International Journal of Environment, Agriculture and Biotechnology
Vol-7, Issue-1; Jan-Feb, 2022

Journal Home Page Available: https://ijeab.com/
Journal DOI: 10.22161/ijeab

Peer Reviewed

Optimization of fermentation conditions of chitosanase enzyme produced by *Aspergillus ornatus*

K. El-Sherbiny[1,2], A. Abdelhameed[1]

[1]Department of Botany and Microbiology, Faculty of Science, Zagazig University, Egypt.
[2]Department of biology, faculty of Science and Arts in baljurashi, Al-Baha University, Al-Baha, Saudi Arabia

Received: 19 Dec 2021; Received in revised form: 03 Feb 2022; Accepted: 10 Feb 2022; Available online: 19 Feb 2022

Abstract**—** *The potentiality of 28 fungal species belonging to 10 genera isolated and identified from Egyptian soils to produce chitosanase in their culture filtrates under submerged culture conditions using colloidal chitosan as the sole carbon source were tested. Aspergilli, particularly Aspergillusornatus were distinguished by its capacity to release exo-chitosanase when grown on a selected medium. Best results were achieved after on 7 days incubation at 30°C and pH 5.0. The impact of aeration, mechanical agitation as well as the volume and age of inoculum upon chitosanase and biomass production were also discussed briefly. The present paper gives an account of observations made on the production of chitosanase and biomass in relation to the chemical constituents of fermentation medium. 1% colloidal chitosan followed by crystal chitosan were more initiative for chitosanase production than any other carbon compounds. Peptone (0.45%) followed by yeast extract were the best nitrogen source for both biomass and chitosanase production. The optimum chitosanase and biomass production were achieved on medium containing 0.1% KH_2PO_4, 0.5% KCl and 0.5% $MgSO_4.7H_2O$. The impacts of the levels of glucose and NaCl on both experimental parameters were also examined. In addition, the supplementation of various B-group vitamin and some trace elements individually to the bioprocess caused no significant effects on chitosanase production. However slight inhibition was obtained with a mixture of tested metal ions. The productivity of chitosanase supported by addition of tween 80 as surfactant agent and enhanced on sand and soil extracts than other natural sources investigated.*

Keywords**— Chitosanase, Chitosan, Glucose amine, Enzyme essay, Fungi.**

I. INTRODUCTION

Chitosan, a linear copolymer composed of β 1, 4-linked glucosamine (GlcN) residues with various degrees of *N*-acetylated residues, is a deacetylated derivatives of chitin, an insoluble linear β 1, 4 linked polymer of *N*-acetylglucosamine (Glc.NAc) and is the most abundant polymer, next to cellulose in nature **(Zhu *et al.* 2003)**. Chitosan is present in the mycelial and sporangiophore walls of many fungi and the exoskeletons of insects and crustacean **(Kim *et al.* 2004)**.Actually, chitosan is applied widely to health food, such as for the treatment of hyperuricemia and as an antimicrobial agent, preservative agent and edible film **(Chen *et al.* 2005)**.Recently, much attention has been paid to converting chitosan to save and functional Chito oligosaccharides. These Chito oligosaccharides produced by hydrolyzing chitosan with chitosanase and have various physiological activities such as antitumer and antimicrobial activities as well as immuno-enhancing effects**(Qin *et al.* 2004 and Chen *et al.* 2005).** The productions of microbial chitosanases have received attention as a step in a proposed bioconversion process to produce low molecular weight chitosans and chitooligomers **(Liu and Xia, 2006)**.

