract of S223 was purified by affinity chromatography with chitin as the binding substrate and the chitinase obtained was found to be around 30 kDa molecular in weight. Purification of chitinase from S224 remained a challenge in spite of 45.6 units/mg of chitobiosidase in the crude extract (data not shown). The higher antifungal activity of the crude extract from S224 suggested the presence of secondary metabolites and chitinases that inhibited the growth of plant-pathogenic fungi.

Isolation of chitinolytic microorganisms from soil and marine ecosystems and the subsequent use of more than one microorganism can expedite the breakdown of lobster shells. Deproteinisation and demineralisation can be performed in sequential steps to remove protein and calcium counterparts of the shell, followed by chitinolysis, rather than in a single process. On the other hand, this requires further optimization and rigorous analysis of each degradation activity.

Co-culturing of microorganisms would be a biodegradation possible option but their compatibility would come into question. One actinomycete S2231 isolated from soil samples, which was morphologically similar to S223 exhibited higher chitin deacetylase activity and demineralisation of lobster shells. The isolate had always been associated with a green bacterium even after subsequent subcultures, which was initially thought to be a contamination and such observation deserves attention in future research. A protocol to detect chitosan in lobster shell media would demonstrate chitin deacetylase activity of the microorganisms, which was hindered by pigments in the present study.

Understanding the mechanisms that regulate the synthesis of microbial chitinases may reveal alternative, specific targets for antibiotic compounds. Sophisticated bioreactors with oxygen controls could facilitate degradation of large quantities of shells under optimum conditions and extract chitin-derived compounds. The chitinolytic system of the soil actinomycetes (S223 and S224) provides an interesting model for further chitinase studies, phenomena that are important in the production of chitinases and enable biodegradation of chitin in the environment. Identification of the actinomycetes as *Streptomyces sp*. was supported by the

earlier research on antibiotic and chitinolytic properties of these Gram-positive, filamentous bacteria.

To shed more light on the determination of microbial degradation activities, a chemical profile of the crude extracts and analysis of digested sediments are required. The extracellular metabolites present in the extracts could be fractionated and studied for their specific antimicrobial properties and plant-defense elicitation compounds.

The second objective focused on the role of the digested extracts in plant defense and induced disease resistance against plant pathogens and is addressed in Chapter 4. A convincing explanation of the predominant reason for reduced disease intensity in phenotype observations of infected Arabidopsis plants is due to combined action of antimicrobial properties of the lobster shell extracts and their function as a potential elicitor of plant defense responses.

The biochemical and gene expression studies confirmed that disease resistance was induced in the plants upon treatment with lobster shell extracts. The increased quantities of chitinase and phenylalanine ammonia lyase and the expression levels of *PR1* and *PDF1.2* genes attribute to the defense responses activated in the plant tissues. The structural similarity of chitin derivatives in the extracts to a pathogen's cell wall and recognition of these non-toxic compounds as pathogen associated molecular patterns by the plants probably stimulated systemic acquired resistance and confered enhanced disease resistance when they were exposed to pathogen infection.

There are differences in the induction of resistance at different stages after treatment. In order to gain a better understanding of the elicitation of plant disease resistance, several genetic and biochemical measures of resistance in plants across different taxonomical groups and

agronomical importance, under a range of environment and resource conditions, are required. Microarray studies of several pathogenesis related genes would provide an understanding of the induced disease resistance and systemic acquired resistance pathways triggered by the application of lobster shell extracts.

Together with the benefits of chitin mentioned in the literature review (see Chapter 2), all the observations that demonstrate microbial degradation of lobster shells and plant disease resistance elicitation, rationalize the results obtained in ~~of~~ the present analysis. The characterization of chitinolytic enzymes from soil actinomycetes offers a precognition for scaling up the degradation studies into an industrial process and provides a tremendous potential economic value for lobster shells. Chitin produced by biological methods is not commercially available yet, but this study provides new insights that can contribute to potential uses in the future. Lobster shell holds the potential to be used as a valuable amendment in crop fields and chitinolytic microorganisms present in soil would continuously recycle the nutrients from the shells, thereby enriching plant growth and protection.

APPENDIX I – MEDIA AND REAGENTS COMPOSITION

Media

All media concentrations are in 1-liter volume and autoclaved at 121 °C for 20 min.

Solid media has 15 g Agar (Bacto) per liter

1. **M9 minimal buffer** pH 7

KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
$Na_2HPO_4 . 7H_2O$	6 g
1 M $MgSO_4$	1 mL (added after autoclaving)

2. **King's B Broth** pH 7 (for *Bacillus* and *Pseudomonas*)

Peptone	10 g
KH_2PO_4	1.5 g
Glycerol	10 mL
1 M $MgSO_4$	1 mL (added after autoclaving)

3. **Tryptone-yeast extract broth** pH 7-7.2

Tryptone	5 g
Yeast extract	3 g

4. **Yeast extract-malt extract agar (YEME agar)** pH 7.2

Yeast extract	4 g
Malt extract	10 g
Dextrose	4 g

5. **Trace salts solution**

$FeSO_4.7H_2O$	1g
$MnCl_2.4H_2O$	1g
$ZnSO_4.7H_2O$	1g

6. **Oatmeal Agar** pH 7.2

Oatmeal	20 g
Trace salts solution	1mL
20 g Oatmeal was cooked or steamed in 1000 mL distilled water for 20 min filtered through cheesecloth.	

7. **Glycerol-asparagine agar** pH 7.4

L-asparagine	1g
Glycerol	1g
Trace salts solution	1mL

8. **Inorganic salts-starch agar** pH 7.4

K_2HPO_4	1g
$MgSO_4.7H_2O$	1g
NaCl	1g
$(NH_4)_2\ SO_4$	2g
$CaCO_3$	2g
Trace Salts solution	1mL
Soluble starch	10g
Starch was added to small amount of cold distilled water and the paste was mixed with the above solution	

9. **Peptone-yeast extract-iron agar** pH 7.2

Yeast extract	1g
Bacto-peptone	15g
Proteose peptone	5g
Ferric ammonium citrate	0.5g
K_2HPO_4	1g
Sodium thiosulphate	0.8g

10. **Tyrosine agar** pH 7.2

Glycerol	15g
L – tyrosine	0.5g
L – asparagine	1g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.5g
NaCl	0.5g
$FeSO_4.7H_2O$	0.01g
Trace salts solution	1mL

Reagents, buffers and reaction substartes

1. **3,5 – Dinitrosalycylic acid reagent** (DNS):

20 mL of 96 mM 3,5- dinitrosalicylic acid is added to 8 mL of 5.3 M of sodium potassium tartarate solution in containing 2 M NaOH. The final volume of 40 mL is made with deionized water.

