CHAPTER FOUR

Discussion

4.1 Qualitative assessment of hydrolytic activity grown at different temperatures

Of total 1093 species of fungi have been reported from Antarctica region (http://www.antarctica.ac.uk//bas_research/data/access/fungi), yet very little study have been done of their enzyme activities. Specific fungal enzymes may contribute to the ability to grow and survive in the extreme environment characterized by high stress condition and limited biodegradation of organic matter (Bradner *et al.,* 1999). Hydrolytic enzyme is described as an enzyme that catalyzes the hydrolysis of a substrate through the addition of water. Few studies have been conducted on chitinase enzyme from Antarctica (Bradner *et al.,* 1999; Fenice *et al.,* 1997; Fenice *et al.,* 1998; Onofri *et al.,* 2000).

Chitinolytic enzymes are found in over 25% of Antarctica fungi isolated from McMurdo Dry Valleys (Ross Desert) of Southern Victoria Land by Onofri *et al*. (2000). The presence of chitinase activity in this area could be related to the large amounts of chitin in ornithogenic soils, reflecting the krill-rich diet of penguins (Onofri *et al.,* 1999). Extracellular chitinase production also has been reported from fungi in the Victoria Land, Continental Antarctica by Fenice *et al.* (1997). Bradner *et al*. (1999) stated *Penicillium* sp. 2 and the *Trichoderma* isolates from Windmill Islands, Continental Antarctica showed strong chitinolytic activity both at 10 °C and 28 °C. Yu *et al.* (2009) reported chitinase activity of *Pseudoalteromonas tetraodonis*, *Pseudoalteromonas elyakovii*, *Bacillus Wrmus* and *Janibacter melonis* isolates from Arctic sea ice all have the ability to consume chitin. No work have been done in

chitinase enzyme activity from King George Island in the Maritime Antarctica. The present study is the first report of chitinase enzyme from *Geomyces* spp. strains isolated from various site of King George Island.

First strain, with significant chitinase activity, *Geomyces* sp. 5 isolated from contaminated lake has human impact. In contrast, *Geomyces* sp. 1 strain isolated from lake GFZ with significant chitinase activity could be related to ornithogentic site which supporting Onofri *et al*. (1999). While, the other four strains isolated from ornithogentic site showed various degree of chitinolytic activity at different temperatures.

4.2 Quantification assay systems for chitinase activity

The present study is the first report of quantification of chitinase enzyme of *Geomyces* spp. Fenice *et al.* (1997) did a preliminary study of *Geomyces pannorum* from Victoria Land at 25 ˚C, he did not quantify chitinase enzyme using RA value but he measured the halo zone resulting halo diameters up to 10 mm. While the present study, the halo zone formation of six strains of *Geomyces* spp. ranged from 3.85 to 0.8 cm, which were more than 10 mm. In this study, quantification of chitinase enzyme was tested using RA value at 25 ˚C and 4 ˚C. As shown on result 25 ˚C showed bigger halo zone than 4 ˚C. When comparing chitinase production it is important to consider the substrate and habitat that the fungi were isolated from. The substrate in this research was colloid chitin while fungal isolates from Fenice *et al.* (1997) grown on chitin. Fungal isolates from Fenice *et al.* (1997) came from a habitat consisting of soil, moss and sand while in this research fungal isolated from soil and moss only.

As a first report towards quantification of chitinase enzyme for *Geomyces* spp., qualitative profiling of hydrolase activities of six strains of *Geomyces* spp. were undertaken at different temperatures. Duncan *et al*. (2008) stated that RA value 1 or greater was classified as significant enzyme activity. In the present study, all *Geomyces*

strains showed ability to produce chitinase enzyme by clear zone formation with RA value ranges from 1.49 to 0.40 cm. These clearing zones in different isolate cultures were observed from day six for different fungi isolates depending on their ability to consume chitin. However, it has observed the haloes diameter were varied among different isolates at different temperature. It is worth noting that the clear zone in *Geomyces* sp. 5 (AK07KGI102 R1-4) strain was found to be the biggest clear zone as compared to other strains at both temperature 25˚C and 4˚C. Therefore, *Geomyces* sp. 5 has high chitinase activity as compare to another strain.

