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***Research Paper***

# IMMOBILIZATION OF CHITINASE FROM *Pseudomonas putida* ON BIOCOMPATIBLE CHITOSAN BEADS AND THE PROPERTIES OF THE IMMOBILIZED ENZYME

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**Abstract**

Chitinase from *Pseuodomonas putida* was immobilized by a biopolymer by using glutaraldehyde conjugation. Gel beads were formed from the enzyme - polymer complex, and the activity of immobilization was compared to the method of encapsulating the enzymes with chitosan. The pH optimum of the immobilized enzyme was shifted to more acidic value compared to that of the free enzymes. This technique holds promise for the development of immobilized enzymes for application in agriculture, biosensors and bio processing.

## INTRODUCTION

Chitin is a high molecular weight linear polymer of β (1, 4) linked N – acetyl glucosamine residues (GlcNAc), is one of the most profuse renewable resources which were obtained from the marine invertebrates such as crustaceans, skin of fish scales and protozoa. It is present in almost 10 % of the global landing of aquatic products consists of organisms rich in chitinous material (10 – 50 % on dry weight basis). The chitin was easily degraded by the enzyme chitinase which was produced chitin degrading microorganism. The roles of chitinase in these organisms are diverse. All organisms that contain chitin also contain chitinase. Fish scale also restrain sum of chitin in it. Chitin is a versatile environmentally friendly modern material (Kanafari *et al.*, 2007). Chitin is a natural biopolymer with a chemical structure similar to that of cellulose and is a major component of the exoskeleton of invertebrates. The crustacean waste is the most important chitin source for commercial use due to its high chitin content and ready availability.

Chitinase are glycosyl hydrolases, which hydrolyze the chitin and which are presumably required for morphogenesis of cell walls and exoskeletons. Other organisms that do not contain chitin may produce chitinase to degrade the chitin in

their environment. Chitinase is more acquisition in consequence for their biotechnological applications. The cost of production and processing of chitinase represents an important factor and it is also necessary to convert the chitin through bioconversion with reduction in the cost of the production. Chitinase is best studied enzymes, which hydrolyze chitin have broad spectrum of distribution in nature including bacteria, fungi, plants, insects, and protozoa, human, animal and yeast. The roles of chitinase in these organisms are diverse. In bacteria, chitinases are usually involved in mineralization of chitin nutrition and parasitism (Gohel *et al*. 2006). Chitinase enzymes have their prospective applications in agro-chemical industry, and the microbes capable of producing chitinases are being used effectively against number of soil-borne fungal pathogens and nematodes (Amin *et al*., 2011). Chitinolytic enzymes have a various potential applications such as preparation of chitooligosaccharides and N-acetyl D-glucosamine which has various biological activities such as antimicrobial, antifungal, immunoenhancers, antitumor, etc.

Bacteria and fungi are thought to be important decomposers of chitin in soil and thereby contribute to the recycling of carbon and nitrogen resources in soil ecosystems. Bacteria produce chitinases to degrade and utilize chitin as carbon and nitrogen source. Isolation of chitinolytic bacteria from chitin-rich soil sources therefore would be a powerful approach for selecting bacteria with high chitinolytic activity and to explore the vast diversity of these bacteria from the soil (Subha *et al*., 2010). *Pseudomonas putida* strain has been extensively characterized as a ubiquitous, aerobic, Gram-negative bacterium that shows great metabolic versatility and non pathogenic in nature. They are easy to isolate and grow in the laboratory and they are not fastidious in their requirement of nutrition and also meant for its non pathogenic in nature. Chitinolytic bacteria play a major role in biocontrol in many systems there by inhibiting the growth of the phytopathogens. The plant growth promoting bacteria were known to enhance the growth of the plant by preventing the proliferation of phytopathogens and facilitating the uptake of nutrients from the soil by producing the plant growth producing components. Fluorescent pseudomonads are ubiquitous bacteria were the natural suppressive of certain soil – borne disease (Lemanceau, 1992).

Enzymes or 'biocatalysts' are remarkable discovery in the field of bioprocess technology. Enzyme immobilization provides an admirable base for escalating their shelf life. Biocatalysts have been widely accepted in diverse sectors owing to their ease of production, substrate specificity and green chemistry. Upholding their structural stability during any biochemical reaction is highly challenging. Subsequently, immobilized enzymes with functional efficiency and enhanced reproducibility are used as alternatives in spite of their expensiveness. Immobilized biocatalysts can either be enzymes or whole cells.

Enzyme immobilization is confinement of enzyme to a phase (matrix/support) different from the one for substrates and products. Immobilization generates continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity. Several methods are used for immobilization and various factors influence the performance of immobilized enzymes. Enzyme may be immobilized by physical methods or by chemical method such as covalent attachment to water – insoluble matrix like chitosan and cross linking with neutral substance. Chen and Chang, 1994 purified chitinase from *Serratia marcescens* and immobilized by covalent binding to a polymer. The immobilized enzyme was able to hydrolyse the chitin which was higher than that of the free enzyme. Immobolization of chitinase produced from *Lysobacter enzymogenes* MG18S on chitosan beads was effective from

the prevention and biological control of phytopathogens (Jang *et al*., 2012). Ghafil (2013) states the immobilization of chitinase on charcoal improves the ability of enzyme to hydrolyse chitin.

## 2. MATERIALS AND METHODS

### 2.1 Immobilization of Chitinase

Chitinase was immobilized on swollen chitosan beads with 7 % (w/v) which contains of 10 U/ml of chitinase in acetic acid solution coagulating solvent (water: methanol: NaOH 4:5:1) (Bhusan, 2000).

The chitosan beads were prepared by cross – linking of glutaraldehyde 5 % in 0.1 M phosphate buffer (pH 7.0) and stirred at room temperature at 120 rpm for overnight then it was stored at 4 ºC (Jang *et al*., 2012).

The immobilization percentage and the amount of the enzyme supernatant of enzyme solutions before and after the immobilization process were calculated from protein concentration measured by Bradford (1976).

### 2.2. Effect of pH on immobilized chitinase

Effect of pH on immobilized chitinase activity was assayed at different pH values (pH 2.0 to 10.0) using different buffers 50 mM such as, Sodium Acetate buffer (pH, 2.6 - 7.0), Sodium phosphate buffer (pH, 7.5 - 8.0), Tris-HCl buffer (pH, 7.0 - 8.5) and Glycine - NaOH buffer (pH, 8.6 - 10.0).