2. **N-Acetylglucosamine** (GlcNAc) **solution**:

0.1 g of N-Acetylglucosamine is dissolved in 10 mL deionized water.

3. **2-Thiobarbituric acid solution**:

A standard solution 0f 0.04 M is prepared by dissolving 0.576 g of reagent in 100 mL distilled water in warm heat for 15 min.

4. **Chitosan stock solution**:

100 mg of chitosan is dissolved in 100 mL of 1% (V/V) acetic acid under constant shaking. From this solution suitable dilutions (25, 50 and 100 μg/mL) were made when required.

5. **p-Dimethylaminobenzaldehyde reagent**:

10 g of p-Dimethylaminobenzaldehyde was dissolved in 100 mL of 10% acetic acid (glacial) with 1.25 mL of conc. HCl. The solution was made fresh before use.

6. **Colloidal chitin** (affinity chromatography)

1 g of chitosan is dissolved in 20 mL of 10% acetic acid by grinding in a mortar. The viscous solution is allowed to stand overnight at 22 °C. Methanol (90 mL) is slowly added and the solution is filtered through Whatman no. 4 filter paper. The filtrate was transferred in to a beaker and 1.5 mL of acetic anhydride is added with magnetic stirring. The resulting gel is left to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel is discarded. Gel pieces will be transferred to a blender, covered with methanol and homogenized at top speed for 4min. The suspension is filtered in a Buchner funnel using Whatman No. 3 filter paper and washed to neutrality. The pellet is resuspended in deionized water (300 mL) containing 0.02% (w/v) sodium azide. The suspension is ball milled using fifty 10 mm and 5mm stainless steel balls for 48 h at 120 rpm. It is filtered again and the final concentration is made to 17mg/mL (dry weight), stored at 4 °C (Dickinson et al., 1989).

7. **Glycol chitin**

The exact procedure above is followed for acetylation of glycol chitosan till the homogenization step. This suspension is centrifuged at 27,000 × g for 15 min at 4°C. The gelatinous pellet is resuspended in about 1 volume of methanol, homogenized and centrifuged as in the preceding procedure. The pellet is washed with water until the pH become neutral and suspended in deionized water (100 mL) containing 0.02% (w/v) sodium azide. This is the final concentration is made to 1% (w/v) stock solution of glycol and stored at 4 °C (Trudel and Asselin, 1989).

8. **Universal pH indicator**

Methyl orange	0.05 g
Methyl red	0.15 g
Bromothymol blue	0.30 g
Phenolphthalein	0.35 g
Ethanol/water	1 L
Indicators dissolved in 10 mL ethanol and made up to 1 liter with water	

Other dehydrated media used:

NB – Nutrient broth (Difco®)

PDB – Potato dextrose broth (Difco®)

TSB – Tryptic soy broth (Difco®)

APPENDIX II – TABLES AND FIGURES IN CHAPTERS 3 AND 4

Figure A.1. Lobster shells were grinded, sieved and stored in glass jars at 4 °C. Cooked (left) and fresh (right) lobster shell powder.

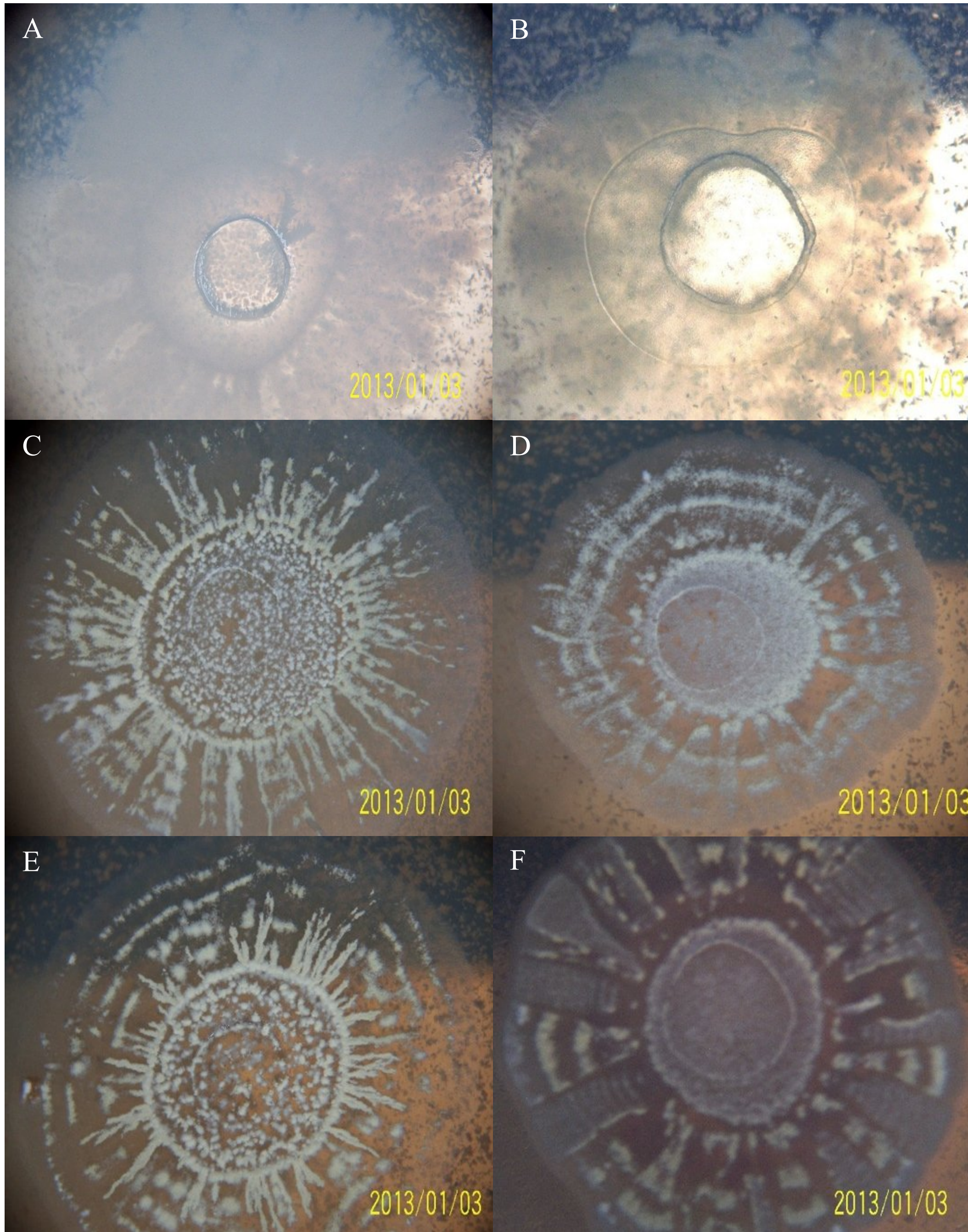

Figure A.2. Observation of clearing zones under a light microscope at 40 X magnification. A) *Bacillus subtilis*, B) S23 C) S223 D) S224 E) S2231 F) S113

