CHAPTER - I

INTRODUCTION

Enzymes are the most efficient bio-catalysts which catalyze many biological reactions. Enzymes are the most important products obtained for human needs through microbial sources. Enzymes are first reported in the second half of the nineteenth century (Godfrey et al. 1996). Since then, its usage has increased manifolds in various industries. In the last three decades with rapid strides in the field of biotechnology, especially in the field of genetic and protein engineering, there has been many exciting research work involving enzymes with the development of new commercially important industrial processes. Enzymes are used in several industries because they are specific, versatile and very effective biological catalysts, resulting in much higher reaction rates when compared to chemically catalyzed reactions under ambient conditions.

Today the enzymes are commonly used in many industrial applications and the demand for more stable, highly active and specific enzymes is growing rapidly. Nearly 60% of the total world supply of industrial enzymes is produced in Europe and the remaining 40% from USA and Japan. Approximately 75% of the industrial enzymes are hydrolases with carbohydrolases being the second largest group. All types of living organisms produce enzyme through metabolic reactions. Out of total enzymes used industrially over half are produced from fungi and yeast, one third from bacterial systems and the remaining from animal and plant sources (Marwaha et al. 2000).

The advantages of microbial enzymes over the plant and animal enzymes are

I. They are economical and can be produced on the large scale within limited space.

II. They are capable of producing a wide variety of enzymes.

III. They can grow in wide range of environmental conditions.

IV. They show genetic flexibility can be genetically manipulated to increase the yield of enzymes.

V. They have short generation times.

VI. They have wide biosynthetic possibilities and high productivity.
Current developments in biotechnology are yielding new applications for enzymes (Zherebtsov et al. 2002). Microbial enzymes are widely used in large number of industries like biochemical, environmental, medicine, agriculture, pharmaceuticals, detergents, food processing industries, leather industries and in the industrial waste treatment. The increasing availability of enzymes will allow their exploitation as potent catalysts to introduce chirality and specificity into compounds in chemical processes. There is ever increasing demand to replace traditional chemical processes with advanced biotechnological processes involving microorganisms and enzymes such as chitinase (Nawani et al. 2005), xylanase (Beg et al. 2000) and lignases (Bajpai et al. 1999). All these not only provide an economically viable alternative, but also more ecofriendly (Viikari et al. 2001).

1.1 Chitinase

Chitin is a polysaccharide composed of β-1, 4 N-acetyl D-glucosamine. It is highly distributed in nature, as a constituent of insect exoskeleton, shells of crustaceans, and fungal cell walls. More than 1x 10^{11} tons are estimated to be produced annually in marine waters alone, mostly by copepods. Chitinase are enzymes capable of hydrolyzing insoluble chitin to its oligo and monomeric components. Chitinase are present in chitin containing microorganisms, bacteria and plants with a diversity of roles such as chitin metabolism in growing hyphae, defense mechanisms in response to pathogens and abiotic stress and in nutrition and parasitism. In addition, chitinase have been used for the production of single cell protein for animal and aquaculture feed, isolation of fungal protoplasts, preparation of bioactive chito oligosaccharides, phytopathogen inhibition and degradation of chitin rich waste materials. In spite of such industrial significance, only few of these applications have been exploited, mainly due to the constraints imposed by the high cost of the enzyme. The interest in chitin degrading enzymes and their application in control of fungal pathogens have advanced significantly, because chitin is a major structural component of fungal cellwall. Chitinase plays key role in biological pest control in food and feed industry (Madhavan Nampoothiri et al. 2004).

Biological control of pathogenic fungi provides an attractive alternative for management of fungal disease without the negative impact of synthetic antifungal agents that can cause environmental pollution and may induce pathogen resistance (Chang et al. 2003). The chitinase producing strains could be used directly in biological control of fungi or indirectly by using chitinase or through gene manipulation.
One of the first biotechnological applications of chitinase concerned is its use in biocontrol of plant pathogens (Someya et al. 2000). *S.marcescens* cultures, its chitinase, and its chitinase genes have shown potential as biocontrol agents in a variety of experimental set ups. A highly chitinolytic strain of *S.marcescens* is found to suppress the growth of *Botrytis* spp. *in vitro*. In a greenhouse setting, *S.marcescens* has been shown to control *B.cinerea*, *Rhizoctonia solani*, and *Fusarium oxysporum f.cyclaminis*, all pathogens of cyclamen. (ELTarabily et al. 2000). *S.marcescens* controlled growth of *Sclerotinia minor*, the casual agent of basal drop disease, in lettuce grown in green house.

