CHAPTER ONE

Introduction

1.1 Overview of Antarctica continent

Antarctica in the geographical sense is the Earth's southernmost continent and, is surrounded by the Southern Ocean (Figure 1.1). Antarctica is about 14 million km^2 making it the fifth largest continent after Asia, Africa, North America and South America, with 98% of it completely covered by ice, average thickness of about 2 km and maximum thickness of over 4 km (Bargagli, 2008).

Antarctica has short term history of human contact and inhabitation (Convey, 2010). Continental Antarctica was first discovered approximately two centuries ago (the northern Antarctic Peninsula). East Antarctica only a little more than a century ago, while the sub-Antarctic islands were most of them landed and their marine-based living resources rapidly over-exploited more than the last two to three centuries.

Antarctica being in the polar region is a continent with almost 99.7% of current terrain covered by permanent ice and snow (Convey *et al.,* 2008). Conversely, only small portion of its area is currently ever free of snow or ice in terrestrial ecosystems including exposed nunataks, cliffs and seasonally snow and ice-free areas (Convey, 2010). Convey (2001) suggested the subdivision of the Antarctic continent into three biogeographic zones namely Sub-antarctic, Maritime Antarctic and Continental Antarctic. The isolated islands of the Southern ocean and archipelagos constitute the sub-antarctic zone. The zone is characterized of low air temperatures with a mean annual temperature above 0 ˚C and high precipitation, and is devoid of seasonal pack or fast ice influences (except in South Georgia). Secondly, the maritime Antarctic includes the western coastal regions of Antarctic Peninsula to Alexander Island, along with the Scotia Arc Island archipelagos, the isolated Bouvetøya and Peter I Øya. This zone

records up to 4 months of positive mean air temperature. Both summer maxima and winter minima are buffered by the surrounding ocean, with possibility of thaws in all winter months. Duration of the active-season could be a few days or weeks, increasing to up to around 3-5 months along the relatively more benign Antarctic Peninsula and the archipelagos of the Scotia Arc (Convey, 1996). The South Sandwich archipelago and Bouvetøya are distinctive areas in Antarctica for being geologically recent and active volcanic islands, with unique biological communities associated with geothermal activity regardless of high precipitation. However, the Continental Antarctica is the largest of the three biogeographic zones. It includes East Antarctica, Balleny Islands and eastern side of the Antarctic Peninsula. Possession of coastal regions makes the zone similar to maritime Antarctic and inland nunataks with positive mean air temperatures recorded for 1 month. Terrestrial habitat of a limited extent and great isolation, except at the Dry Valleys region of Victoria Land.