Table A.1. Colony growth of microorganisms on agar plates containing chitin, cooked and fresh lobster shell powder measured on day 7 after incubation at 25 °C [n=3, $P < 0.0001$]

Number	Microbe	Media constituent	Colony diameter (cm)	Letter groupings
1	Bs	Fresh lobster shells	3	A
2	S2231	Fresh lobster shells	2.67	AB
3	S223	Fresh lobster shells	2.4	BC
4	S113	Fresh lobster shells	2.33	BCD
5	S223	Cooked lobster shells	2.1	CDE
6	S2231	Cooked lobster shells	2.07	CDE
7	S113	Cooked lobster shells	2	DE
8	S224	Fresh lobster shells	2.03	DE
9	S113	Chitin	1.83	EF
10	S224	Cooked lobster shells	1.8	EF
11	S223	Chitin	1.77	EFG
12	S2231	Chitin	1.77	EFG
13	S224	Chitin	1.63	FGH
14	S232	Fresh lobster shells	1.6	FGH
15	Bs	Cooked lobster shells	1.53	FGHI
16	S23	Cooked lobster shells	1.53	FGHI
17	Pf	Fresh lobster shells	1.5	FGHIJ
18	S23	Fresh lobster shells	1.5	FGHIJ
19	S23	Chitin	1.43	GHIJK
20	Lba	Fresh lobster shells	1.43	GHIJK
21	Bs	Chitin	1.4	HIJK
22	S232	Cooked lobster shells	1.3	HIJKL
23	Lba	Cooked lobster shells	1.23	IJKLM
24	Pf	Cooked lobster shells	1.17	JKLM
25	S232	Chitin	1.13	KLM
26	Pf	Chitin	1.03	LM
27	Lba	Chitin	0.9	M

Data points sharing the same letters are not significantly different (Tukey's HSD at $\alpha = 0.05$). (Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*)

Table A.2. Protease activity measured in the culture supernatants of microbes grown in fresh lobster shells from one to seven days after incubation at 25 °C [n=3, P < 0.0001]

Number	Microbe	Day	Protease (units / mg of protein)	Letter groupings
1	Bs	3	115.53	A
2	S223	7	103.79	AB
3	Bs	4	101.75	AB
4	Bs	5	86.06	ABC
5	S223	6	81.19	ABC
6	Bs	6	65.43	ABC
7	S223	4	65.39	ABC
8	S223	5	63.51	ABC
9	S223	3	63.25	ABC
10	Bs	2	29.3	ABCD
11	S224	7	27.28	ABCD
12	Bs	7	22.92	ABCD
13	S224	6	16.27	BCD
14	S224	5	4.57	CDE
15	Th	3	1.9	CDE
16	Th	4	1.19	CDE
17	S223	2	-0.72	CDE
18	Th	6	-34.9	DEF
19	Th	5	-37.86	DEF
20	Th	7	-42.55	DEF
21	S224	4	-55.64	DEF
22	Th	1	-58.4	DEF
23	Th	2	-83.06	EFG
24	S224	3	-119.37	FG
25	S224	2	-121.35	FG
26	S223	1	-177.55	GH
27	S224	1	-248.29	H
28	Bs	1	-489.21	I

Data points sharing the same letters are not significantly different (Tukey’s HSD at $\alpha = 0.05$). (Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*)

Table A.3. pH changes during incubation with fresh lobster shell powder at 25 °C

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Bs	7.13	7.37	7.42	7.44	7.55	7.63	7.63
Pf	7.27	7.51	7.58	7.61	7.71	7.77	7.81
S23	7.26	7.35	7.54	7.52	7.62	7.63	7.69
S232	7.31	7.41	7.54	7.51	7.67	7.69	7.74
S113	7.29	7.47	7.62	7.6	7.72	7.75	7.74
S224	7.29	7.46	7.54	7.49	7.58	7.61	7.65
S223	7.28	7.41	7.51	7.51	7.62	7.64	7.64
S2231	7.31	7.46	7.56	7.5	7.59	7.66	7.67
Lba	7.27	7.37	7.56	7.59	7.71	7.75	7.81
Th	7.31	7.29	7.42	7.39	7.5	7.52	7.61
Shells	7.36	7.57	7.69	7.8	7.89	7.96	7.99

Table A.4. pH changes during incubation with cooked lobster shell powder at 25 °C

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Bs	7.39	7.35	7.43	7.51	7.56	7.61	7.58
Pf	7.41	7.48	7.57	7.66	7.74	7.81	7.89
S23	7.45	7.49	7.54	7.59	7.61	7.63	7.65
S232	7.42	7.45	7.49	7.52	7.55	7.58	7.64
S113	7.4	7.45	7.5	7.55	7.58	7.61	7.64
S224	7.39	7.45	7.51	7.57	7.58	7.59	7.59
S223	7.36	7.42	7.46	7.5	7.56	7.62	7.65
S2231	7.41	7.58	7.55	7.53	7.59	7.66	7.61
Lba	7.37	7.6	7.62	7.64	7.69	7.74	7.83
Th	7.36	7.47	7.5	7.54	7.6	7.66	7.67
Shells	7.43	7.59	7.69	7.79	7.88	7.98	8.04

(Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*)