### 2.3. Effect of temperature on immobilized chitinase

The residual activity of chitinase was assayed at different temperatures ranging from 20 ºC – 90 ºC at pH 5.5 in Tris-HCl buffer (25 mM) 1 h.

### 2.4. Compatibility with pesticide

The relative activity was determined by incubating the pesticide of allosamidin with a concentration ranging from 10 - 100 µg/ml. The immobilized enzymes were also used for comparative study and results were shown graphically.

### 2.5. Substrate specificity

The effect of various substrates like colloidal chitin, fish scale chitin and fish scales were studied to determine the substrate specificity of chitinases. The activity was expressed as released reducing sugars by the method described in enzyme assay section. The released N-acetyl-β-D-glucosamine was measured.

### 2.6. Kinetic studies

To study the enzyme kinetics purified chitinase was incubated in colloidal chitin as substrate at concentration of 0.25 mg to 10 mg and reducing sugars was determined at optimum temperature of pH 5.0 and incubation of 1h. Michelis Menten's ($K_m$) constant value analysis and maximum speed ($V_{max}$). The $K_m$ and $V_{max}$ values were determined by Line weaver – Burk's plot. From the $V_{max}$ values the $k_{cat}$ values were also determined.

### 2.7. Chitin digestion by chitinase

#### 2.7.1. Viscometric assay (Dahiya, 2005)

Purified chitinase (50 µg) was added to the 50 ml of substrate solution (colloidal chitin) and the mixture was kept for digestion at 37 ºC. The same procedure was followed for fish scales which were in 50 mM of citrate phosphate buffer, at pH 5.5. Aliquots were removed at interval of time and subjected to viscosity measurement for the digestions of fish scales were ensured.

#### 2.7.2. Chitin degradation and hydrolysis pattern of chitinase – TLC and HPLC

The enzyme solution was incubated with 1.0 % of colloidal chitin and fish scales as the substrates at 37 ºC for 5 h, the hydrolytic product chitin were resolved by TLC

and HPLC (Shimadzu, USA). TLC was performed according to the method Lewis and smith 1969. The hydrolysate were spotted onto a Silica gel 60 and developed in a solution of n-butanol: methanol: 25 % ammonia solution: water (5: 4: 2: 1). The spots were detected with aniline phthalate reagent. Then hydrolyzed products were checked through the HPLC (Shimadzu, USA). The samples were eluted with 75 % acetonlitrile in water with a flow rate of 1.0 ml / min. 10 ml of the sample was injected to it.
UV absorbance of the hydrolyzed products was measured at 210 nm, and the peaks were identified using N - acetyl glucosamine as the standard from Sigma.

### 2.8. Applications of Immobilized chitinase

#### 2.8.1. *In vitro* test – chitinase enzymes on antifungal activity

This assay was performed by using 1 % of fish scale and immobilized enzymes with concentration of 5, 10, 15 and 20 U/ml. The soil samples was collected and tested for its components by Ben-Dor and Banin (1989). One ml of free, immobilized enzymes was added in concentration with 1 g of soil samples. The other tested with the presence of fish scales. The control was kept with the chitosan without enzyme in acetate buffer was maintained. All the tubes were inoculated with 1 ml of *F. oxysporum* suspension obtained from a 12 days old culture. Immediately after tube inoculation, enzyme activity, as wells as the fungal growth was calculated. The same was measured after incubation at room temperature for $5^{th}$, $10^{th}$, $15^{th}$, $20^{th}$, $25^{th}$, $30^{th,}$ $35^{th}$ and $40^{th}$ day.

## 3. RESULT AND DISCUSSION

### 3.1. Immobilization of chitinase

Chitinase was immobilized with chitosan beads and sodium alginate. Chitinase which was immobilized in chitosan beads shows the highest activity and stability when compared to others. So the chitinase was immobilized in chitosan beads to study further activity. When fresh untreated sample was kept as control, other set of the samples were kept frozen, immobilized with the chitosan and calcium alginate mixture. In the present investigation, it was observed that chitinase reacts with chitosan and binds with it and increases the activity of chitinase. It was shown in the fig. 1.

When compared with control, chitinase activity was increased and more stable when compared with calcium alginate also. So, it has been predicted that immobilization of chitinase could increases the activity of chitinase and its stability as well. Immobilization of chitinase with chitosan is fulfilled by physical adsorption. Chitosan contains amino and hydroxyl group which helps for the sorbent for transition metal ions and organic species. Thus they were used as immobilization of chitinase by increasing the activity of the enzyme property when compared with sodium alginate.