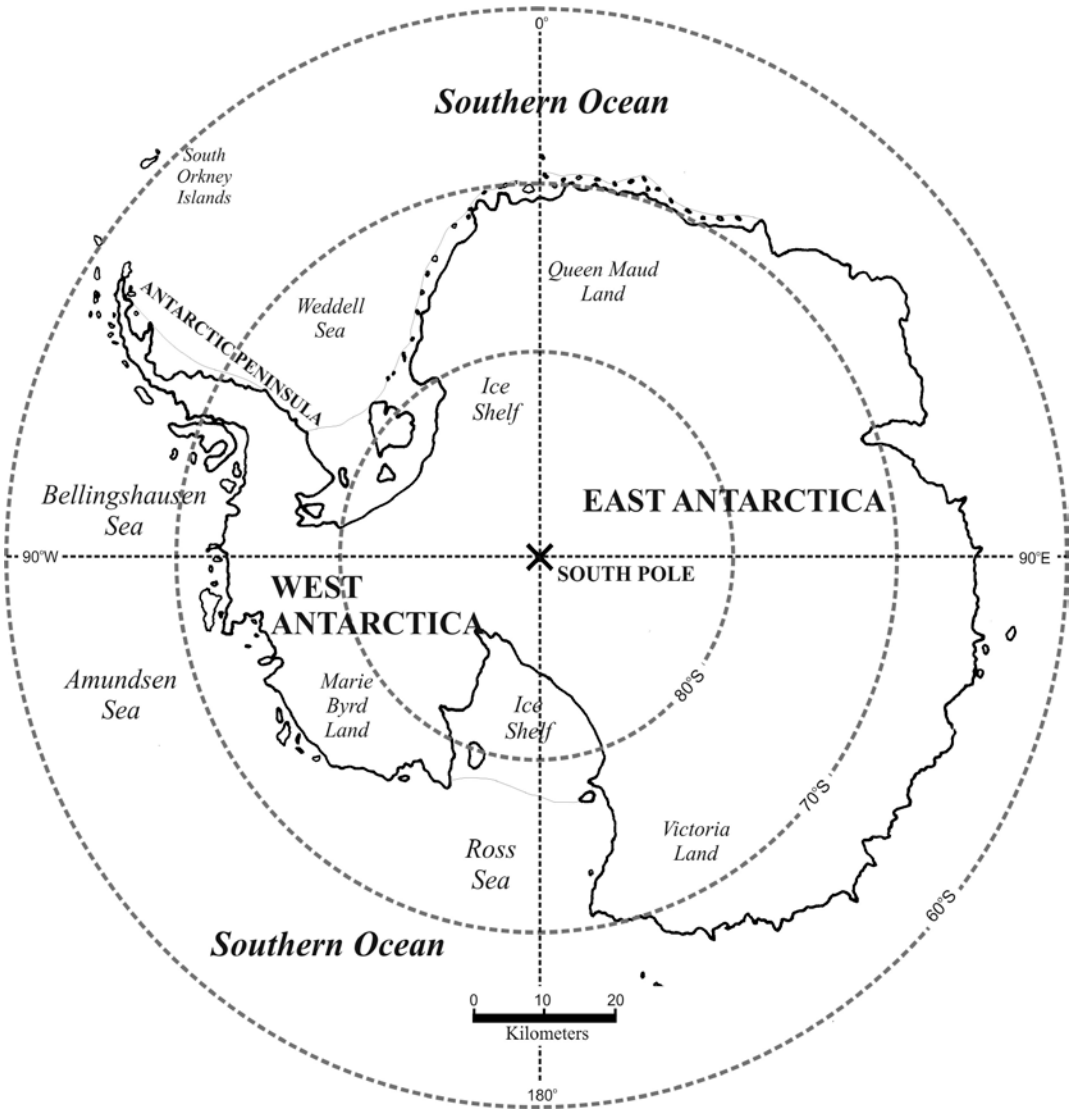

Figure 1.1 Geographic map of Antarctica Continent.

1.1.1 Terrestrial Microbial Communities in Antarctica

Antarctica soils are poorly developed, variable and sensitive by freeze-thaw activity, with low organic matter (Davey and Rothery, 1993; Bolter *et al.,* 1994; Blume *et al.,* 2002). Antarctic microorganisms demonstrate high level of adaptation and ability to withstand extreme conditions which significantly constitutes limiting factors for plant and animal life. For examples, low temperature, low water availability, frequent freeze-thaw cycles, strong winds with low annual precipitation, in addition to the high sublimation and evaporation, and high incidence of solar, especially the ultraviolet radiation (Wynn-Williams, 1996; Bradner *et al*., 2000; Brett *et al*., 2006 and Ruisi *et al*., 2007). Terrestrial ecosystems in Antarctica include a great variety of habitats from ice-free areas, where microbial community development is significantly limited to three types of habitats: endolithic communities inside rocks, freshwater communities in transient water bodies, and hypersaline ice-covered lakes (Wynn-Williams, 1990).

Wynn-Williams (1996) reviewed that autotrophic cyanobacteria and algae were the primary colonist's communities in Antarctica, followed by bacteria, fungi and protozoans. However, communities like algae and cyanobacteria were found to develop filaments and mats within water bodies and the superficial layers of humid soils. These communities are significantly colonize within the maritime region, and are often a climax community of the continental zone. As reported there is no evidence of life on the surface of soils or rock of large parts of Dry Valleys, continental Antarctica (Convey, 2001). In terms of association, the symbiotic lichen of fungi and algae is the most successful in Antarctica (Onofri *et al*., 2007).

It has been observed that fungal communities are more diverse and more abundant in sub-Antarctic islands, more humid and temperate, as compared to other Antarctic regions. This has been correlated to the organic matter, soil water content, pH

and total nitrogen on the sub-Antarctica Signy Island (Yergeau *et al*., 2006). Consequently, cryptoendolithic communities constitute very simple communities with only a few species of Antarctic cryptoendolithic microorganisms. On the other hand, lichen dominated community is the most common and well studied group found in sandstone (Ruisi *et al*., 2007). Through observation of several studies on Antarctica fungal communities are dominated by cold tolerant rather than cold adapted fungi as a result in diverse fungal community (Yergeau *et al*., 2006).