However, it is noted that the enzyme activity was better at 25ºC rather than at 4ºC. Most of the isolates showed a significant activity at 25ºC as compared to 4°C. Based on these result, best incubation temperature for the isolated fungal strains of *Geomyces* at 25°C. In the present study, only *Geomyces* sp. 5 (AK07KGI102 R1-4) was able to show significant enzyme activity. These results indicate that chitinase from *Geomyces* spp. has typical features of cold-active enzymes: relatively high catalytic activity at low temperature and great interest of thermosensitivity (Feller *et al.,* 1996).

To date, there is no defined medium has been discovered for chitinase production from different microbes (Park *et al.,* 2010). Each organism requires special conditions to produce high level of chitinase (Dahiya *et al.,* 2005). During cultivation of fungi in the shake flask, the process of pellet formation of the isolated strains was observed. The pellets obtained in shake-flask cultures showed distinct layers of mycelial density with only the thin outer layer consisting of a dense mycelia network having pale-yellow coloration. However, it is necessary to maintain the shaking-rotation at low energy dissipation rates for successful pellet formation. A shaking speed of either 100 or 200 r.p.m. in an orbital incubator was satisfactory and biomass yield responded to an increase in chitin substrate concentration though yield declined at higher concentrations level, possibly due to mass transfer limitation. As reported by Kellly *et al.* (2004), in

fungal cultivation, biomass growth and enzyme activity were correlated with energy dissipation.

During Bradford method, the increase in optical absorbance depends on the isolates strain. For example, *Geomyces* sp. 5 has highest optical absorbance as compared to *Geomyces* sp. 1. In order that, protein concentration from *Geomyces* sp. 5 was higher than protein concentration from *Geomyces* sp. 1. Nevertheless, protein concentrations from synthetic medium for chitinase production after ten days of inoculated by both strains of *Geomyces* spp. were very little values. Similar result reported by Magan (2007). He stated the Antarctic fungi grown under high osmotic water stress significantly affect nutrient uptake, protein biosynthesis and slow down of enzyme activity. Moreover, this is also probably due to the media for chitinase production, which used in this experiment, contained little amount of nitrogen source. Yeast extract contains nitrogen compound, several growth factor and oligomeres of GlcNAc. The presence of the yeast extract which are rich of protein increases the soluble protein concentration (Nawani and Kapadnis, 2005). Enzymes are protein but not all proteins are enzyme, therefore the more enzyme being produced, the more protein also detected. Similarity, Lopes *et al*. (2008) reported chitinase production in microorganisms is influenced the nitrogen source like yeast extract. He showed the extracellular chitinase activity was increased by the yeast extract when combined with NAG.

During enzyme assay, the production rate of *N*-acetylaglucasamine from *Geomyces* sp. 5 was relatively higher than the rate from *Geomyces* sp. 1. This could probably due to the more adaptability of the strain *Geomyces* sp. 5 to both cultivation media and fermentation parameters such as temperature and pH. Nevertheless, it considered to be low amount as compare to *Lecanicillium muscarium* producing high amounts of extracellular chitinolytic enzymes (Fenice *et al.,* 1998), this is perhaps due

to Antarctic microfungi are not able to grow on synthetic media probably because of the absence of some essential grow factor as reported by (Onofri *et al.,* 2004b). Another reason may be due to inappropriate fermentation circumstances as many factors may affect such as temperature, incubation time, inoculums size.

4.3 Optimization studies of cold active chitinase from *Geomyces* spp.

The optimum pH is the point where enzyme is most active. Chitinase production is pH sensitive (Felse and Panda, 2000). It was found that most chitinase were produced in an acidic to neutral conditions. In the present study, the optimum pH for chitinase produced by psychrotolerant *Geomyces* sp.5 was pH 6.5. Similar observation has been reported that the optimum pH for cold-active chitinase produced by the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 was pH 6.5 (Park *et al*., 2010). While the optimum pH for chitinase production from psychrotolerant bacterium *Vibrio* sp. strain Fi:7 isolated from Antarctica was pH 8.0 (Bendt *et al*., 2001).