Chitosanases (EC.3-2.1.132) are glycosyl hydrolases that catalyses the hydrolysis of β 1, 4 glycosidic bonds of chitosan and have been found in a variety of microorganisms, including bacteria and fungi **(Somashekar and Joseph, 1996, Yun *et al.* 2005 and**

Chen *et al.* 2006). Fungal chitosanolytic enzymes have been produced and characterized from different species of the following genera; *Aspergillus***(Kim *et al.* 1998, Cheng and Li, 2000, Zhang *et al.* 2000, Eom and Lee, 2003 and Chen *et al.* 2005)**, *Fusarium* **(Shimosaka*et al.* 1993)**, *Mucor***(Alfonso *et al.* 1992)**, *Paecilomyces***(Chen *et al.* 2005)**, *Penicillium***(Fenton and Eveleigh, 1981)** and *Trichoderma***(Nogawa*et al.* 1998)**.Also fermentation conditions of microbial chitosanase production have been reported **(Somashekar and Joseph, 1996, Zhang *et al.* 2000 and Zhu *et al.* 2003)**. It appears from the literature that chitosanase production by moulds received less attention than bacteria. Thus, the present study aimed to investigate the chitosanolytic activity of some Egyptian soil fungi as well as the optimal fermentation conditions leading to maximum yield of exo-chitosanase by the most active fungal isolate.

II. MATERIAL AND METHODS

Materials: Chitosan and glucosamine were purchased from Sigma (St-Louis, MO, USA). Commercial chitosan was prepared from shrimp chitin in our laboratory as described by **Hejazi and Amiji (2003).** Colloidal chitosan was prepared by the method of **Fen *et al.* (2006).**Folin reagent was purchased from LOBA chemie (Mumbai, India). All other chemicals used were of analytical grade.

Isolation and identification of chitosan degrading fungi: Different fungal isolates were isolated from soil as well as agriculture waste samples collected from Sharkia Governorate, Egypt (Table,1) .The dilution plate method essentially as described by **Johnson *et al.* (1959)** with some modification was employed for isolation of fungal species. Chitosan (1%) Czapek's Dox agar medium was used for chitosanase producing fungi **(Zhang *et al*. 2000)** to which were added both rosebengal (65 ppm) and dihydrostreptomycin (20 ug/ml) as bacteriostatic agents, **(Smith and Dawson, 1944)**, was used as an isolation medium . The plates were incubated at 30ºC for up to 10 days, during which the developing fungi were isolated. The pure fungal isolate were identified by consulting, **Barron (1968), Ellis (1971), Raper and Fennell (1977), Pitt (1979), Carmichael *et al.* (1980), Domsch*et al.* (1980), Nelson *et al.* (1983)** and other. The fungal cultures were maintained on potato dextrose (Difco) agar plates and incubated at 30ºC for 7 days **(Zhang *et al.* 2000).** The conidial suspension was prepared by suspending the conidia from the slants in 10 ml of sterilized distilled water.

Fermentation medium and culture conditions : The fermentation medium used for chitosanase production was modified Czapek- Dox's medium containing chitosan as carbon source **(Zhang *et al.* 2000)** ,with some modifications , composed of (g/L): 10.0 colloidal chitosan , 2.0 $NaNO_3$, 5.0 yeast extract, 1.0 KH_2PO_4 , 0.5 $MgSO_4.7H_2O$, 0.5 KCl, 0.01 $FeSO_4.7H_2O$ and 1000 ml distilled water . The pH adjusted to 5.0. The fermentation medium was dispensed in 250 ml conical flasks, each containing 50 ml. The flasks were inoculated with 1ml spore suspension of each tested fungal species. The submerged flasks were shaken at 3.7 H_Z and 30ºC for 7 days (using shaker incubator, ***New Brunswick Scientific, EdisonN.J.USA***).

Estimation of biomass production : Each fungal culture was filtered on Whatman No 1 filter paper and the pellets were washed several times , then dried at 90ºC till constant weight and the dry biomass was estimated in g/L of productive medium **(Shindia,1997)**.

Chitosanase assay: Assay of chitosanase activity was carried out as described by **Fenton and Elveleigh (1981)** as well as **Ushida and Ohtakara (1988)** with some modification. The culture filtrate was centrifuged at 5000 r.p.m for 10 min at 40ºC and the supernatants were used as crude enzyme. The standard assay mixture containing 1 ml of crude enzyme preparation and 1ml of 1% colloidal chitosan in citrate phosphate buffer (pH 5.0) was incubated at 40 ºC for 1h. The amount of glucosamine of the enzymatic reaction mixture was then determined using the Nelson-Somogi method **(Nelson, 1944)**. The intensity of the colored solution was quantified in spectrophotometric (Spekol- spectrocolorimeter) at 700 nm. The reducing sugar concentration produced in the reaction mixture was measured based on standard curve obtained with glucosamine as standard. One chitosanase unit (U) is defined as the amount of enzyme that liberates 1µ mol of reducing sugar per minute at 40ºC **(Zhang *et al.* 2000).**

Determination of protein: The protein content of the crude enzyme preparation was estimated colorimetrically according to the method adapted by **Lowery *et al.* (1951)**.