1.1.2 Soil Fungi in Antarctica

Fungi distribution in Antarctica are subjected to a number of potential limiting factors such as soil, rocks, vegetation which basically consist of plants, bird feathers and dung, and lichen (Bridge and Worland, 2004; Jumpponen *et al.*, 2003; Leotta *et al.*, 2002; Tosi *et al*., 2002). Psychrophilic and psychrotrophic fungi play important role in the biodegradation of organic content. In addition to their important roles in permanently cold areas, these organisms are efficient in habitats which experience seasonal variation in temperature during late fall and spring (Ray *et al*., 1992). In northern Victoria Land, the most frequently isolated filamentous soil fungi are species of *Alternaria*, *Aspergillus*, *Cladosporium* (typically *C. cladosporioides* (Fresen.) G.A. de Vries and *C. herbarum* (Pers.) Link), *Geomyces* (almost exclusively *G. pannorum*, often reported as *Chrysosporium pannorum*), *Phialophora* (typically *P. fastigiata* (Lagerb. and Melin) Conant, often reported as *Cadophora fastigiata*), *Phoma* (often *P. herbarum* Westend.), *Thelebolus microsporus* (Berk. and Broome) Kimbr. and *Mortierella antarctica* Linnemann (Del Frate and Caretta, 1990; Zucconi *et al*., 1996; Fenice *et al*., 1997; Tosi *et al*., 2002, Tosi *et al*., 2005).

However, species level diversity on Antarctica soil is low (Convey, 2010). A few species that are represented in Antarctic soils are mainly *Acremonium, Aspargillus, Cladosporium*, *Fusarium* and *Trichoderma* known as soil fungi (Singh *et al*., 2006).

Despite the low diversity, they have unique mycelial characters that had abundant intercalary, swollen, thick-walled cells that sometimes produce a clear mycelial cord besides chlamydospores. The occurrence of ten species, consists of five Hypohomycetes, two Ascomycetes, one Coelmycetes, one Zygomycetes and one yeast isolated from soil sample taken in Windmill Island have been reported by Alias and Azlina (2004). They are *Aureobasidium* sp., *Cadophora malorum* (Kidd and Beaum) W. Gams, *Geomyces cretaceus* Traaen, *Trichosporiella cerebriformis* (de Vries and Kleine-Natrop) W. Gams, Unidentified sp.9, *Antarctomyces sp*., *Thelebolus* sp., *Phoma* sp., *Mucor* sp. and *Mrakia frigida* (Fell *et al.)* Yamada and Komagata.

Moreover, the common isolated from soils in coastal regions is filamentous fungi, with many species documented from bryophyte-dominated areas around Terra Nova and Wood Bays by several Italian expeditions (Adams *et al*., 2006). Unlike filamentous fungi, only few species of yeasts have been isolated from northern Victoria Land soils, but species of *Cryptococcus* (typically *C. albidus* (Saito) C.E. Skinner) and *Rhodotorula* (typically *R. minuta* (Saito) F.C. Harrison) are significantly isolated from soils in the northern region (Del Frate and Caretta, 1990; Tosi *et al*., 2002, Tosi *et al.*, 2005).

The marked ability of *Geomyces pannorum*, which used in the present study, to degrade a variety of substrates and specifically able to consume nutrients resulting it to grow fast and reproduction on the exotic materials confirms it as the most abundance genus isolated from Ross Island soil (Arenze *et al.,* 2010). Since, this cosmopolitan fungus has been suggested to be indigenous Antarctica microfungi based on their widespread (Vishniac, 1996). It has been isolated so frequently in association with feathers in soil and keratinophilic fungus (Marshall, 1998). It seems to be more frequently presented compared to other fungi species in harsh environment, including the Arctic (Bergero *et al*., 1999; Ozerskaya *et al.*, 2008). This organism also appear to

have a very broad capacity to hydrolyse starch and produce extracellular chitinase, urease and lipase at temperatures lower than 25ºC confirms it as psychrotolernet (Zucconi *et al.*,1996).