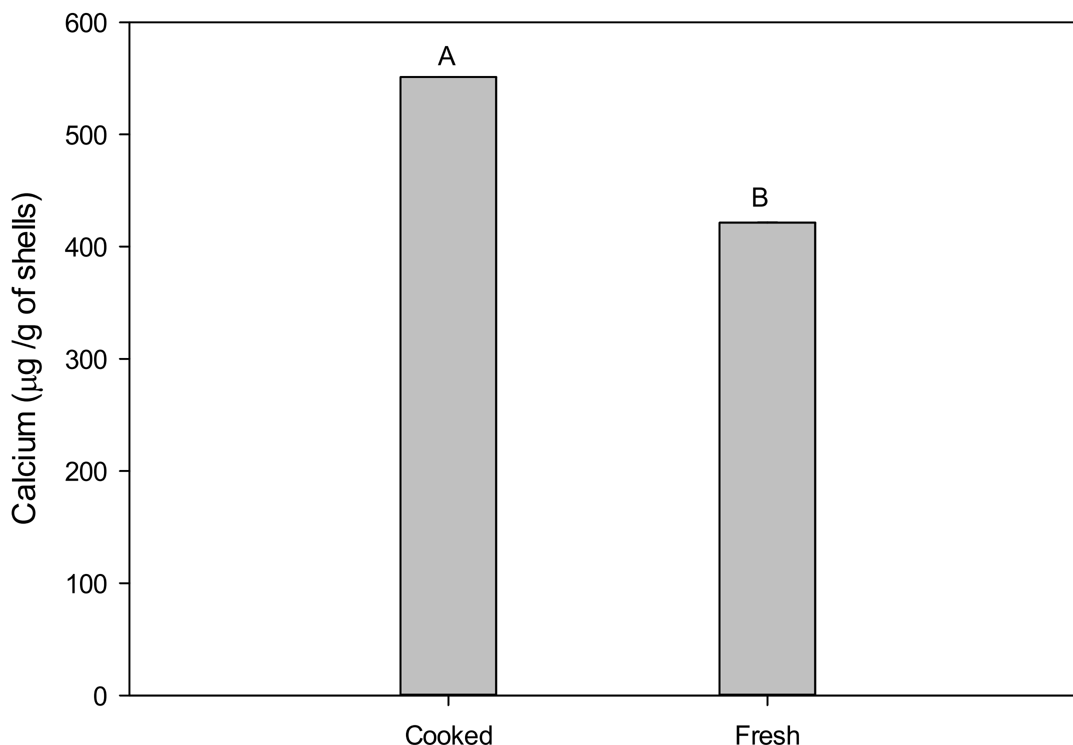

Figure A.3. Calcium content of cooked and fresh lobster shells determined by ash test. The ashen samples of the shells were dissolved in HCl and then fed into a Varian® Atomic absorption spectrometer. Values are mean ± SE of four replicates and and values sharing the same letter are not significantly different (Tukey's HSD at α = 0.05).

Table A.5. Calcium content measured in the culture supernatants of microbes grown in cooked and fresh lobster shells on day seven after incubation at 25 °C [n=3, P < 0.0001]

Number	Microbe / Shells	Type of shells	Calcium (µg / mL)	Letter groupings
1	S224	Cooked	1.57	A
2	S223	Cooked	1.4	ABC
3	S232	Cooked	1.36	ABCD
4	Lba	Cooked	1.15	BCDE
5	S113	Cooked	1.15	BCDE
6	Th	Cooked	1.11	BCDE
7	Shells	Cooked	1.1	BCDE
8	S23	Cooked	1.1	BCDE
9	S2231	Cooked	1.02	CDE

Data points sharing the same letters are not significantly different (Tukey's HSD at α = 0.05). (Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*) continued on next page

Table A.5. Calcium content measured in the culture supernatants of microbes grown in cooked and fresh lobster shells on day seven after incubation at 25 °C [n=3, P < 0.0001]				
Number	Microbe / Shells	Type of shells	Calcium (µg / mL)	Letter groupings
10	Bs	Cooked	1	DE
11	Pf	Cooked	0.93	E
12	Bs	Fresh	1.48	AB
13	S2231	Fresh	1.39	ABCD
14	S223	Fresh	1.23	ABCDE
15	S23	Fresh	1.19	ABCDE
16	S232	Fresh	1.17	BCDE
17	Th	Fresh	1.16	BCDE
18	S224	Fresh	1.12	BCDE
19	Lba	Fresh	1.12	BCDE
20	S113	Fresh	1.1	BCDE
21	Pf	Fresh	1	DE
22	Shells	Fresh	0.87	E

Table A.6. N-Acetylglucosamine quantified in the culture supernatants of microbes grown in chitin at 25 °C [n=3, P < 0.0001]				
Number	Microbe / Shells	Days after incubation	GlcNAc (µg/ml of sample)	Letter groupings
1	Pf	3	26.91	A
2	S224	7	27.3	A
3	S223	3	23.83	B
4	Pf	5	23.39	B
5	Pf	7	20.96	C
6	Pf	1	19.59	CD
7	S223	1	18.55	D
8	S23	3	16.81	E
9	S224	5	12.88	F
10	S23	7	12.3	FG
11	S23	5	10.93	GH
12	S23	1	10.15	H
13	S223	5	9.78	H
14	S223	7	10.1	H
15	S2231	7	5.12	I

Data points sharing the same letters are not significantly different (Tukey's HSD at $\alpha = 0.05$). (Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*) continued on next page

Table A.6. N-Acetylglucosamine quantified in the culture supernatants of microbes grown in chitin at 25 °C [n=3, P < 0.0001]

Number	Microbe / Shells	Days after incubation	GlcNAc (µg/ml of sample)	Letter groupings
16	S113	5	4.61	IJ
17	S113	7	4.13	IJK
18	S224	3	3.2	JKL
19	Bs	5	2.95	KL
20	S232	7	2.58	LM
21	S113	3	2.44	LMN
22	Lba	7	1.7	LMNO
23	S232	5	1.07	MNOP
24	Lba	1	0.98	NOP
25	Lba	3	0.93	NOP
26	Lba	5	0.98	NOP
27	S232	1	0.84	OP
28	S224	1	0.8	OP
29	S113	1	0.47	OP
30	Th	1	0.45	OP
31	S2231	3	0.82	OP
32	S2231	3	0.82	OP
33	Bs	3	0.82	OP
34	S232	3	0.45	OP
35	Th	5	0.26	OP
36	Th	7	0.86	OP
37	Bs	7	0.73	OP
38	S2231	1	0.01	P
39	Bs	1	-0.01	P
40	Th	3	0.01	P
41	S2231	5	-0.2	P