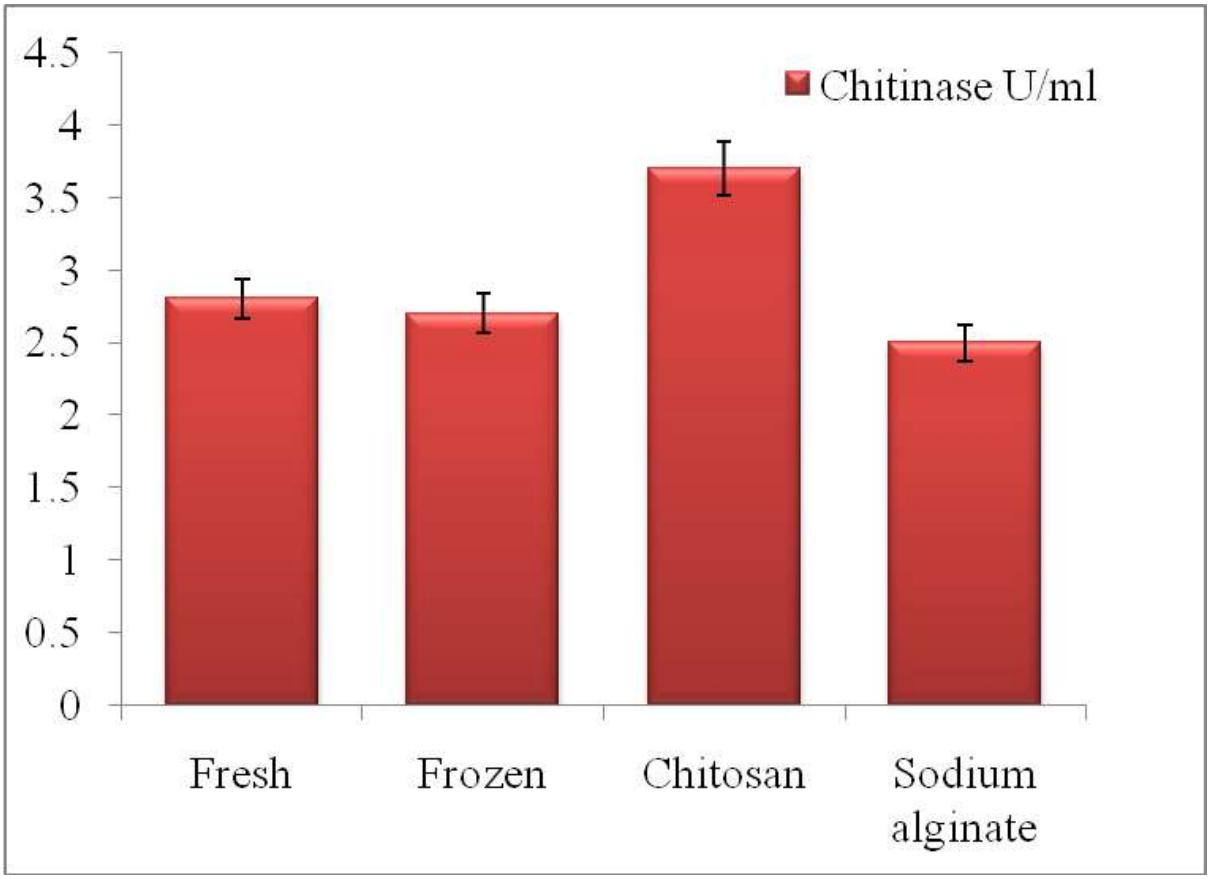



**Fig. 1 Immobilization of chitinase**

Bhushan (2000) immobilized chitinase by chitosan beads which leads to 35 % increase in activity compared with free enzyme and also predicted that the chitosan bead gives favourable and more protective microenvironment for the enzymatic activity. The stabilization of chitinase in chitosan beads is due to non – covalent links between amino group in chitosan backbone and enzymes (Dumitriu and Chornet, 1991).

3.2. **Shelf life - stability**

Stability profile (fig. 2) of chitinase illustrated that the enzymatic activity was stable for 8 weeks of incubation by immobilization. Chitinase activity retains its activity to 60 % after incubation of 8 weeks. Chitinase enzyme devoid of immobilization was able to stable only for 2 weeks. Chitinase was active in the presence of the preservative sodium azide but the activity declines as the number on days when compared with immobilized enzyme.