1.1.3 Thermal Classes of Antarctic Fungi

Microbial life in Antarctica has been described as being psychrophilic, psychrotolerent or mesophilic (Margesin *et al*., 2007). Some fungi are psychrotolerant more than psychrophilic (Onofri *et al.,* 2004a). Psychrophilic fungi have special adaptations to their enzymes, membranes and other cellular components, enabling them to grow at optimum growth at 15˚C or lower and cannot grow above 20˚C, while psychrotolerant (also called psychrotrophic, particularly in the food industry) fungi, which can also grow close to zero, have optima and upper limits above these temperatures and may well grow above 20˚C (Russell, 2006).

According to Morita (1975) the term of psychrotrophic used for cold-tolerant organisms, previously referred as facultative psychrophiles, the optimum growth temperature of 20 ºC or being above. The main different between the two group is that psychrotolerants have a much broader growth temperature rate (30- 40 ^{0}C) than do psychrophilles (~20 ^{0}C). Therefore, psychrotolerants may grow as fast as psychrophiles at low temperatures but both are sensitive to warming at moderate temperatures (Russell, 2006).

Psychrophilic and psychrotolerant fungi contribute significantly to the processes of nutrient uptake, biomass production, and decomposition cycle in cold ecosystems (Margesin *et al.,* 2007). Species of *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Geomyces,* and *Lecanicillium,* isolated from Antarctic soil are identified as psychrotolerant (Kostadinova *et al*., 2009). In contrast, *Thelebolus microspores* is psychrophilic, ascomycete has been frequently isolated from skua and penguin dung

and from soil frequented by birds, which it look indigenously Antarctica fungi (Corte *et al.*,1993).

1.1.4 Extracellular enzyme activity from polar region

Microorganisms are known to produce enzymes and secondary metabolites of immense biotechnological potential (Singh *et al.,* 2006). There are listed of enzymes isolated from Antarctica such as amylase, protease, β-galactosidase, cellulose, pectinase, xylanase, keratinase and chitinase (Fenice *et al*., 1997; Ray *et al*., 1992; Raza *et al*., 2000; Duncan *et al*., 2008). Isolates play an important role in the degradation of insoluble macromolecules such as tributyrin, starch, chitin, skim milk and cellulose (Yu *et al*., 2009). Cold active enzymes are produced by psychrotrophic microorganisms with low activation energies and high activities at low temperature (Jackman *et al.,* 1983; Feller *et al*., 1990). In another hand, psychrophilic or cold enzymes work efficiently at low temperature, that showing specific activity at low and moderate temperature higher than mesophilic organisms (Gerday *et al*., 1997).

Little studies have been done in order to test the enzymatic competences of different Antarctic fungal strains. For instance, Antarctica strain of *Lecanicillium muscarium* (Petch) Zare & W. Gams CCFEE 5003 producing high levels of extracellular chitinolytic enzymes at low temperature which is active in a wide range of temperature (Fenice *et al*., 1998). An extracellular cold-active chitinase with psychrotolerant characteristics was recently purified from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 (KCTC 13143) and coding sequence for the enzyme was obtained from genomic DNA (Park *et al*., 2009). High levels of chitinase produced by bacterial strains and molds are widely reported (Muzzarelli 1989; Sahai and Manocha, 1993). From another site, Yu *et al*. (2009) listed different bacterial strains isolated from Arctic sea ice all have the ability to consume chitin. Fungal production of

amylase has been reported by Fenice *et al.* (1997) from Victoria Land. Cotarlet *et al.* (2009) investigated that cold adapted amylase can obtained from polar strain coded *Streptomyces* 4 Alga, isolated from vegetation samples from East Antarctica. Moreover, this enzyme has also been produced from bacteria and actinomycetes from Terra Nova Bay and Edmonson Point Victoria Land (Gesheva, 2009).

Cellulase produced by fungi has been reported from historic discovery hut on Ross Island, Antarctica (Duncan *et al*., 2008). Fenice *et al*. (1997) also reported cellulase activity from fungi from Victoria Land. Also, this enzyme investigated from soil microorganisms isolated from Syowa Station and Langhovde, Antarctica (Yamamaoto *et al.,* 1991). Actinomycetes and bacteria isolated from Terra Nova Bay also possessed cellulase activity (Gesheva, 2009).