Temperature is one of the most important factor that effects the enzymatic activity. Cold- active enzyme production and/or activity is significantly below the "optimal" growth temperature of the enzyme producer, which reflects the thermal characteristics of the secretion process (Margesin *et al*., 2007). According to numerous studies, chitinase are active at temperatures ranging from 20 to 50 ºC (Frändberg and Schnürer, 1994; Huang *et al*., 1996; Bhushan and Hoondal, 1998; Wiwat *et al*., 1999; Bendt *et al*., 2001). Chitinolytic enzymes of bacteria and fungi production have been displayed the highest activity at 40-50 ºC. In the present data, the optimum temperature for chitinase was 37 ºC. In contrast, Chitinolytic enzyme produced by Antarctic strain (A3) of *Verticillium lecanii* was active in a wide range of temperatures (5- 60 ºC) with optimum activity at 40 ºC (Fenice *et al.,* 1998). Similar result was obtained by Bendt *et al.* (2001) showed high chitinolytic activity from psychrotolerant bacteria *Vibrio* sp. at temperatures ranging from 30 to 45 ºC.

To date, no practical information has been available on optimization of cold-active chitinase production by *Geomyces* species. The present study provides change of media to a suitable medium and operating conditions for improving chitinase production by *Geomyces* spp. On the basis of the results reported in this research, *Geomyces* strains can be regarded as great interest for utilization in applied research. In fact, for its ability to grow and to release chitinase activity at different temperatures. It could be important for different applications such as the treatment of particular industrial chitin rich waste materials in cold climates. Obviously, the potential of *Geomyces* strains must be reinforced investigated either with laboratory application or by field tests.

4.4 Importance application of cold- adapted enzyme

Cold adaptation is the most studied biological aspect related with the effect of environmental factors on occurrence and distribution of Antarctica fungi (Onofri *et al.,* 2004a). Antarctic microbes are efficient not only in permanently cold habitats, but also microbial extracellular enzyme with optimal activity at low temperature provide the chances of the adaptation of life in cold habitats and the potential for biotechnological exploitation (Aguilar, 1996). Cold adapted enzymes are generally characterized by two main characteristics; high turnover rates (k_{cat}) and catalytic efficiency measured as (k_{cat}/K_m) at low and moderate temperatures (Gerday *et al.*, 2000), and reduced stability at moderate and high temperatures. In addition to their ecological role in the natural environment, cold adapted enzymes have application in the industry and products. For examples of technology affected by these studied cleaning agents, degradation of xenobiotic compounds in cold environments, leather production, food product (cheese manufacture, bakery, fruit and vegetable products), pharmaceuticals like organic synthesis of enantiomerically pure drugs also, in molecular biology as seen in heterogonous gene expression (Huston, 2008).

Moreover, there are various application fields of cold-active, thermolabile protease (Cotarlet *et al.,* 2009). For instance, such enzymes could be useful new tools in molecular field, food processing and bioremediation. Also, amylase adapted at low temperature can be valuable source in detergents, food industry and in bioremediation process (Cotarlet *et al.,* 2009). Glucose oxidase is important for its role in several fields of specific application such as food biotechnology and bioanalysis (Fenice *et al.,* 1997). Fenice *et al.* (1998) reported *Verticillium cfr. Lecanii* can be considered as a promising organism for future research. This is probably due to its ability to grow and to produce chitinase activity at low temperature, it could be useful for biocontrol of microbial spoilage of refrigerated foods and used as mycoparasite of phytopathogenic fungi in cold areas. High production of fungal chitinase could have some uses such as the biological treatment of chitin-containing wastes, and mild degradation of chitin to prepare chito-oligosaccharides for medical application (Fenice *et al.,* 1997).

Ultimately, the following recommendations could be useful for future research:

- Use of more than one culture media broth for chitinase production, will probably provide enzyme activity.
- Purify cold-active chitinase enzyme from *Geomyces* spp. may provides more options for several fields of practical application
- Test the isolates for other biotechnological valuable metabolites that may produce another enzyme or antimicrobial agent.