III. RESULTS AND DISCUSSION

Potentiality of production of chitosanase among test fungi: The aim of this experiment was to investigate the capacity of 28 fungal species to produce chitosanase in their culture filtrates. Twenty eight fungal species belonging to 10 genera were examined (Table, 1). The results show that the chitosanolytic activity among the fungal species of the same genus is quite different. Moreover, not all the fungal species possess the same chitosanolytic activity. *A. ornatus* was obviously the best producer of highest chitosanase activity followed by *A. ochraceous*, *P. citrinum*, *Trichoderma viride*, *A. fumigatus*, *P. chrysogenum*, *Paecilomycesvarioti* and *Trichoderma* sp. The fungal isolates with chitosanase activity below 25% of

that *A. ornatus* were considered low producers of enzyme and will not be considered for further discussion. Four fungal isolates showed no chitosanase activity (Table, 1). In connection with our screening study, several species particularly Aspergilli were listed by other investigators as chitosanase producers during their course of screening of fungal strain with chitosanase activity such as *A. oryzae*, *Aspergillus*sp J22-326, *A. fumigatus*, *Aspergillus*sp Y2K and *A. flavus* and others (**Zhang *et al.* 2000 and 2001, Cheng and Li, 2000, Eom*et al.* 2003, Liang *et al.* 2005, Chen *et al.* 2005).** The studies of other investigators **(Fenton and Eveleigh, 1981, Alfonso *et al.* 1992, Shimosaka*et al.* 1993, Nogawa*et al.* 1998 and Chen *et al.* 2005)** support our conclusions about chitosanolytic activities of the reported fungal strains. These activities that showed a wide range of variation and these natural differences proved the complementary action of these organisms towards each other in their living ecosystem. This may be attributed to the inherited differences in the biological activities of different fungal strains surviving single environmental niche. On the other hand, it is revealed that the most isolated fungal species in our screening have been recorded as chitosanolytic fungi by the aid of compendium of soil fungi **(Domsch*et al.* 1980).**

Aspergillus ornatus gave the highest chitosanase activity under bioprocess conditions. These findings justified the selection of *A. ornatus* for further experimentation in order to enhance its productive capability.

Time course of growth and chitosanase productivity of *Aspergillus ornatus*: The growth of *Aspergillus ornatus* and its production of chitosanases in culture filtrates were determined during the incubation period which prolonged for 14 days. The result in Fig (1) reveals a correlation between level of chitosanases production and extent of fermentation period. Negligible production of chitosanases was observed in the initial 48 h of fermentation process, though adequate growth was maintained under these conditions. Optimum chitosanase production was achieved after 7 days of fermentation. Beyond this period the chitosanase was found to drop gradually with further extension of the fermentation period to 14 days. The data also appeared that, no correlation was observed between the released chitosanase in culture filtrates and the biomass of *Aspergillusornatus*. Optimum biomass was obtained after 8 days of fermentation periods, above which the fungal biomass production dropped. The late appearance of chitosanases could be the result of induction as colloidal chitosan eventually becomes available after consumption of proteins. Previous investigators found that an incubation period from 4 to 5 days was optimal for chitosanase production by some *Aspergillus*sp (**Cheng and Li, 2000 and Chen *et al.* 2005**) and *Bacillus*sp**(Choi *et al.* 2004)**. On the other hand, cultures of *Aspergillus Oryzae* showed the highest chitosanase and growth rate after 60 hours of incubation **(Zhang *et al.* 2000)** .These differences in optimum production of chitosanases by different fungal and bacterial species may ascribed to either the condition of cultivation or special differences.