Protease production has been reported from Ross Island (Duncan *et al.,* 2008), also from Windmill Islands (Bradner *et al.,* 1999). Fungal production of protease has also been reported by Fenice *et al.* (1997) from Victoria Land. Gesheva (2009) stated protease activity from bacteria and actinomycetes from Terra Nova Bay and Edmonson Point. Cotarlet *et al.* (2009) reported the new polar streptomycete strain coded *Streptomyces 4Alga* able to produce protease adapted at low temperature (10°C and 20°C). Extracellular protease production has been reported from the Antarctic yeast *Candida humicola* (Ray *et al*., 1992).

Yu *et al*. (2009) stated the temperature dependences for extracellular lipase activity were determined for five psychrophilic and six psychrotolerant bacteria isolated from Arctic sea ice. Also, a keratinolytic enzymes produced by actinomycetes isolated from Antarctic soils has been studied by Gushterova *et al*. (2005).

1.1.5 Biotechnological application of cold-active and cold adapted enzyme

Cold active enzymes have great interest of economical value because enzymatically driven reaction can be observed within a temperature range 0-20˚C (Morita *et al.,* 1997). Enzymes purified from Antarctic microorganisms have structural and functional properties, some properties that might be correlated with the environmental temperature. Enzymes from cold-adapted microorganisms are used for most industrial processes due to their energy-saving for example can be used such as additives in detergents for cleaning at low temperatures (Morita *et al.,* 1997). Psychrophilic or cold enzymes have interesting properties due to the high flexibility and stability of these enzymes at low and moderate temperature higher than mesophilic organisms (Gerday *et al*., 1997). Obviously, psychrophilic and psychrotrophic fungi have been potentially use in biotechnology. For example, Psychrotrophic microorganisms are used as starter culture and in the cool ripening and flavour improvement of cheeses and other dairy products (Russell, 2006). Many enzymes from cold-adapted microorganisms are used for most industrial processes, including food production, mining, waste processing, environmental bioremediation such as chemicals, agriculture, and medicine application (Russell, 2006). Some cold adapted microbes are an attractive alternative source of polyunsaturated fatty acids for instance docosahexaenoic acid and arachidonic acid, which are important for human health and significantly used in the food production, health and cosmetic fields (Russell, 2006). Therefore, it would be of interest to study the nature of extracellular chitinase enzyme secreted by cold adapted fungi isolated from soils of Fildes Peninsula, King George Island, Maritime Antarctica.

1.2 Chitin and Chitinolytic Activity

1.2.1 Properties of Chitin and its derivatives

After cellulose, Chitin is the second largest of the polysaccharides, which are abundant in nature. Chitin is present in the exoskeleton of arthropods, coelenterates, flatworms, protozoa, molluscs, and crustaceans (Suzuki *et al*., 1998). Their special properties include polyxylate formation, ability to form films, chelate metal ions and optical structural characteristics. Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It can soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohol in conjugation with aqueous solutions of minerals acid and dimethylacetamide containing 5% lithium chloride. The nitrogen content in chitin ranged between 5 to 8% depending on the extent of deacetylation (Yalpani *et al.,* 1992). Chitin can be fully acetylated by acetic anhydride and linear aliphatic N-acetyl groups above propionyl, permit rapid acetylation of hydroxyl groups. Highly benzoylated chitin is soluble in benzyl alcohol, dimethyl sulfoxide, formic acid and dichloroacetic acid. Cellulose and chitin are highly crystalline, intractable materials and only a limited number of solvents are known which are applicable as reaction solvents. Chitin and chitosan degrade before melting, which is typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve chitin in an appropriate solvent system to impart functionally. For each solvent system, polymer concentration, pH, counterion concentration and temperature effects on the solution viscosity must be known. The comparative data from solvent to solvent are not available. As a general role, researchers dissolve the maximum amount of polymer in a given solvent that still retained homogeneity and then regenerated it in the required from. Basically, coagulant is required for polymer regeneration or solidification. Muzarelli (1973) showed that the nature of the polymer coagulant is also highly dependent on the solvent and solution properties as well as the polymer used.