Fungal plant diseases are one of the major concerns to agricultural production. It has been estimated that total losses as a consequence of plant diseases reach 25% of the yield in western countries and almost 50% in developing countries. Of this, one third is due to fungal infections (Bowyer, 1999). So there is a pressing need to control fungal diseases that reduce the crop yield so as to ensure a steady and constant food supply to ever increasing world population. Conventional practice to overcome this problem has been the use of chemical fungicides which have adverse environmental effects causing health hazards to humans and other non target organisms, including beneficial life forms. Hence there is an increasing concern towards the toxicity and biomagnification potential of these chemicals in agriculture. Currently practices based on molecular biology techniques which involve development of transgenic plants which are resistant to plant pathogens, are being used. However there have been few reports of fungal infestations in resistant varieties Chitin has broad spectrum distribution in the biosphere (like in the shells of crustaceans, such as crab, shrimp and lobster, exoskeleton of marine zoo-planktons).

Chitinase are reported to play a protective role against fungal pathogens (Boller, 1985). Besides its ability to attack the fungal cell wall directly, chitinase release oligo-N-acetyl glucosamines that function as elicitors for the activation of defense-related responses in plant cells. Chitin is the second most abundant polysaccharide in nature. It is insoluble in water, dilute and concentrated alkalis, alcohol and other organic solvents. It forms the major structural component in the shells and cuticles of arthropods, crustaceans and insects and in cell walls of fungi. The major contribution of chitin to nature is in the form of animal biomass.
1.2 Fermentation

1.2.1 Submerged Fermentation (SmF)

Chitinase enzymes are produced efficiently by submerged fermentation. The submerged fermentation offers many advantages. The component of the media remains uniform throughout the fermentation. The temperature, pH and other fermentation conditions remains uniform throughout the fermentation medium. The analysis of substrates, products, intermediates and biomass are easier when compared to solid state fermentation. But microbial chitinase are produced mainly by the fungal cultures. The fungal cultures like *Trichoderma harzianum*, *Trichoderma viride* and *Aspergillus fumigates*, bacteria like *Serratia marcescens* are highly filamentous and the medium becomes highly viscous. The biomass produced is also very thick and are agglomerated. The biomass is not uniformly distributed in the fermentation medium during the fermentation period. The mass transfer is very difficult. Chitinase production using fungal cultures by submerged fermentation becomes difficult and hence solid state fermentation is preferred. Most of the commercial chitinase are inducible enzymes and substrates rich in chitin must be added to the medium to stimulate enzyme production. For reasons of economy, chitin is not used as such in the production media but it is substituted by dried sugar, citrus peel and apple pomace.

Laboratory studies indicate that very high levels of chitinase may be obtained with the medium containing mixed carbon sources, eg, glucose with chitin, sucrose with chitin and lactose with chitin. Control of pH is also very important for higher enzyme production and inactivation occur at high pH levels.

1.2.2 Solid State Fermentation (SSF)

Solid State Fermentation is generally defined as the growth of the microorganisms on solid material in the absence or near absence of free water. In recent years, SSF has shown much promise in the development of several bio process and products.

Solid state fermentation process is the most suitable technique for chitinase production using fungal cultures utilizing cheaper substrates. SSF provides intimate contact of microbes with substrates and maximum production of chitinase can be obtained. The enzyme production cost is less and there is less chance for contamination in SSF. For chitinase production, various agro wastes like crab shell, Colloidal chitin, flake chitin, crab shell chitin, mushroom stalk, fungal cell wall, wheat bran, rice bran (Dahiya et al. 2005)
Swollen chitin (Nawani et al. 2005), cell walls, crab and shrimp shells (Madhavan Nampoothiri et al. 2004) are used as substrates in solid state fermentation. They are mixed with water according to the specific formulation. The bran absorbs all the water and nutrients. They must be enclosed either in large closed chambers or long horizontal rotating drums. After inoculation with spore suspension, air is circulated through the system. The enzyme production time varies from 3 to 7 days (Fogarty et al. 1983).