Table.1: Chitosanolytic activity and protein production of different fungal isolates grown on chitosan as carbon source at 30°C.

No.	Fungal isolate	Chitosanase activity (U/ml)	Protein (mg/ml)
1	*Aspergillus awamori*	1.11	0.15
2	*A. carbonarius*	1.28	0.17
3	*A. carneus*	1.95	0.2
4	*A. flavus*	3.21	0.31
5	*A. fumigatus*	2.36	0.26
6	*A. niger*	1.17	0.15
7	*A. ochraceous*	2.93	0.22
8	*A. ornatus*	4.46	0.4
9	*A. restrictus*	1.26	0.17
10	*A. tamari*	3.11	0.31
11	*A. terreus*	2.06	0.21

12	*Chaetomium globosum*	-	0.05
13	*Chaetomium sp*	-	0.10
14	*Penicillium chrisogenum*	2.34	0.23
15	*P. citrinum*	2.68	0.26
16	*P. elegans*	1.70	0.19
17	*P. funiclulosum*	2.0	0.21
18	*Cladosporium cladosporioids*	-	0.08
19	*Fusarium moniliforme*	1.66	0.18
20	*F. oxysporum*	1.95	0.2
21	*Paecilomycesvarioti*	2.31	0.22
22	*Trichoderma konigii*	2.10	0.21
23	*Trichoderma viridie*	2.50	0.24
24	*Trichoderma*sp	2.23	0.22
25	*Cunninghamellaechinulata*	-	0.06
26	*Mucor circinoloids*	0.5	0.1
27	*M. racemosus*	0.7	0.11
28	*Rhizopus oryzae*	0.1	0.09

Effect of initial pH value: Chitosanase and dry biomass profile with respect of initial pH of the fermentation medium is shown in Fig (2). It is evident that, the growth of *Aspergillusornatus* and its ability to produce chitosanase respond differently to the reaction of the basal medium. The results revealed that an initial pH of 5.0 was found to be optimal for both growth of *Aspergillusornatus* and chitosanase production. Above and below this pH value the yield of biomass and chitosanase was substantially lower. It is also clear from the data that, the experimental fungus has the ability to survive and release extracellular chitosanase in its culture filtrate at wide range of pHs value. These observations agree with the findings of **Alfonso *et al.* (1992)** who found that the pH 5.0 was the best for growth and chitosanases production by *Mucor rouxii*. The most of fungal chitosanases showed optimum productivity close to 5.0 **(Zhang *et al.* 2000 and Chen *et al.* 2005).**

Effect of incubation temperature: The incubation temperature has a significant influence on both chitosanase production and growth of *A. ornatus* (Fig, 3). The optimum temperature for efficient fermentation was found to be 30º C. Above and below this temperature the biomass and chitosanase production dropped. It is also found that the tested organism failed completely to develop mycelia and hence no chitosanase activity at 50ºC. Therefore, all further optimizing efforts using *A. ornatus* were tried at 30ºC which seems to be the optimum for chitosanase production and dry biomass of tested fungus. In accordance with these findings are those obtained by **Somashekar and Joseph (1992), Tanabe *et al.* (2003), Choi *et al.* (2004) and Chen *et al.* (2005)** who reported that 30ºC was optimal for the growth and chitosanase production by different microorganisms.

Effect of aeration rate: Data showing the influence of aeration rate (volume of fermentation medium/volume of fermentation flask) on biomass yield and chitosanase production by *A. ornatus* are presented in Fig (4). Under our experimental conditions, 50 ml of fermentation medium in 250 ml flask (1/5 v/v) allowed optimal dry biomass output and extracellular chitosanase biosynthesis. This may be due the compromises between mass transfer and shearing stress at this volume. Further increase in the volume of fermentation medium (decreased O_2 levels) causes gradual decrease in biomass production as well as extracellular productivity. As the volume of fermentation medium increased, the shearing stress may be increased and causal hazardous effect on fungal pellets **(Liu *et al.* 2003).** These observations are in line with that previously reported **(Tanabe *et al.* 2003 and Kim *et al.* 2004).**