Interestingly, chitosan is taken from chitin. It is a modified carbohydrate polymer derived from the chitin component of the shells of crustacean, such as shrimp, crab and cuttlefish. Chitosan, the acetylated product of chitin is soluble in very dilute acids like acetic acid, formic acid and others. The nitrogen content in chitosan is mostly in the form of primary aliphatic amino groups. Therefore, it undergoes the reaction typical to amines, of which N-acetylation and Schift reaction are the most important.

Chitosan derivatives are easily obtained under mild condition and can be considered as substituted glucans. N-acetylation with acid anhydrides or acyl halides introduces amino groups on chitosan. At room temperature, chitosan forms aldimines and ketimines with aldehydes and ketones, respectively. Reaction with ketoacids followed by reaction with borohydride produces glucans carrying protein and non-protein amino groups. N-carbomethyl chitosan is obtained from glyoxylic acid. Chitosan and simple aldehydes produce N-alkyl chitosan upon hydrogenation. The presence of the more or less bulky substituent weakens the hydrogen bonds of chitosan. Therefore, N-alkyl chitosan swell in spite of the hydrophobicity of alkyl chains. They retain in film forming property of chitosan (Yalpani *et al*., 1992).

1.2.2 Chitin structure

As demonstrated by Prashanth and Tharanathan (2007), Chitin is a highly insoluble biopolymer, is composed of linear chains of β-1,4-linked 2-acetamido-2-deoxy-β-D-glucose (GlcNAc) resides that are highly cross-linked by hydrogen bonds, similar to cellulose. It belongs to carbohydrate as one kind of polysaccharide and is the sole cationic macromolecular material in nature that can be decomposed by organisms (Figure 1.2).

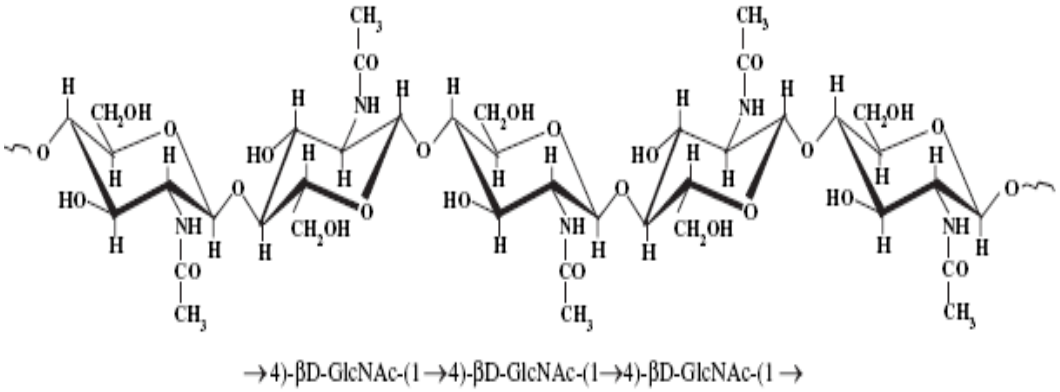

Figure 1.2 Primary Structure of Chitin.

1.2.3 Chitinase enzyme and its origin

Chitinase is a hydrolytic enzyme cleaving β-1, 4-linkages of chitin. Chitinase catalyzes the conversion of chitin to its monomeric or oligomeric components of N-acetylglucosamine. Chitinases can be produced by a number of organisms including bacteria, fungi, plant and yeast, and they are capable of catalyzing the hydrolysis of chitin into smaller chitooligosaccharides (Po-Min *et al.*, 2009). Chitinase-secreting microorganisms are widespread in our environment and play specific roles in various organisms. In arthropods such as insects and crustaceans, chitinase act in the ecdysis. Snails, hunting spiders and fish digest the chitinous foods by chitinases. Bacteria also produce the enzymes to consume chitin for up taking the hydrolysates into the cells as nutrient. Also, chitinase was found in human serum and protozoa, which plays important role of protection against fungal infections and penetration of the mosquito peritropic membrane by the malaria parasite respectively (Souza *et al.*, 2003).

1.2.4 Characterization of Chitinase

Chitinase can be characterized via;

1) microbial chitinase characteristics.
2) enzymatic properties of chitinase.
3) depolymerized process of chitin and its derivatives by chitinase.