Data points sharing the same letters are not significantly different (Tukey's HSD at $\alpha = 0.05$). (Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*)

Table A.7. Chitosan content quantified in the culture supernatants of microbes grown in chitin at 25 °C [n=3, P = 0.0002]

S.No	Microbe / Shells	Days after incubation	Chitosan (µg/ml of sample)	Letter groupings
1	S223	7	5.02	A
2	S223	6	3.72	B
3	S2231	7	3.51	B
4	S224	7	3.45	B
5	S2231	6	2.94	BC
6	S113	7	2.27	CD
7	S224	6	2.13	CD
8	S113	6	1.5	DE
9	Bs	7	1.43	DE
10	Pf	7	1.17	EF
11	Th	7	1	EF
12	S23	7	0.82	EF
13	Bs	6	0.65	EF
14	S23	6	0.61	EF
15	Pf	6	0.57	EF
16	Th	6	0.56	EF
17	Lba	6	0.48	F
18	Lba	7	0.43	F
19	S232	7	0.35	F
20	S232	6	0.3	F

Data points sharing the same letters are not significantly different (Tukey's HSD at α = 0.05). (Bs – *Bacillus subtilis*, Pf – *Pseudomonas fluorescens*, Lba – *Lactobacillus acidophilus*, Th – *Trichoderma harzianum*)

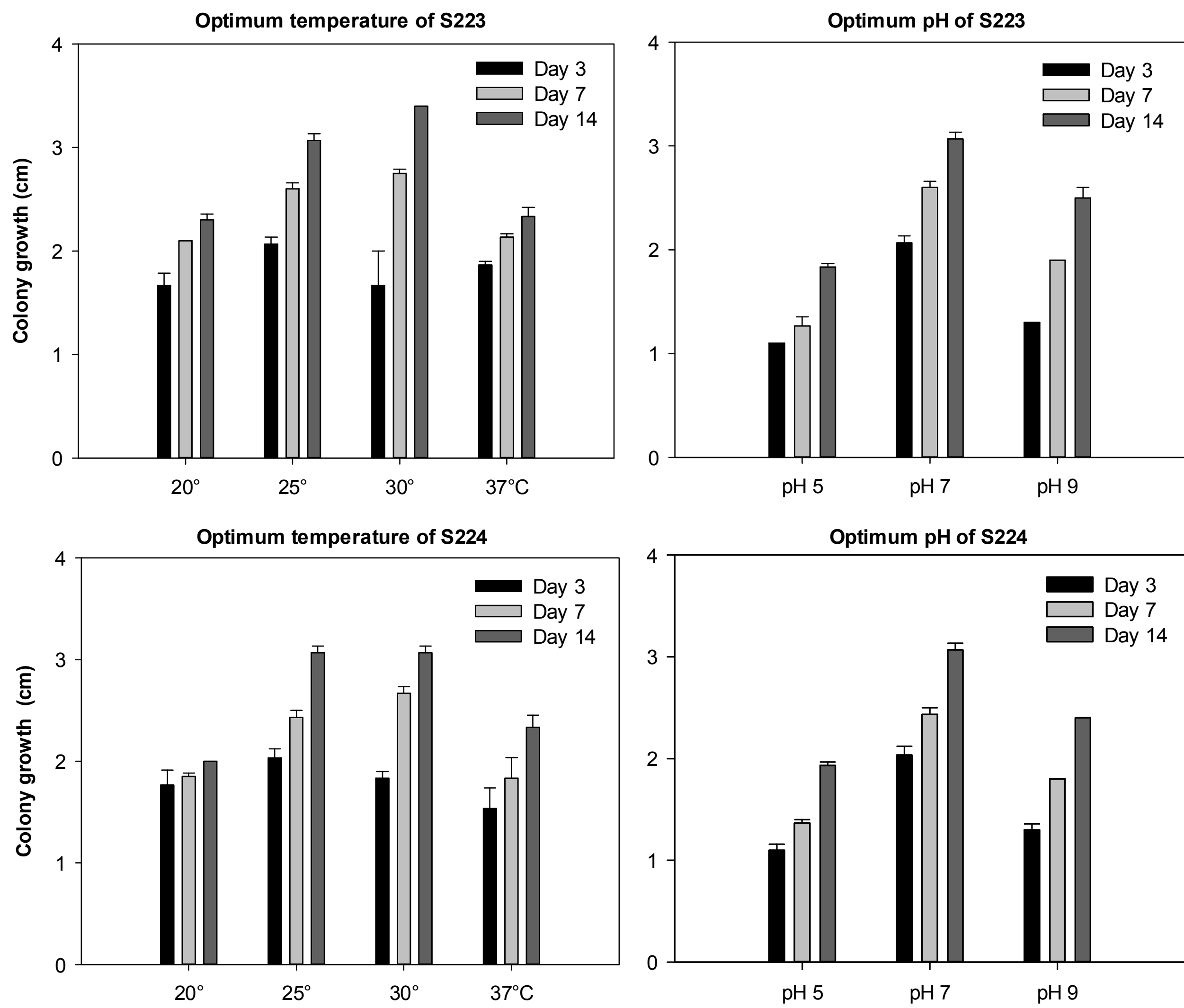

Figure A.4. Optimum temperature and pH for the growth of S223 and S224 observed by measuring colony diameter on day 14 after incubation on agar plates containing fresh lobster shells (0.5% w/v).