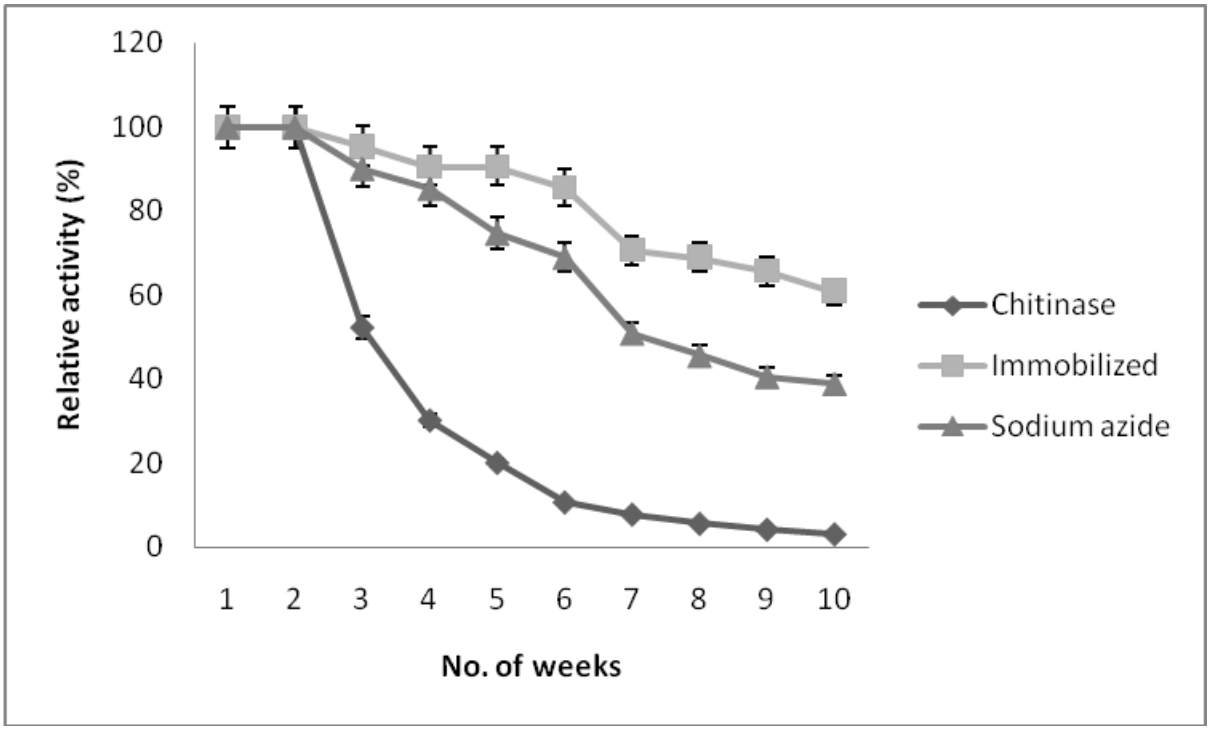



**Fig. 2 Stability of chitinase by immobilization**

### 3.3. Effect of pH on immobilized chitinase

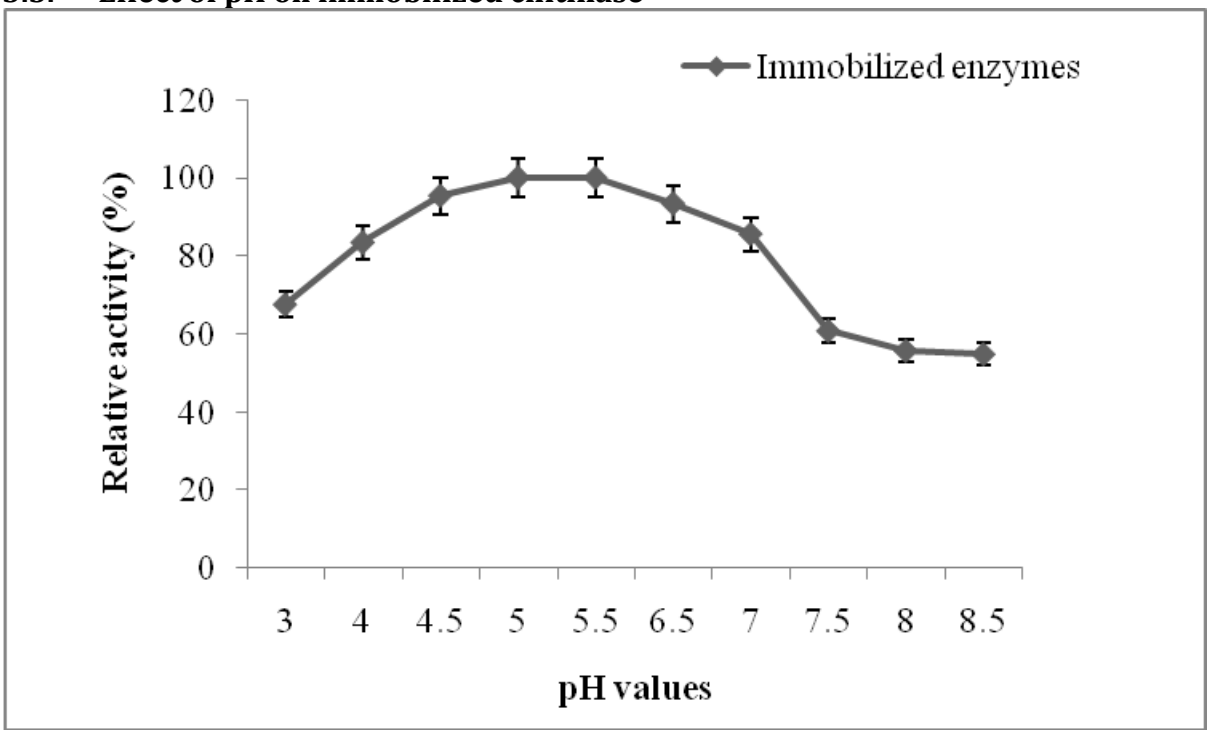



**Fig. 3 Effect of pH after immobilization of chitinase enzymes**

The pH optima for the immobilized chitinase were same as those of free enzymes, *i.e*. at fig. 3. Similar results were seen when pH optimum of alkalophilic *Bacillus* sp. remains same as those of free (Bhushan and Hoondal, 1998). Following immobilization, stability of chitinase towards various pH and temperature were found significantly increased. By immobilizing the chitinase with chitosan beads, stability of pH ranges from 4.0 - 7.0. It has a wide range of pH stability when it was immobilized.

Even in alkaline conditions, the chitinase was found stable up to 60.86 % of its relative activity (fig.56). Similarly the chitinase on chitosan beads increases the activity of chitinase at a wide range of pH 5.0 – 10.0 (Bhushan, 2000).

### 3.4. Effect of temperature on immobilized chitinase

Following immobilization, stability of chitinase towards various temperatures was found significantly increased. By immobilizing the chitinase with chitosan beads, stability of has a wide range of temperature. It ranges from 20 °C to 90 °C. It was stable at high temperature. Similarly the chitinase on chitosan beads increases the activity of chitinase at a wide range of temperature (Bhushan, 2000). It was shown in the fig. 4.