The culture conditions are more similar to the natural habitat of filamentous fungi, so that these are able to grow and excrete large quantities of enzymes. Solid state fermentation process is the most suitable technique for chitinase production using fungal cultures utilizing cheaper substrates. SSF provides intimate contact of microbes with substrates and maximum production of chitinase can be obtained. The enzyme production cost is less and there is less chance for contamination in SSF. Since 1986, in Brazil, a series of research projects for the value addition of tropical agricultural products and sub products by SSF have been developed due to the high amounts of agricultural residues generated by this country (Soccol et al. 2003).

In recent years SSF has received more and more interest from researchers, since several studies on enzymes (Pandey et al. 1999), flavours (Ferron et al. 1996), colourants (Johns et al. 1991) and other substances of interest to the food industry have shown that SSF can give higher yields (Tsuchiya et al. 1994) or better product characteristics (Acuna-Arguelles et al. 1995) than submerged fermentation. In addition, costs are much lower due to the efficient utilisation and value-addition of wastes (Robinson et al. 2003). Castilho et al. (2000), have performed a detail economic analysis of the production of Penicillium restrictum lipase in both SmF and SSF. They found that for a production scale of 100 m$^3$ lipase concentrate per year, total capital investment needed for SmF was 78% higher than that needed for SSF. Also, SSF unitary product cost was 47% lower than the selling price. The main advantage of SSF process is it utilizes the cheap raw materials as main substrates like rice bran, sugarcane bagasse, wheat bran, palm kernel cake, palm tree wastes, tea wastes, tapioca and sago wastes, fruit wastes and the municipal solid waste etc. They are produced in huge quantities annually, which have low value in the market. Therefore, SSF is certainly a good way of utilizing nutrient rich solid wastes as a substrate. Both food and agricultural wastes are produced in huge amount and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production of bulk chemicals and enzymes using SSF technique. The nature of the solid substrate employed is the most important factor
affecting SSF processes and its selection depends upon several factors mainly related with cost and availability and thus may involve the screening of several agro-industrial residues. In SSF process the solid substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells.

Research on the selection of suitable substrates for SSF has mainly been focused on agro industrial residues due to their potential advantages for filamentous fungi, which are capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium (Ramachandran et al. 2004). In addition the utilization of these agro industrial wastes, on one hand, provides alternative substrates and on the other helps in solving problems with their disposal (Pandey et al. 1999a). The present research study aims in utilizing these agro industrial residues which are abundantly available in India for the production of chitinase enzymes under optimized conditions.

In the solid state fermentation technique for enzyme production, the medium is prepared using solid substrates and number of other ingredients. They are mixed with water according to the specific formulation. The substrate absorbs all the water and nutrients. They must be enclosed either in large closed chambers or long horizontal rotating drums. After inoculation with spore suspension, air is circulated through the system.

1.2.3 Extraction and Recovery

When the chitinase enzymes are produced by submerged fermentation, no extraction process is required other than to separate cell mass by centrifugation or filtration. In solid-state fermentation the enzyme is to be extracted using water or other suitable chemicals as a solvent. This method provides relatively clear solution. Alternatively, the semi-solid may be dried at low temperatures. This dried material may be used as a crude commercial preparation or it may be stored and the enzyme is extracted subsequently.

The clear enzyme solution prepared by the method mentioned above may be concentrated or precipitated by salt or organic solvents. Following precipitation, enzyme cake is centrifuged or filtered and then dried at low temperatures or spray dried. Subsequently, it is ground to particular particle size and is used to prepare commercial enzyme formulations. Resulting powder is either sold as a concentrate on the basics of its activity or diluted with various agents to give a standard activity.
1.3 Applications of Chitinase

Chitinase have shown an immense potential for increasing the production of several useful products in the most economic way. The major applications of chitinase are discussed in the following subsections. Chitinases are bioconversions of chitin waste to single cell proteins, ethanol and fertilizers. Industrial applications of chitinase have been governed mainly by key factors such as cost production, shelf life stabilities and improvement in enzyme properties by immobilization (Bhushan, 2000). Chitinase producing marine bacteria plays an important role in the degradation on chitin in the oceans (ShinHyePark et al. 2000).