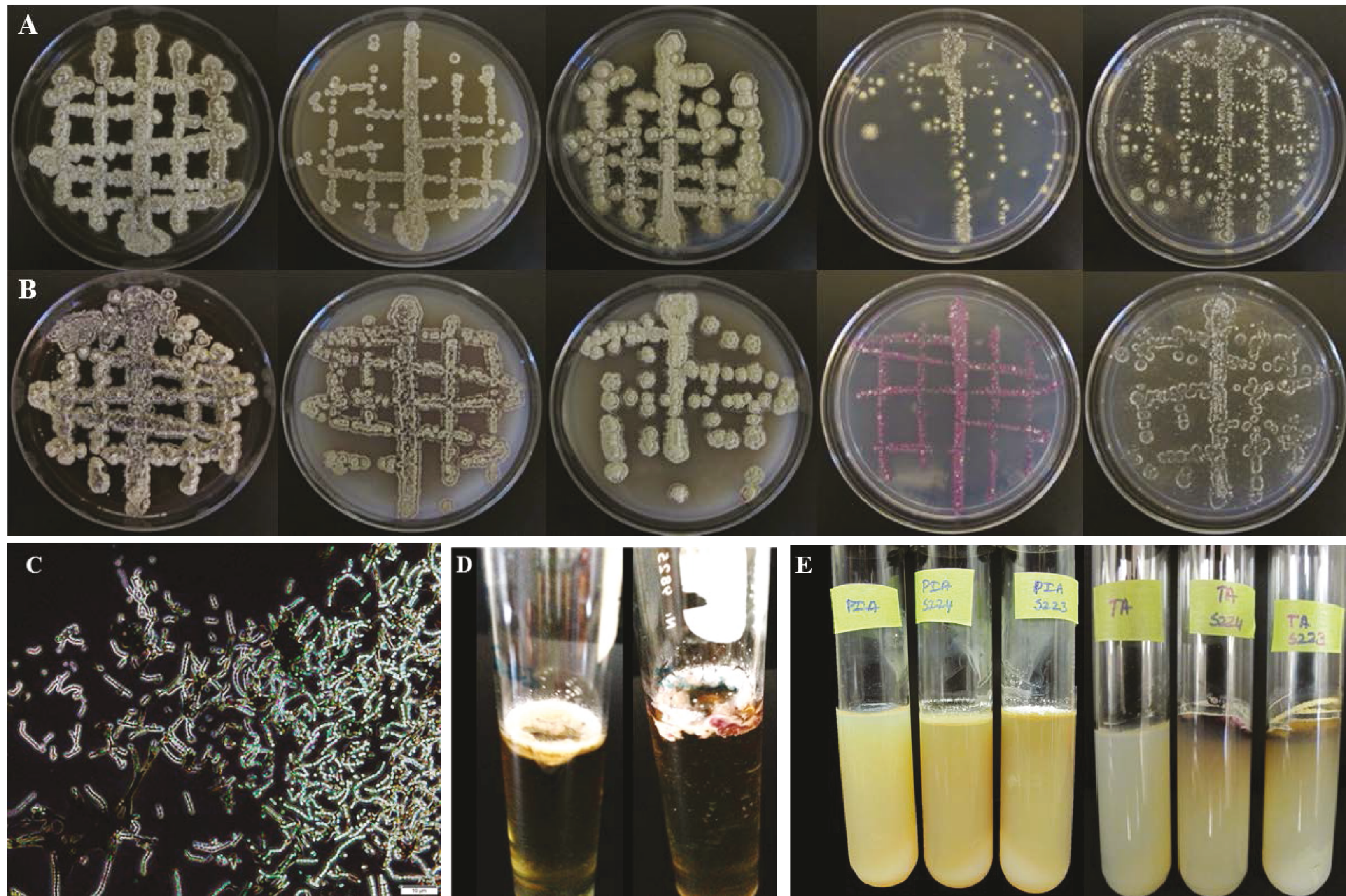

Figure A. 5. (A) and (B) are Colonies of S223 and S224 on YEME, oats, starch, glycerol and lobster shell agar plates (left to right) respectively. (C) Spore chains of S223 under 1000X magnification (D) Thioglycollate test (E) Melanin production in peptone-iron and tyrosine agar slants.

Table A.8. Morphological characteristics of S223 and S224				
Morphology characteristics on growth media	**S223**		**S224**	
	Colour of aerial mycelium	Colour on the reverse side	Colour of aerial mycelium	Colour on the reverse side
YEME	White	Brown	White	Deep pink
Oats	Milky white	Brown to green	White	Pink
Starch	Greenish white	Green to brown	White	Pink to dark purple
Glycerol	White	Creamy white	Pink	Pinkish purple
Lobster	White	Creamy white	White	White to pale pink

Table A.9. Physiological characteristics of S223 and S224		
Physiological characteristics	**S223**	**S224**
Peptone iron	Brown	Yellow to brown
Tyrosine	Dark green to brown	Dark pink to brown
Oxygen class Thioglycollate test	Aerobic	Aerobic

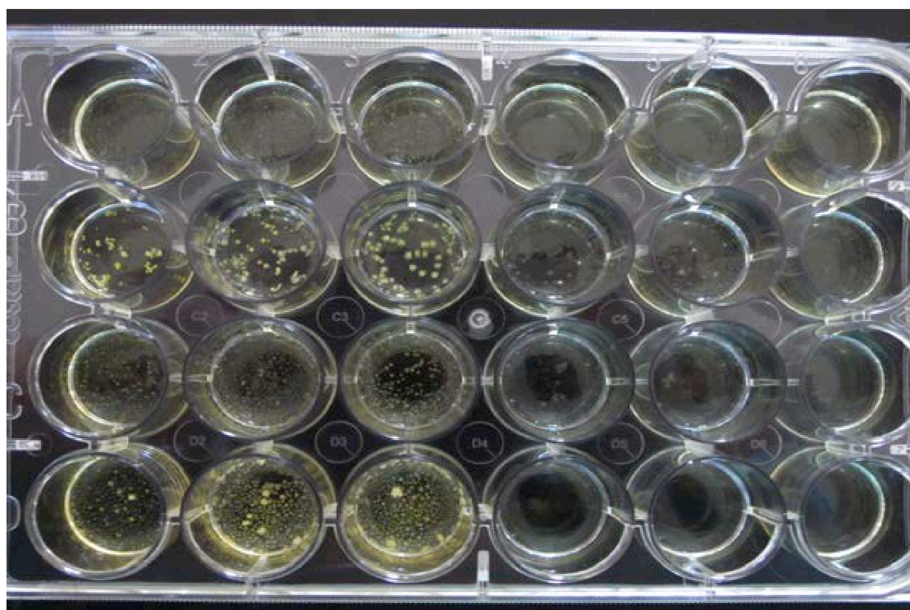

Figure A.6. Cultures of S223 and S224 growing on nutrient sources in 24-well plates.