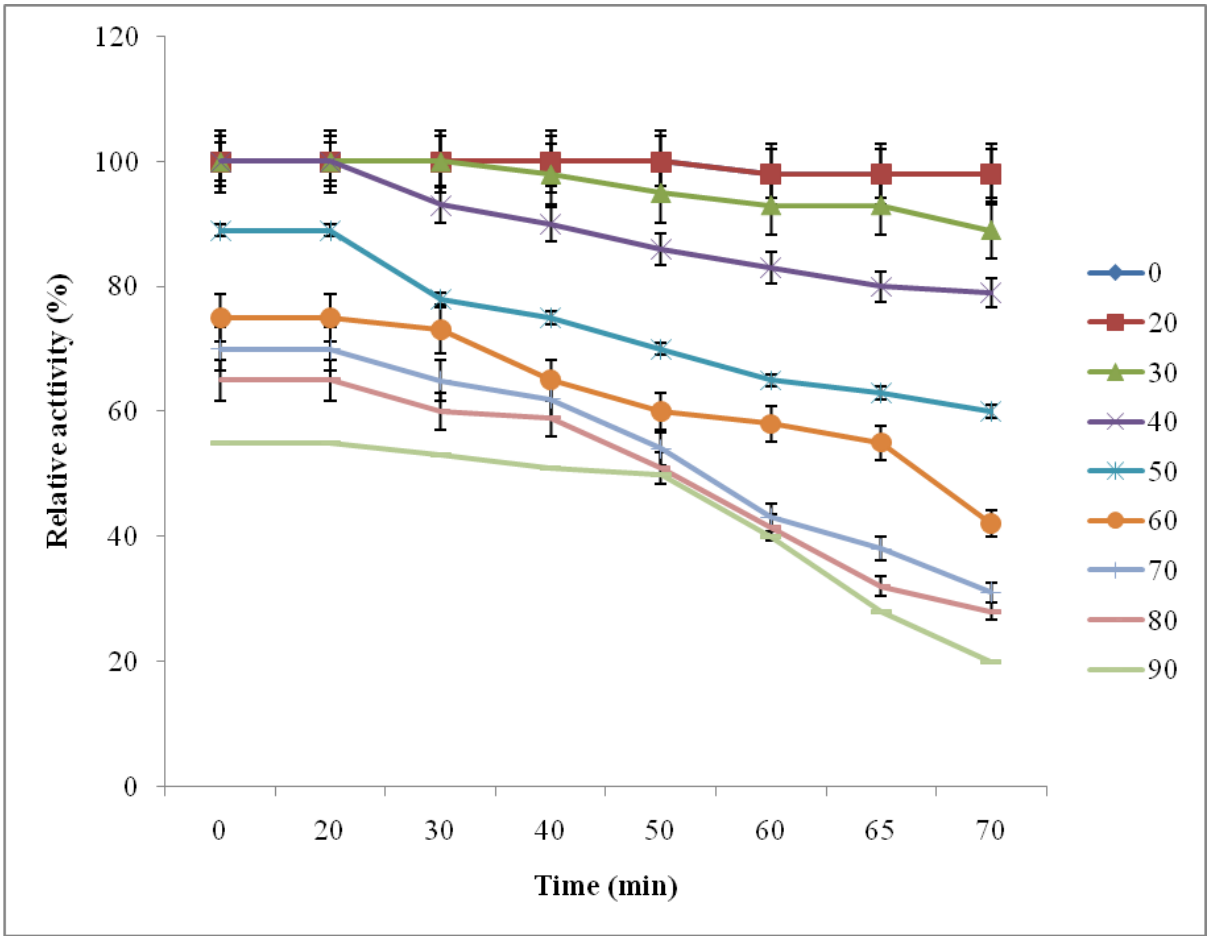



**Fig. 4 Effect of temperature after immobilization of chitinase**

### 3.5. Compatibility of immobilized enzymes with pesticide

Allosamidin was added to enzyme solution with a concentration range of 10 to 100 µg/ml. A comparative study of chitinase and immobilized enzymes mixed with pesticide of different concentration. It was observed that the enzyme concentration was inversely proportional to the concentration of pesticide. At 100 µg/ml concentration of allosamidin, known specific inhibitor of chitinase inhibited chitinase from *P. putida* by 80 %. But when we used immobilized enzyme mixed at 100 µg/ml concentration of allosamidin by 30 %. So the rate of inhibition was reduced when the enzyme was immobilized.

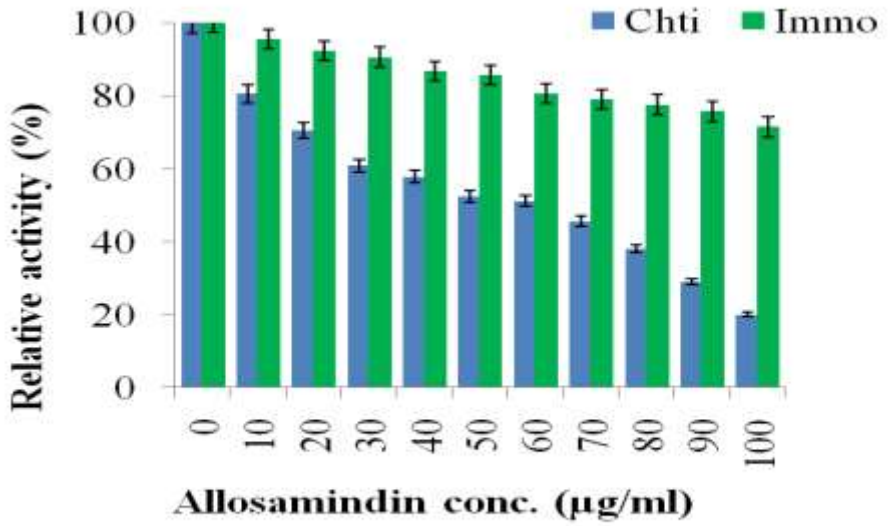



**Fig. 5 Comparative study of chitinase and immobilized enzymes on the pesticide Allosamidin**

Dhahiya *et al*. (2005) reported that allosamidin inhibited chitinase activity by 65.7 % at 100 µg/ml. Chitinase enzyme was compatible with many commercially available fungicide and insecticides. It may be used as one of the active ingredients of chemical pesticides to enhance potency. Chitinase from *P. putida* was able to resistant to the action of allosamidin ($IC_{50}$ = 52 µmol/l). Chitinase from *Bacillus* sp. was able to resistant to the action of allosamidin at 48 µmol/l (Bhushan and Hoondal, 1999).

### 3.6. Substrate binding specificity and kinetic studies

The enzyme showed activities and substrate binding towards colloidal chitin, glycol chitin, swollen chitin, fish scale chitin, fish scales. But they exhibited no activity towards cellulose and starch. The chitinase activity by using colloidal chitin as the substrate was taken as maximum of 100. The activity with fish scale increases the chitinase activity by 24 %. Metal ions and molecules present in fish scales increases the chitinase activity and degrade chitin present in scales. Chitinase enzyme readily hydrolyzes colloidal chitin and glycol chitin as compared to crab shell chitin and the enzyme was classified as endochitinase. It was revealed in the fig.6.