1.3.1 Chitinase as a Target for Biopesticides

Chitin is present in the exoskeleton and gut lining of insects. The molting enzyme chitinase has been derived from Bombyx mori (silkworm), Manduca sexta (tobacco hawkmoth), and several other species. Similarly, chitinase have been implicated in different morphological events in fungi (VillagomezCastro et al. 1996). Allosamidin, a potent inhibitor of chitinase, was found to be inhibitory to the growth of mite (Tetranychus urticae) and a housefly larva (Musca domestica) after ingestion (Sakuda et al. 1987). Chitinase inhibitors can be explored as potential biopesticides. Chitinase (50μ liter, 0.3 to 3μM) are individually administrated orally into the digestive tube of the pine sawyer beetle and most of the beetles died in 20 hrs. Thus chitinase have potential as a pest-control agent (kavikarunya et al. 2011).

1.3.2 Estimation of Fungal Biomass

A variety of methods have been described to quantify fungi in soil. The techniques include direct microscopic observation and extraction of fungus specific indicator molecules such as glucosamine ergosterol. A strong correlation has been reported between chitinase activity and fungal population in soils. Such correlation is not found for bacteria and actinomycetes. Thus, chitinase activity appears to be a suitable indicator of actively growing fungi in soil. Miller et al. (1998) reported the correlation of chitinase activity with the content of fungus-specific indicator molecules 18:2ω6 phospholipid fatty acid and ergosterol using specific methylumbelliferyl substrates. Similarly, chitinase and chitin-binding proteins can be used for the detection of fungal infections in humans (Laine and Lo, 1996).
1.3.3 Mosquito Control

The worldwide socioeconomic aspects of diseases spread by mosquitoes made them potential targets for various pest control agents. In case of mosquitoes, entomopathogenic fungus such as *Beauveria bassiana* could not infect the eggs of *Aedes aegypti*, a vector of yellow fever and dengue, and other related species due to the aquatic environment. The scarabaeid eggs laid in the soil were found to be susceptible to *B. bassiana* (Ferron, 1985). *M. verrucaria*, a saprophytic fungus, produces a total complex of an insect cuticle degrading enzyme (Shaikh *et al.* 1993). It has been seen that both first and fourth instar larvae of mosquito *A. aegypti* can be killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonça *et al.* 1996). Though 100% mortality is observed within 48 h, purified endochitinase lethal times (LT50) are 48 and 120 h for first and fourth instar larvae, respectively. However, the time period is found to be decreased, corresponding to 24 h and 48 h, when the purified chitinase is supplemented with lipolytic activity.

1.3.4 Morphogenesis

Chitinase plays an important role in yeast and insect morphogenesis. (Kuranda *et al.* 1991) reported the role of chitinase in cell separation during the growth of *S. cerevisiae*, and (Shimono *et al.* 2002) studied the functional expression of chitinase and chitosanase and their effects on morphogenesis in the yeast *S. pombe*. When the chiA gene is expressed in *S. pombe*, yeast cells grow slowly and cells become elongated, but when the choA gene is expressed, cells become swollen. Expression of both chiA and choA genes results in elongated fat cells.

1.3.5 Medical Applications

Chitinase can be employed in human health care, such as making ophthalmic preparations with chitinase and microbiocides. A direct medical use has been suggested for chitinase in the therapy for fungal diseases in potentiating the activity of antifungal drugs (Pope *et al.* 1979; Orunsi *et al.* 1985). They can also be used as potential additives in antifungal creams and lotions due to their topical applications.

1.3.6 Control of Plant Pathogenic Fungi

Biological control or the use of microorganisms or their secretions to prevent plant pathogens and insect pests offers an attractive alternative or supplement for the control of
plant diseases. Therefore, biological control tactics have become an important approach to facilitate sustainable agriculture (Wang et al. 2002). Chitin application increased the population of chitinolytic actinomycetes, fungi, and bacteria. The increase is shown to be correlated with the reduction in pathogenic fungi and nematodes and more importantly, with the reduction of infectivity and hence crop damage (Wang et al. 2002). The chitinase produced by *Enterobacter sp.* NRG4 is highly active toward *Fusarium moniliforme*, *A. niger*, *Mucor rouxi*, and *Rhizopus nigricans* (Dahiya et al. 2005a). The chitinase from *Alcaligenes xylosoxydans* inhibited the growth of *Fusarium udum* and *Rhizoctonia bataticola* (Vaidya et al. 2001).