Effect of agitation rate: From the result in the Fig (5), it can be noticed that the growth of *A. ornatus* was induced with agitation speed compared to static culture

and gave the maximum biomass at 150 rpm (5.0 g/L) after which gradually decreased were obtained up to 300 rpm . At the same agitation rate (150 r.p.m) the optimum chitosanase activity was also recorded by tested organism and dropped thereafter. Generally, the major roles of providing agitation rate were in improving the mixing, mass and heat transfer in submerged bioprocess. This is compatible with the findings previously concluded by **Yoon *et al.* (2001)** and **Chen *et al.* (2005)** for optimal chitosanases production by *B.* sp CK4 and *A.* sp CJ22-326 respectively. However, at higher agitation rates the biomass and chitosanase production by tested fungus decreased. This may be due to over increasing in mass and heat transfer or/and may have negative effects on morphological states such as rupture cells, vaculation and outlysis as well as changes in fungal pellets **(Cui *et al.* 1997) .**

Effect of the amount and the age of the inoculum: The strength ofthe inoculum and age distribution of mycelium is known to impact the growth rate as well as the enzymatic activities of fungi grown under special conditions (**Gottleib and van Etten, 1965)**. The aging of inoculum used to inoculate fermentation medium, (Fig, 6) exerted changes in biomass and chitosanase production. The optimum yields of both parameters were obtained by using 8 day-old inoculum of *A. ornatus*. On the other hand, chitosanase activity of this fungus was not influenced greatly by the size of inoculum in the range from 2 to 10% (Fig, 6). Also, the yield of biomass was not influenced appreciably within the range of 4 to 8% of inoculum but above and below this range a marked decline in production of biomass occurred. It seemed probable that certain substances present in large amounts of inocula may tend to inhibit the growth of microorganism. These result coincide with these previously reported for different fungal species and their chitosanolytic activity **(Zhang *et al.* 2000 and 2001, Zheng and Xiao, 2004)**.

Effect of different carbon sources: As shown in Table (2), the relation between the carbon substrates and extracellular chitosanase production as well as growth rate of the experimental organism were investigated. The data indicated that the optimum exochitosanase levels were detected as *A. ornatus* was grown on media containing chitosan, but not with any other tested carbon compounds. Colloidal chitosan induced the best chitosanase productivity of tested organism followed by crystalline chitosan and fungal chitosan. While, cell free filtrate of culture growth with non chitosan substrates were devoid of this activity. On the other hand, the monomer glucose and glucoseamine supported fungal growth, but chitosanase production was not detectable in culture filtrates of tested fungus.

Table.2: Effect of different carbon sources on growth and chitosanase production by Aspergillus ornatus:

Carbon source	Chitosanase activity (U/ml)	Dry wt. (g/L)
Glucose	-	12.4±0.03
Fructose	-	7.8±1.03
Mannose	-	7.6±1.1
Xylose	-	8.6±0.68
Glucoseamine	-	11±1.1
Sucrose	-	8.5±2.0
Lactose	-	6±1.1
Maltose	-	10±1.0
CMC	-	2.1±1.0
Cellulose	-	0.94±0.76
Pectin	-	6.1±0.05
Starch	-	6.9±0.08
Crystalline chitin	-	1.3±0.006
Colloidal chitin	-	3.9±0.21
Crystalline chitosan	2.2±0.1	2.2±0.07
lloidal chitosan	4.46±1.8	5.0± 0.13
Fungal chitosan	1.6±1.0	3.2±0. 5

The selectivity in action of carbon sources led us and others **(Kim *et al.* 1998 and Chen *et al.* 2005)** to conclude that the apparent increased represence of the enzymes to the carbon sources was an inducible one. The preference in the usage of one carbon source by different chitosanolytic microorganisms was reported by several investigators **(Mitsutomi*et al.* 1998, Zhu *et al.* 2003, Kim *et al.* 2004 and Chen *et al.* 2006)**. For example, **Cheng and Li (2000)** indicated that high chitosanase production was found only in culture of *A.* sp Y2K supplied with soluble chitosan. The highest production of enzyme with colloidal chitosan may be related to greater accessibility to enzymatic attack probably resulted from the largest surface area of solubilized chitosan. Like other inducible enzyme systems chitosanase formation can be repressed by excess soluble metabolites in the presence of inducer **(Davis and Eveleigh, 1984 and Tanabe *et al.* 2003)**. The induction of *A. ornatus* chitosanase, in the present study is repressed by addition of different concentrations of glucose (at zero time) to the chitosan basal medium as shown in Fig (7). These results suggest that *A. ornatus* chitosanase is controlled by an inducer repressor system. These finding