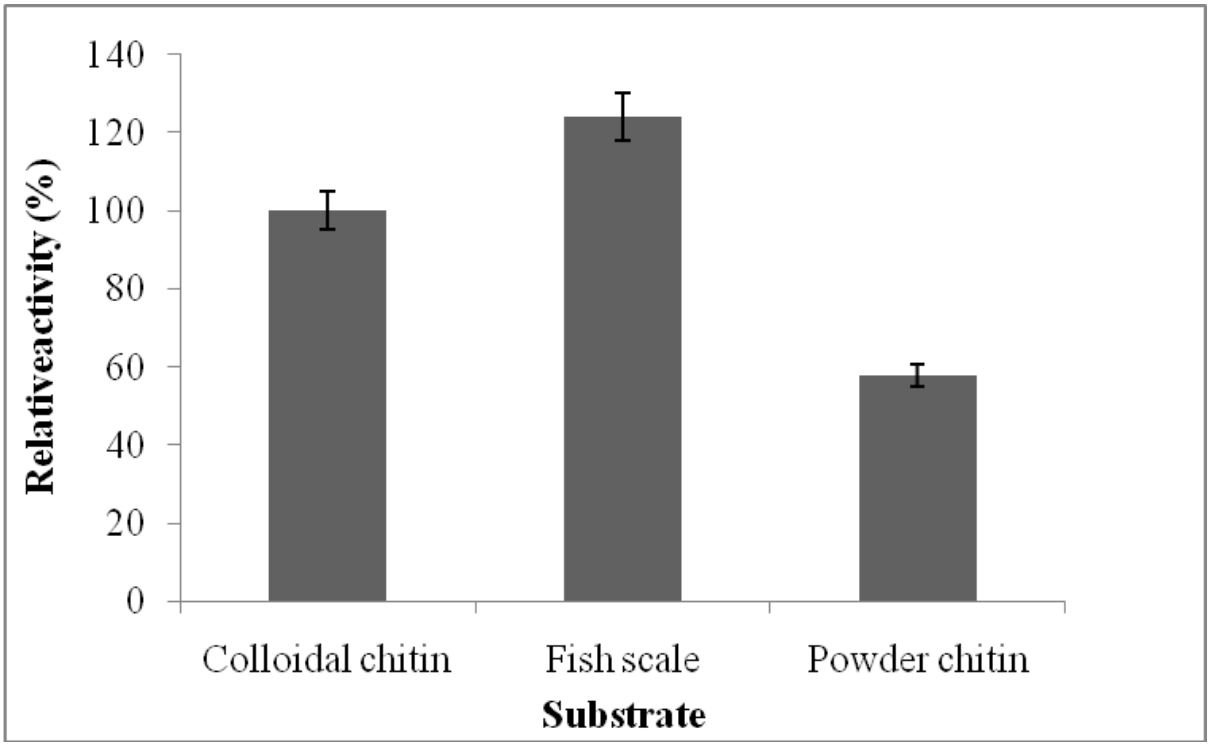
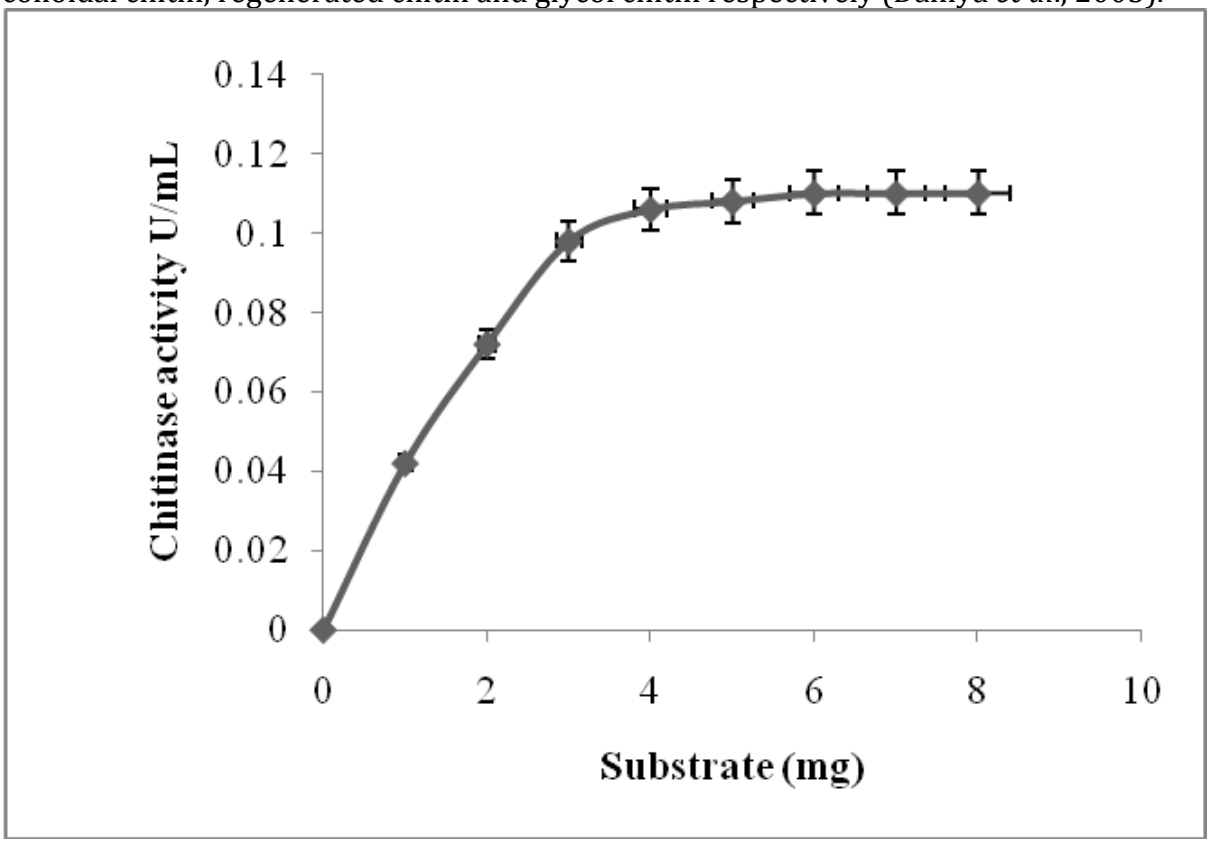



**Fig. 6 Substrate binding specificity**

The $K_m$ and $V_{max}$ values for *P. putida* were found to be 1.42 mg/ml and 111.11 µM µg$^{-1}$ h$^{-1}$, with colloidal chitin as the substrate. Similar result were found in *Enterobacter* sp. NRG4 chitinase against different substrates were the $K_m$ and $V_{max}$ values were 1.43 mg/ml, 1.41 mg/ml, 1.8 mg/ml and 2.0 mg/ml, respectively with swollen chitin, colloidal chitin, regenerated chitin and glycol chitin respectively (Dahiya *et al.*, 2005).