Table A.10. Nutrition utilization tests on carbon and nitrogen sources

Characteristic changes	Legend symbols
Highly acidic	++
Positive utilization	+ (turn acidic)
Neutral	*
Negative utilization	- (turn alkaline)
Highly alkaline	-
No growth	∇
Strong utilization	♦

Nutrient sources	S223	S224	Nutrient sources	S223	S224
D – Arabinose	++ ♦	*	D – Malic acid	++ ♦	++ ♦
D – Xylose	+ ♦	+	Putrescine	* ♦	∇
D – Trehalose	* ♦	* ♦	Glycine	*	*
L - Rhamnose	* ♦	* ♦	L – Proline	+ ♦	+ ♦
D – Raffinose	*	*	L – Valine	*	* ♦
D – Sucrose	*	*	L – Methionine	*	* ♦
D – Maltose	* ♦	-	Alanine	*	*
D – Ribose	+ ♦	- ♦	Cysteine	* ♦	* ♦
Inulin	*	-	L – Arginine	*	* ♦
Cellulose	-	-	L – Lysine	*	* ♦
Chitin	- ♦	- ♦	L – Isoleusine	* ♦	* ♦
Glycogen	+ ♦	-- ♦	L – Leucine	* ♦	*
D – Mannitol	*	∇	L – Serine	*	* ♦
D – Sorbitol	* ♦	* ♦	L – Asparagine	* ♦	* ♦
D – Adonitol	*	* ♦	L – Threonine	* ♦	*
Citric acid	++ ♦	++ ♦	L – Phenylalanine	+ ♦	+ ♦
D – Lactic acid	++ ♦	++ ♦	L – Histidine	*	*
Ascorbic acid	+	+ ♦	L – Tryptophan	*	* ♦
D – Galacturonic acid	++ ♦	++ ♦	Glutamic acid	++ ♦	++ ♦
Niacin	*	*	Aspartic acid	++ ♦	++ ♦
Pyridoxine	++ ♦	++	Riboflavin	+	+ ♦
Thiamine	+ ♦	+	Folic acid	+	+

Table A.11. Inhibition of *Pseudomonas syringae* pv. *tomato* DC3000 by addition of microbe digested lobster shell extracts [n=6, P < 0.0001]

S.No	Treatments	Hours after incubation	Optical density measured (λ 600nm)	Letter groupings
1	S224-1	24	96.29	A
2	S224-1	48	95.89	A
3	S224-ct	48	90.85	AB
4	S224-ct	24	72.66	ABC
5	S224-0.5	48	67.02	BCD
6	S224-0.5	24	56.97	CD
7	Chitosan	48	44.66	DE
8	Shells-0.5	24	27.93	EF
9	Shells-1	24	27.34	EF
10	S223-ct	48	24.41	EFG
11	S223-1	24	22.66	EFG
12	S223-control	24	21.16	EFG
13	S223-0.5	24	7.42	FGH
14	S223-0.5	24	2.02	FGH
15	Control	24	-0.07	GH
16	Control	48	-0.12	GH
17	S223-1	48	-13.32	H
18	Shells-1	48	-51.06	I
19	S223-0.5	48	-66.02	I
20	Shells-0.5	48	-71.24	I

Control – water, Chitosan – positive control, S224-CT – extract of S224 grown in YEME broth, S223-CT – extract of S223 grown in YEME broth, S224-0.5 – extract of 0.5% of lobster shells digested by S224, S224-1 – extract of 1% of lobster shells digested by S224, S223-0.5 – extract of 0.5% of lobster shells digested by S223, S223-1 – extract of 1% of lobster shells digested by S223, Shells-0.5 – extract of 0.5% of undigested lobster shells, Shells-1 – extract of 1% of undigested lobster shells. Data points sharing the same letters are not significantly different (Tukey's HSD at α = 0.05).

Table A.12. Inhibition of *Botrytis cinerea* by addition of microbe digested lobster shell extracts [n=6, P = 0.0028]

S.No	Treatments	Hours after incubation	Optical density measured (λ 595nm)	Letter groupings
1	S223-1	24	99.69	A
2	S223-1	48	92.57	A
3	S224-1	48	87.3	A
4	S224-1	24	84.11	AB
5	S224-0.5	48	78.78	ABC
6	Chitosan	48	73.05	ABCD
7	S223-ct	48	55.95	BCDE
8	Shells-0	48	55.45	BCDE
9	S224-0.5	24	54.53	BCDE
10	Shells-1	48	53.86	BCDE
11	S223-0.5	48	51.73	CDEF
12	S223-0.5	24	44.25	DEFG
13	Chitosan	24	37.27	EFGH
14	Shells-0	24	30.65	EFGHI
15	Shells-1	24	22.25	FGHIJ
16	S224-ct	48	21.41	FGHIJ
17	S223-ct	24	18.18	GHIJ
18	S224-ct	24	9.52	HIJ
19	Control	24	0	IJ
20	Control	48	-0.74	J

Control – water, Chitosan – positive control, S224-CT – extract of S224 grown in YEME broth, S223-CT – extract of S223 grown in YEME broth, S224-0.5 – extract of 0.5% of lobster shells digested by S224, S224-1 – extract of 1% of lobster shells digested by S224, S223-0.5 – extract of 0.5% of lobster shells digested by S223, S223-1 – extract of 1% of lobster shells digested by S223, Shells-0.5 – extract of 0.5% of undigested lobster shells, Shells-1 – extract of 1% of undigested lobster shells. Data points sharing the same letters are not significantly different (Tukey's HSD at α = 0.05).

Table A.13. Enumeration of *Pst* DC3000 cfu in infected Arabidopsis leaves after treatment with lobster shell extracts [n=3, P < 0.0001]

S.No	Treatments	Days after infection	Log cfu / mg fresh weight of leaves	Letter groupings
1	Control	4	7.41	A
2	Shells-0.5	4	7.09	AB
3	Chitosan	4	7.05	AB
4	S223-0.5	4	6.67	ABC
5	Shells-1	4	6.65	ABC
6	S224-0.5	4	6.59	ABCD
7	S224-0.5	3	6.1	ABCDE
8	Chitosan	3	6.05	ABCDE
9	S224-1	4	6	ABCDE
10	Shells-0.5	3	5.97	ABCDE
11	Shells-0.5	2	5.87	ABCDE
12	S223-0.5	3	5.87	ABCDE
13	Shells-1	3	5.86	ABCDE
14	S223-1	4	5.77	ABCDE
15	Control	3	5.76	ABCDE
16	Chitosan	2	5.75	ABCDE
17	Control	1	5.64	BCDE
18	Control	2	5.45	BCDE
19	S223-1	3	5.25	CDE
20	S224-0.5	2	5.02	CDEF
21	S224-1	3	5	CDEF
22	S223-0.5	2	4.89	DEF
23	S223-1	2	4.63	EFG
24	Shells-1	2	4.61	EFG
25	S224-1	2	3.47	FGH
26	Chitosan	1	3.17	GHI
27	S223-1	1	2.68	HIJ
28	S223-0.5	1	2.29	HIJ
29	S224-0.5	1	1.77	HIJ
30	S224-1	1	1.67	IJ
31	Shells-0.5	1	1.34	J
32	Shells-1	1	1.29	J

Control – water, Chitosan – positive control, S224-0.5, S224-1 – extract of 0.5% and 1% of lobster shells digested by S224, S223-0.5, S223-1 – extract of 0.5% and 1% of lobster shells digested by S223, Shells-0.5, Shells-1– extract of 0.5% and 1% of undigested lobster shells. Data points sharing the same letters are not significantly different (Tukey's HSD at $\alpha = 0.05$).