were correlate well with the previous observations of **Kim *et al.* (1998) and Chen *et al.* (2005)**. In the presence of adequate concentration of easily metabolites monosaccharides, a number of catabolite pathways involved in hydrolysis of polysaccharides are repressed **(Atlas, 1984 and Angell *et al.* 1992)**. In contrast to these inducible enzymes, chitosanase which are produced constitutively are also reported for different microorganism **(Alfonso *et al.* 1992 and Somashekar and Joseph, 1992)**.

The results in Fig (8) show that the excretion of chitosanase and growth of *A. ornatus* were not only affected by the kind of carbon source supplied but also were sensitive to the concentration of the specific carbon used (colloidal chitosan). It was found that 1.0% of colloidal chitosan gave the maximum production of enzyme while the lowest productivity was recorded at 3.0% chitosan. It was reported that the yield of chitosanase depend on microorganism as well as nature of chitosan and its levels **(Somashekar and Joseph, 1996)**. For maximum chitosanase productivity, the optimal concentration of colloidal chitosan was 1.0% for different microorganisms **(Zhu *et al.* 2003 and Chen *et al.* 2005)**.

Effect of different nitrogen sources: The impact of a range of nitrogen sources on chitosanase productivity and growth rate of *A. ornatus* was shown in Table (3). All 8 nitrogenous tested compounds were able to support growth; the range in variation in final biomass yield was only 3.63 g/L except for urea nitrogen containing media, in which it is reduced about 7 fold from 5.8 to 0.78 g/L of biomass. This may be due to its toxic effect in high doses used or/and the pH variations occurring after the addition of it **(Reid, 1983)**. The final extracellular chitosanase production varied considerably, however, maximum production of enzyme was evident in presence of peptone followed by yeast extract.

Table.3: Effect of different nitrogen sources on growth and chitosanase production by Aspergillus ornatus:

Nitrogen source	Chitosanase activity (U/ml)	Dry wt. (g/L)
Without	1.9± 3.4	0.75± 0.06
Control*	4.46±1.8	5.0± 0.13
$NaNO_3$	2.1± 1.5	2.17± 0.09
NH_4Cl	2.3± 0.2	2.3± 0.006
KNO_3	2.76± 2.8	2.96± 0.02
$(NH_4)NO_3$	3.2± 2.1	4.4± 0.12
$(NH_4)_2SO_4$	2.2 ±0.0	2.26± 0.14
Urea	1.9 ± 0.0	0.78±0.08
Peptone	4.47± 1.9	5.8± 0.24
Yeast extract	4.13± 0.43	5.65± 0.14

*Mixture of yeast extract (0.5%) and $NaNO_3$ (0.3%).

Minimum chitosanase production was obtained only in the presence of urea. Other tested inorganic and organic nitrogen compounds were favourable for chitosanase production by tested organism but, comparatively less inducible than control. It is interest to note that, in absence of exogenous supply of nitrogen compound, the biomass and chitosanase productivity of *A. ornatus* posed low yield, indicating that, it is able to utilize the colloidal chitosan as carbon and nitrogen sources. These observations confirmed the early reports that certain microorganisms produced large amounts of extracellular chitosanases in the presence of complex organic nitrogen such as peptone and yeast extract **(Mitsutomi*et al.* 1998 and Yoon *et al.* 2001)**.The prominent effect of peptone may be attributed to the fact that such complex organic nitrogen gives on hydrolysis a number of some intermediate compounds structurally available as precursors of enzymes biosynthesis as well as major, minor element, and growth factors that may be used for inducing growth and biosynthesis of enzymes **(Anonymous, 1958)**. Also, the preferability of one nitrogen source by chitosan degrading microorganisms has been reported **(Fenton and Eveleigh, 1981. Cheng and Li, 2000, Zhang *et al.* 2000 and Kim *et al.* 2004)** and their findings were in connection with our results.