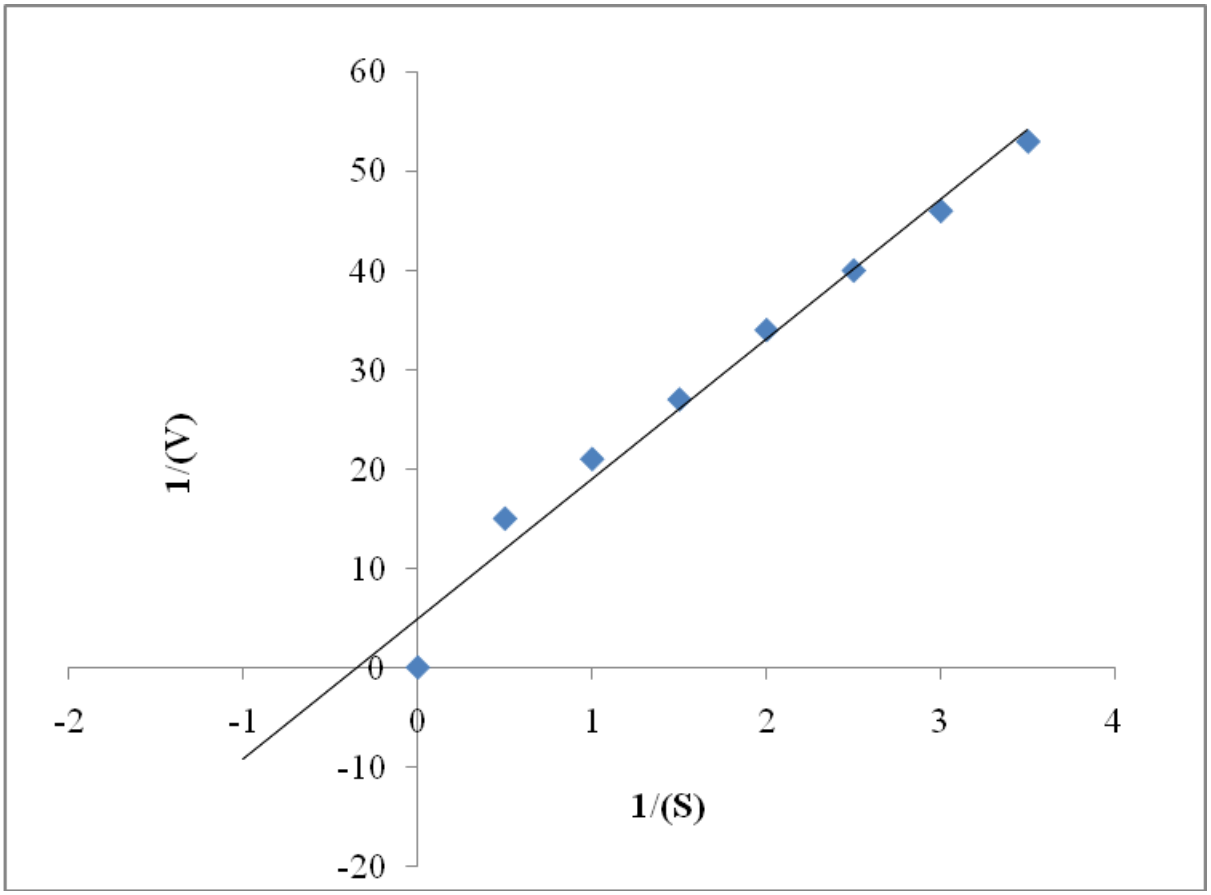



**Fig. 7 Substrate specificity by Michaelis - Menten's curve and Line weaver Burk plot.**

**A. Michaelis - Menten's curve relating *P. putida* chitinase reaction to the substrate.**

**B. Line weaver Burk plot relating reciprocal of velocity and substrate.**

The $K_m$ values of chitinase from different organisms were, 2.88 mg/ml for *Enterobacter* aerogenes (Tang *et al.,* 2001), 1.4 mg/ml and 0.8 mg/ml for chitinase C1 and C3 from *Vibrio alginolyticus* H-8 against squid chitin (Ohishi *et al*., 1996), 3.0 mg/ml for *Alcaligenes xylosoxydans* chitinase (Vaidya *et al*., 2003) and *Bacillus* sp. WY22 chitinase (Woo and Park, 2003), 12 mg/ml for *Bacillus* sp. BG-11 chitinase (Bhushan and Hoondal, 1998).

Park *et al*. (1997) stated that the substrates like ethyl glycol chitin, glycol chitin and colloidia chitin are best substrates for endotype chitinase. The enzyme showed activities and substrate binding towards colloidal chitin, glycol chitin, swollen chitin, fish scale chitin, fish scales. But they exhibited no activity towards cellulose and starch. The activity with fish scale increases chitinase activity. Chitinase enzyme readily hydrolyzes colloidal chitin and glycol chitin as compared to crab shell chitin so the enzyme was classified as endo-chitinase. The endotype chitinase from *Enterobacter* sp. was able to hydrolysis the colloidal chitin, swollen chitin, flake chitin and glycol chitin at high rate (Dahiya *et al*., 2005).

**Table 1 Comparison of chitin substrate specificity with fish scales and their $K_m$ value, $V_{max}$ and $K_{cat}$**

| Substrate | $K_m$ (mg ml-1) | $V_{max}$ (µmole µg$^{-1}$h$^{-1}$) | $K_{cat}$ (µmole µg$^{-1}$min$^{-1}$) |
|---|---|---|---|
| **Colloidal chitin** | **1.42** | **111.11** | **52.9** |
| **Swollen chitin** | **1.51** | **89.9** | **44.5** |
| **Fish scales** | **1.38** | **99.89** | **49.45** |
| **Regenerated chitin** | **2** | **30.98** | **15.33** |
| **Glycol chitin** | **2.5** | **20.98** | **10.38** |

Colloidal chitin, as the substrate for purified chitinase gives the $K_m$ and $V_{max}$ values of 1.42 mg/ml and 111.11 µM µg$^{-1}$ h$^{-1}$ which was proved by Michaelis - Menten's curve and Lineweaver - Burk plot which is shown in the figure. Michaelis -Menten's ($K_m$) constant value analysis and maximum speed ($V_{max}$) was shown in plot results the qualitative relationship between speed of reaction and substrate saturation (fig 4.47). The chitinase enzyme had high affinity towards colloidal chitin, swollen chitin, fish scale, regenerated chitin and glycol chitin. This indicates that the chitinase form *P. putida* was highly specific to substrate. When the value of $K_m$ is very low the affinity towards chitin is very high. The $K_m$ value is low for fish scales and next to colloidal chitin so the chitinase which is isolated from *P. putida* is highly affinity towards them. The $K_{cat}$ Value was calculate for all the substrate and it was also shown the fig below. The $K_{cat}$ values for fish scales were found to be 49.45 µmole µg$^{-1}$min$^{-1}$, which was also compared with other substrate.

### 3.7. *In vitro* test – chitinase enzymes on antifungal activity

The effect of treatments applied on fungus growth, the quantity of colony forming units (CFU) was estimated by means of standard microbiological techniques. The results of control tests and assays with immobilized enzymes and fish scales were mixed with the enzymes were added to the soil with 5 U and 10 U of chitinase concentration. By comparing the CFU/g of soil detection in the in immobilized enzymes and immobilized enzyme mixed with fish scales waste.

The results revealed that the percentage inhibition of *F. oxysporum* was maximum, when treated with the mixture of fish scales and immobilized chitinase enzyme with the concentration of 10 U/ml. The test was performed with and without the presence of the fish scales. The antifungal activity was increased with the presence of fish scale waste. There was about 98 % of inhibition of fungal growth when enzyme mixture and fish waste was introduced. Moreover the immobilized enzyme increased the chitinase activity and stability of chitinase was also increased.