Table A.14. Size of leaf lesion measured on days 3 and 5 post inoculation at the inoculation spot of *Botrytis* on Arabidopsis leaves after treatment with lobster shell extracts [n=3, P < 0.0001]

S.No	Treatments	Days after infection	Size of leaf lesion (mm)	Letter groupings
1	Control	5	4.17	A
2	Control	3	3.42	AB
3	Shells-1	5	3.22	ABC
4	Shells-0.5	5	2.97	ABCD
5	Chitosan	5	2.89	ABCD
6	Shells-1	3	2.83	ABCD
7	S223-0.5	5	2.78	ABCD
8	S224-0.5	5	2.72	ABCD
9	Shells-0.5	3	2.53	ABCD
10	S223-1	5	2.47	ABCD
11	Chitosan	3	2.31	BCD
12	S224-1	5	1.86	BCD
13	S224-1	3	1.75	BCD
14	S223-1	3	1.69	CD
15	S223-0.5	3	1.56	CD
16	S224-0.5	3	1.44	D

Control – water, Chitosan – positive control, S224-0.5 – extract of 0.5% of lobster shells digested by S224, S224-1 – extract of 1% of lobster shells digested by S224, S223-0.5 – extract of 0.5% of lobster shells digested by S223, S223-1 – extract of 1% of lobster shells digested by S223, Shells-0.5 – extract of 0.5% of undigested lobster shells, Shells-1 – extract of 1% of undigested lobster shells. Data points sharing the same letters are not significantly different (Tukey’s HSD at $\alpha = 0.05$).

Table A.15. Chitinase quantification at 48 h in Arabidopsis leaf samples treated with lobster shell extracts [n=3, $P < 0.0001$]

S.No.	Treatments	Infected / Non-infected	Chitinase (units/mg protein)	Letter groupings
1	Chitosan	*Botrytis*-48 h	1.34	A
2	S223E	*Botrytis*-48 h	1.17	AB
3	S223E	*Pst* DC3000-48 h	0.95	ABC
4	S224E	Mock-48 h	0.77	BCD
5	S223E	Mock-48 h	0.75	BCD
6	S224E	*Pst* DC3000-48 h	0.71	BCDE
7	Chitosan	*Pst* DC3000-48 h	0.7	BCDE
8	ShellsE	*Botrytis*-48 h	0.7	BCDE
9	S224E	*Botrytis*-48 h	0.69	BCDE
10	Control	*Botrytis*-48 h	0.61	CDE
11	Control	*Pst* DC3000-48 h	0.59	CDE
12	ShellsE	Mock-48 h	0.51	CDE
13	ShellsE	*Pst* DC3000-48 h	0.34	DE
14	Chitosan	Mock-48 h	0.3	DE
15	Control	Mock-48 h	0.21	E

Control – water, Chitosan – positive control, S224E – extract of 1% of lobster shells digested by S224, S223E – extract of 1% of lobster shells digested by S223, ShellsE – extract of 1% of undigested lobster shells. Data points sharing the same letters are not significantly different (Tukey's HSD at $\alpha = 0.05$).

Table A.16. Phenylalanine ammonia lyase estimation at 24 and 28 h in Arabidopsis leaf samples treated with lobster shell extracts [n=3, P < 0.001]

S.No.	Treatments	Infected / Non-infected	Time	Cinnamic acid (µmoles/mg protein)	Letter groupings
1	Chitosan	*Pst* DC3000	48 h	24.62	A
2	S223	*Pst* DC3000	48 h	22.47	AB
3	S224	*Pst* DC3000	48 h	22.12	ABC
4	Shells	*Pst* DC3000	48 h	21.47	ABCD
5	Chitosan	Mock	48 h	19.84	ABCDE
6	S223	Mock	48 h	19.03	ABCDEF
7	S224	Mock	48 h	18.95	ABCDEF
8	Control	Pst DC3000	48 h	18.34	ABCDEFG
9	Chitosan	*Botrytis*	48 h	17.58	ABCDEFG
10	Shells	Mock	48 h	17.08	ABCDEFGH
11	S223	*Botrytis*	48 h	16.16	ABCDEFGH
12	S224	*Botrytis*	48 h	14.3	ABCDEFGH
13	Chitosan	*Pst* DC3000	24 h	13.3	BCDEFGH
14	Shells	*Botrytis*	48 h	12.95	BCDEFGH
15	Control	Mock	48 h	12.85	BCDEFGH
16	S224	*Pst* DC3000	24 h	12.43	BCDEFGH
17	Control	*Botrytis*	48 h	12.29	BCDEFGH
18	Shells	Mock	24 h	12.21	BCDEFGH
19	S223	*Pst* DC3000	24 h	11.63	CDEFGH
20	Chitosan	Mock	24 h	11.16	DEFGH
21	Shells	*Pst* DC3000	24 h	10.7	EFGH
22	Control	*Pst* DC3000	24 h	10.16	EFGH
23	S224	Mock	24 h	10.15	EFGH
24	Chitosan	*Botrytis*	24 h	9.67	EFGH
25	S223	Mock	24 h	9.18	EFGH
26	Control	Mock	24 h	8.81	FGH
27	S223	*Botrytis*	24 h	8.69	FGH
28	S224	*Botrytis*	24 h	8.43	FGH
29	Shells	*Botrytis*	24 h	8.11	GH
30	Control	*Botrytis*	24 h	6.63	H

Control – water, Chitosan – positive control, S224E – extract of 1% of lobster shells digested by S224, S223E – extract of 1% of lobster shells digested by S223, ShellsE – extract of 1% of undigested lobster shells. Data points sharing the same letters are not significantly different (Tukey's HSD at $\alpha = 0.05$).

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