Different levels of peptone (0.25-0.5%) as the best nitrogen source were tested (Fig, 9). The best levels of peptone for maximum extracellular chitosanase release and biomass production by tested organism were found to be 0.45%. Above and below this optimal concentration of nitrogen source, the biomass and chitosanase productivity almost reduced. Others indicated that both the nature of nitrogen source and its level in bioprocess are important in controlling fungal development and biosynthesis of enzymes **(Shindia*et al.* 2001)**. Also, similar results have been reported by several workers connecting the nitrogen concentration dependence of both chitosanase and biomass of microorganisms **(Zhang *et al.* 2000 and Chen *et al.* 2005).**

The effects of different levels of essential salts in culture medium ($MgSO_4.7H_2O$, KH_2PO_4 and KCl), one at a time, were also investigated (Fig, 10).

It well known that phosphorous especially KH_2PO_4 plays an important roles in fungal cell metabolism

particularly utilization and metabolism of carbohydrates **(Jennings, 1995)**. The highest chitosanase activity (4.74 u/ml) was obtained with 0.1 g/L of KH_2PO_4 (Fig, 10) as source of phosphate in bioprocess with more than two-fold increase than the phosphate depleted medium. Further increase in phosphate levels supply led to a decrease in both chitosanase and biomass production by experimental organism. Other investigators reported different optimal levels according to the kind of phosphorus source as well as their tested microorganisms **(Fenton and Eveleigh, 1981, Cheng and Li, 2000, Zhu *et al.* 2003 and Chen *et al.* 2005)**. Also, **Zhang *et al.* (2000)and Tanabe *et al.* (2003)** found that, the maximum chitosanase production by *A. oryzae*IAM 2660 and *Streptomyces griseus* HUT 6037 were obtained in the presence of 0.1% KH_2PO_4 in fermentation media.

The vital importance of Mg^{+2} ions and other bivalent ions as growth factors had been discussed by **Jennings (1995)**. The optimal levels of $MgSO_4.7H_2O$ supporting the highest chitosanase activity and biomass yield dropped markedly with higher salt levels. Others reported that, the optimal Mg^{2+} ions concentration employed varied with the different organism being 0.5g/L for *A. oryzae*IAM 2660 and *A.* sp CJ22-326 **(Zhang *et al.* 2000 and Chen *et al.* 2005)** and 0.7 g/L for *P. islandicum***(Fenton and Eveleigh, 1981)**.

Similarly the presence of KCl in bioprocess exerted a significant impact on chitosanase activity and biomass production (Fig, 10). The presence of 0.5g/L KCl maximum chitosanase and mycelial dry weight production by *A. ornatus* were recorded with an increase of abut 3.7-fold compared to the medium omission of the salt. Similar results have been previously recorded by **Zhang *et al.* (2000)** who found that, the suitable amount of *A. oryzae* IAM 2660 chitosanase was achieved in the presence of 0.5 g/L KCl in bioprocess.

Trace elements have been shown to exert a low impact on chitosanase production as well as on growth of tested organism, in general, as individually added (Fig, 11). No significant increase in chitosanase and biomass production was observed by the addition of $CoCl_2$ and $FeSO_4.7H_2O$ to the fermentation medium. However, a remarkable inhibition in both fermentation parameters were obtained by the addition of mixture of tested heavy metals and $ZnSO_4.7H_2O$ and ZnCl salts. These results partially were in agreement with those obtained by **Zhang *et al.* (2000) and Kim *et al.* (2004).** The support of some heavy metals ions in the enzyme yield and growth of microorganisms is either related to the actual increase in the enzyme yield or simply to enhancement of enzyme activity **Harper *et al.* (1977).**

Generally, fungi need only water soluble vitamins of B-complex series **(Bilgrami andVerma, 1981)**. Thus, the impact of some vitamins on the production of chitosanase and growth of *A. ornatus* were investigated as shown in Table (4). No significant increase in enzyme production was observed by the addition of each vitamin individually to the fermentation medium. Both chitosanases and biomass production are ineffective by B_2 but retarded by B_{12}, thiamin and B_6 in fermentation media. These findings are in connection with those previously reported for different enzymes **(Shindia*et al.* 2001)**.