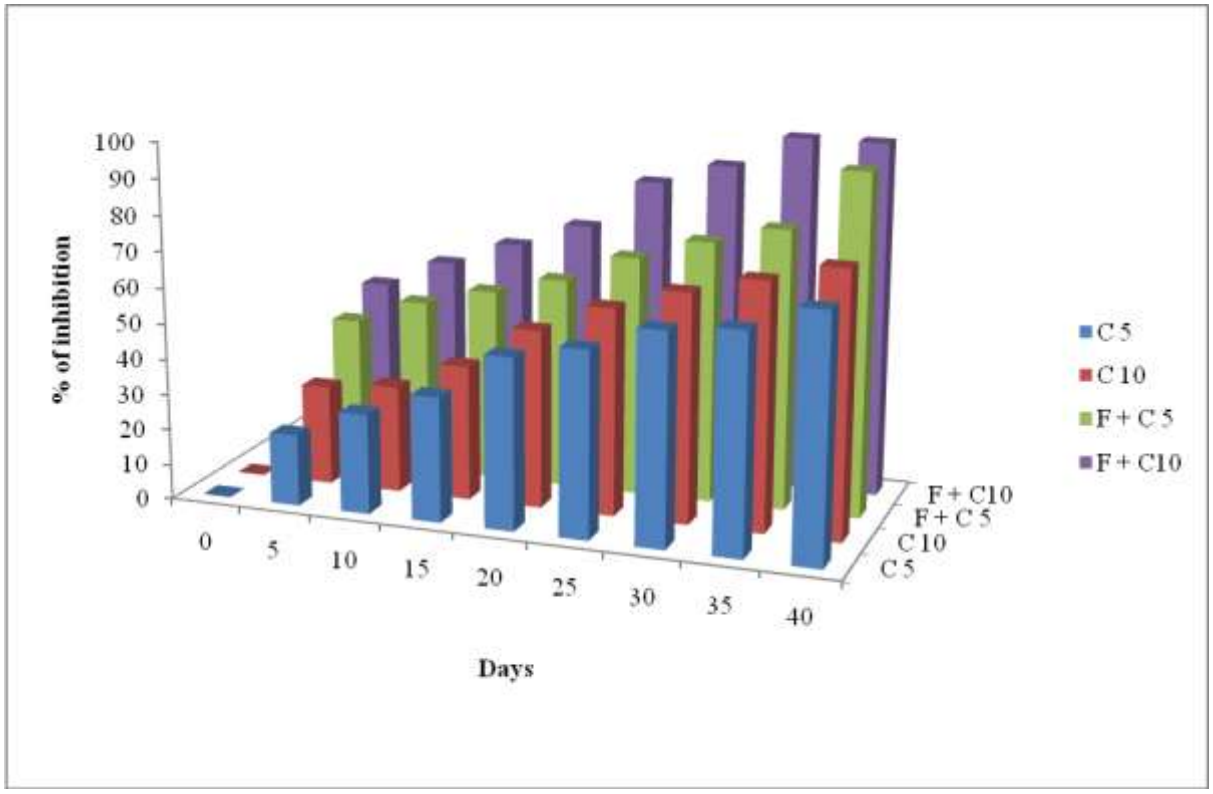



**Fig. 8 Soil test for antifungal activity. F is the fish scale waste. C 5 represents the chitinase enzymes at concentration 5 U/ml and C 10 represents the chitinase at concentration 10 U/ml**

Immobilized chitinase with fish scale waste protects the active site and inhibits the growth of the fungal cell wall. With the presence of the chitinase enzyme alone the inhibition of *F. oxysporum* was low when compared with the mixture of the fish scales. This reveals that the fish scale waste could be used for the enhanced and effective production of chitinase.

## CONCLUSION

Chitinase which was immobilized in chitosan beads which shows the highest activity and stability when compared to others. So the chitinase was immobilized in chitosan beads to study further activity.Stability profile of chitinase illustrated that the enzymatic activity was stable for 8 weeks of incubation by immobilization. Chitinase activity retains its activity to 60 % after incubation of 8 weeks. Chitinase enzyme devoid of immobilization was able to stable only for 2 weeks. Chitinase was active in the presence of the preservative sodium azide but the activity declines as the number on days when compared with immobilized enzyme. By immobilizing the chitinase with chitosan beads, stability of pH ranges from 4.0 - 7.0. It has a wide range of pH stability when it was immobilized. Even in alkaline conditions, the chitinase was found stable up to 60.86 % of its relative activity. A comparative study of chitinase and immobilized enzymes mixed with pesticide of different concentration. It was observed that the enzyme concentration was inversely proportional to the concentration of pesticide. At 100 µg/ml concentration of allosamidin, known specific inhibitor of chitinase inhibited chitinase from *P. putida* by 80 %. But when we used immobilized enzyme mixed at 100 µg/ml concentration of allosamidin by 30 %. So the rate of inhibition was reduced when enzyme was immobilized.

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The $K_m$ and $V_{max}$ values for *P. putida* were found to be 1.42 mg/ml and 111.11 μM $\mu g^{-1}$ $h^{-1}$, with colloidal chitin as the substrate. The activity with fish scale increases chitinase activity. Chitinase enzyme readily hydrolyzes colloidal chitin and glycol chitin as compared to fish scale chitin so the enzyme was classified as endo-chitinase. The chitinase enzyme has the potential to inhibit the growth of fungal mycelium was recorded for 12 days of incubation of the fungal and the percentage of the inhibition increases with the concentration. The percentage of inhibition was recorded higher on $9^{th}$ day of incubation with 8 U/ ml and 10 U/ ml of chitinase to digest the mycelium of fungi.

The effect of treatments applied on fungus growth, the quantity of colony forming units (CFU) was estimated by means of standard microbiological techniques. The results of control tests and assays with immobilized enzymes and fish scales were mixed with the enzymes were added to the soil with 5 U and 10 U of chitinase concentration. By comparing the CFU/g of soil detection in the in immobilized enzymes and immobilized enzyme mixed with fish scales waste. The results revealed that the percentage inhibition of *F. oxysporum* was maximum, when treated with the mixture of fish scales and immobilized chitinase enzyme with the concentration of 10 U/ml. The test was performed with and without the presence of the fish scales. The antifungal activity was increased with the presence of fish scale waste. There was about 98 % of inhibition of fungal growth when enzyme mixture and fish waste was introduced. Moreover the immobilized enzyme increased the chitinase activity and stability of chitinase was also increased.

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