Mahadevan et al. (1997) reported the antagonistic action of *Streptomyces lydicus* WXEC108 against *Pythium ultimum* and *Rhizoctonia solani*, which cause disease in cotton and pea. Horsch et al. (1997) suggested the use of N-acetylhexosaminidase as a target for the design of low molecular weight antifungals. Chitinase can be added as a supplement to the commonly used fungicides and insecticides not only to make them more potent but also to minimize the concentration of chemically synthesized active ingredients of the fungicides and insecticides that are otherwise harmful to the environment and health. Bhushan et al. (1998) studied the compatibility of a thermostable chitinase from *Bacillus sp.* BG-11 with the commonly used fungicides and insecticides.

Mathivanan et al. (1998) reported chitinolytic enzymes of *T.harzianum* are found to be inhibitory to a wide range of fungi than similar enzymes from other sources (Lorito et al. 1993). Govindsamy et al. (1998) reported the use of purified preparation of *M. verrucaria* chitinase to control groundnut rust *P.arachidis*. *Penicillium janthinellum* P9 caused mycelial damage in *Mucor plumbus* and *Cladosporium cladosporiodes* (Giambattista et al. 2001). Partially purified chitinase from *T.harzianum* destroys the cell wall of *Crinipellis perniciosa*, the casual agent of witches’ broom disease of cocoa (DeMarco et al. 2000). Chitinase from *B.cereus* YQ 308 inhibited the growth of plant pathogenic fungi such as *Fusarium oxysporum*, *Fusarium solani*, and *P.ultimum* (Chang et al. 2003).

1.4 Objectives of the Present Work

Due to environmental concerns, during the last two decades there has been an increased interest in using agro industrial residues for the production of enzymes or other bio products. In India large amount of agro industrial residues are produced whose disposal poses
a great problem and also they occupy enormous space. Among these agro industrial residues, wheat bran, rice bran and sugarcane bagasse are abundantly available in southern part of India and are selected for the present research work as substrates. These agro industrial residues contain many valuable nutrients and minerals and are very useful for the production of enzymes or other useful bio products. The use of agro industrial residues by solid-state fermentation results in the reduction of overall enzyme production cost.

The objectives of the present research work are given below:

1. To study the chitinase enzyme production using (i) *Trichoderma viride* (MTCC-167) (ii) *Serratia marcescens* (MTCC-97) (iii) *Trichoderma harzianum* (MTCC-792) by solid-state fermentation using cheaper substrates like rice bran, wheat bran, and sugarcane bagasse.

2. To identify the most significant medium components affecting chitinase enzyme production using Plackett-Burman Design in Solid State Fermentation (SSF) for the three microorganisms.

3. To optimize the selected media components for the enhanced production of chitinase by Response Surface Methodology (RSM).

4. To optimize the process variables for the maximum production of chitinase enzyme by Solid State Fermentation using Central Composite Design (CCD).

5. To study the performance of packed bed reactor in chitinase production at various air flow rate and packing density.

6. To study the product formation kinetics using Michaelis-Menten equation.

7. To model the chitinase production using Artificial Neural Network (ANN).

1.5 Organization of the Thesis

**Chapter 1 - Introduction** deals with the definition, classification and industrial importance of chitinase enzyme. It also explains the need for carrying out the present research work. The objectives of the present research work are given.

**Chapter 2 - Literature Review** describes a complete and exhaustive review of literature about the various sources, characterization and industrial applications of chitinase enzyme. This also covers the production of chitinase enzyme by yeast, bacteria and fungi by solid state fermentation.
This covers the conventional and statistical method of optimization for chitinase production by solid state fermentation. The kinetics on solid state fermentative production of chitinase is also discussed.

**Chapter 3** – “Materials and Methods” describes about the substrate preparation, microorganisms used their maintenance and the analytical procedure for determination of chitinase activity. The experimental setup and experimental procedure are also described.

**Chapter 4** – “Results and Discussion” describes the results of the research work carried out for solid-state fermentative production of chitinase. This includes (i) screening of nutrients for maximum chitinase production by Plankett-Burman design, (ii) optimization of selected media components using RSM, (iii) optimization of process variables using CCD. The production of chitinase is carried out using packed bed reactor at various flow rates and packing density.

**Chapter 5** – “Kinetics and Modelling” Michaelis-Menten kinetics is used to study the kinetics of chitinase production using microorganisms *T.viride, S.marcescens* and *T.harzianum* under optimized conditions. Comparison of experimental values with RSM and ANN for the production of chitinase is carried out.

**Chapter 6** – “Conclusions” summarizes the results obtained in the present research work and suggestions for future studies of chitinase production using agro-industrial wastes.