Microbial characteristics are elucidated through the inducibility potential of microorganisms. While majority of microorganisms could express chitinase induction regulated by inducible system, some others can produce chitinase without inducers. Basically chitin, chitosan, chitiooligosaccharides (COs) and low molecular weight chitooligomers (LMWC) are excellent inducers rather than NAG. The secretory property is important because extracellular chitinase from microorganisms are mostly secreted by inducer. Significantly diversity is another factor in microbial chitinase characteristics because chitinase from different microorganisms differ in sorts, components and properties.for example *Saccharomyces cerevisiae* can synthesize two kinds of endochitinase while *Serratia marcescens* can produce five kinds of chitinases.

Enzymatic properties of chitinase are evident in their molecular weight. Generally MW of chitinase range from 20 kDa to 120 kDa. Though MW of chitinase from actinomycetes are often 30 kDa or less, the fungi chitinase is generally 30 kDa and above (Guan *et al*, 2006). Chitinase can also depolymerize chitin and its derivatives. This is achieved because chitinase has to potential to specifically hydrolyze substrates such as colloidal chitin, glycol chitin, soluble chitin, glycol chitosan, and carboxymethyl cellulose (CMC) as well as soluble chitooligosaccharides but cannot catalyze hydrolysis of maltose, starch and cellulose which are composed of $(GlcNAc)_2$ by β-(1,4) glycosidic bond.

1.2.5 Application of Chitinase

There are various applications of chitinase either in industrial or in agricultural and several applications of chitinase were identified including:

1.2.5.1 Chitinase in biocontrol of plant pathogenic fungi and insects

In many plant species, local invasion of pathogen induces production of pathogenesis related proteins like chitinase, β-1,3-glucanases, protienasea, proteinase inhibitors and others. As pathogenic fungi and insects contain chitin in their protective covers, induction of chitinase in plants is the main defense response. Most of these chitinases are induced in the vegetative plant organs by infection but some are also present in seeds. As demonstrated by Hadwiger and Beckman (1980), that extracts of the pea endocarp contain chitinase and chitobiose activity. Most of the chitinase prefer cleave highly acetylated substrates and the activities decrease with the decrease in the degree of acetylation. Therefore, increase in the deacetylation level on the surface of hyphae may be useful for the fungus to resist plant chitinases. Based on the studied of Roberts and Seliternnkof (1988) chitinase isolated from the grains of wheat, barley and maize functioned as endchitinases and inhibited hyphal elongation of test fungi. In contrast, bacterial chitinases from, *Serratia marces*, *Serratia griseus* and *Pseudomonas stutzeri* act as exo-enzymes and had no effect on hyphal extension of test fungi like *Trichoderma reesei* and *Phycomycess blackesleeanus*.

1.2.5.2 Mosquito control

Mosquitoes are a vector agent that carries disease-causing viruses and parasites from person to person without catching the disease themselves and this makes them potential targets for various pest control agents. In the case of mosquitoes, entomopthogenic fungus like *Beauveria bassiana* could not infect eggs of *Aedes*

aegypti, a vector of yellow fever and dengue, and other related species may be due to aquatic environment. The scarabaeid eggs laid in the soil found to be susceptible to *B. bassiana* (Ferron, 1985). The soil saprophytic fungus *Myrothecium verrucaria* produces a total complex of an insect cuticle degrading enzymes (Shaikh and Deshpande, 1993). It has been observed that both first (I) and fourth (IV) instar larvae of a mosquito, *A. aegypti*, can be killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonsa *et al*., 1996).

1.2.5.3 Single cell protein production

Reva-Moiseev and Carrod (1981) used chitinase from *Serratia marcescens* and yeast *Pichia kudriavzevii* to hydrolyse chitinous material to single cell protein (SCP) that acceptable for aquaculture. The criteria used to evaluate SCP production are growth yield, total protein and nucleic acid contents. The best reported organism was *S. cerevisiae* that exhibited 60% protein and 1 to 3% nucleic acid contents. Vyas and Deshpande (1991) used *Myrothecium verrucaria* chitinase preparation for chitin hydrolysis and *Saccharomyces cerevisiae* for SCP. The total protein content was reported to be 61% with very low contents of nucleic acids (3.1%).

1.3 Research Objectives

This research was conducted with the following objectives;

1. To screen the presence of chitinase activity from *Geomyces* strains based on RA value.
2. To quantify chitinase enzymes of selected strains of *Geomyces*.
3. Optimization of chitinase production from *Geomyces* sp. 5