The results (Fig, 12) revealed that, the chitosanase and biomasss production by *A. ornatus* gradually decrease with an increasing the levels of NaCl up to 0.5%. This is not compatible with the results reported by **Cheng and Li, (2000)** who found that 0.05% NaCl enhanced the productivity of *A.* sp Y2K chitosanases.

Supplementing the bioprocess with individual surface agents exerted different effects on both chitosanase and biomass production by tested organism (Table, 5). Addition of 2.0% tween 80 supported fair amount of chitosanase and biomass output, however, Tween 40 retarded the two fermentation parameters. Tween 80 stimulates enzyme production, release of enzyme and enhancement of the O_2 supply to the fungal cells as discussed by **Perdih and Lestan, (1993)**. Similarly, **Kim *et al.* (2004)** found that the addition of tween 80 enhanced the release of chitosanase in fermentation media.

The experiments were extended to investigate the impact of natural products on the chitosanase and biomass production by tested fungus (Table, 6). The results of this study showed that sand and soil extracts incorporated separately, exhibited comparable values of both chitosanase and biomass production as control. However, malt extract recorded a general lower productivity of both parameters. The superiority of sand and soil extracts might attribute to the availability of appreciable amounts of soluble salts as well as oligomers that induce enzymes and growth of microorganisms **(Sabry *et al.* 1992)**.

Finally, from the above results, it could be concluded that, *A. ornatus* proved to be the most active fungus for chitosanase production and its productivity affected by environmental and nutritional conditions of the culture media.

REFERENCES

[1] **Alfonso, C., Martinesz, M.J and reyes, F (1992)**. Purification and properties of two endochitosanases from *Mucor rouxii* implicated in its cell wall degradation. FEMS Microbiol. Lett. 95:187-194.

[2] **Angell, S.; Schwarz, E. and Bibb, M.J. (1992):** The glucose kinase gene of *Streptomyces coelicolor*A3 (2): its nucleotide sequence, Transcriptional analysis and role in glucose repression. Mol. Microbiol., 6: 2833-2844.

[3] **Anonymous, (1958):** Ingredients of culture medium in (Difco Manual) ،9th ed Difco laboratories Inc., Detroit M. I.

[4] Atlas, R.M. (1984): DNA, RNA and protein synthesis; the replication and repression of genetic information. In: Microbiology fundamentals and Application. Mac Millon Publ. Comp., New York. USA.

[5] **Barron, G.L. (1968):** The genera of hyphomycetes from Soil. Williams and Wilkins, Baltimore USA.

[6] Bilgrami, K.S. and Verma, R.N. (1981): Physiology of fungi. 2, nd eds., Vikas Publishing. PVT., Ltd. Indian, P. 23-27.

[7] **Carmichael, J.W.; Brycekendrick, W.; Conners, I.L. and Siglar, L. (1980):** Genera of Hyphomycetes. The Univ. of Alberta Press Edmonton, Alberta, Canada.

[8] **Chen, X. Wenshui, X. and Xiaobin, Y. (2005).** Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326.Food Research International, 38 : 315-322.

[9] **Chen, Y-L, Su, C. and Chiang, B.H (2006).** Optimization of reversed micellar extraction of chitosanases produced by *Bacillus cereus*. Process Biochemistry. 41:752-758.

[10] **Chen, Y-Y., Cheng, C-Y., Haung, T-L., Li and Yaw-Kuen. (2005).** A chitosanase from *Paecilomyceslilacinus* with binding affinity for specific chito-oligosaccharides. Biotechnol and Appl. Biochem. 41 (2): 145-150.

[11] **Cheng, C.Y. and Li, Y.K. (2000).** An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. Biotechnol. Appl. Biochem. 32(3): 197–203.

[12] **Choi, Y.J., Kim, E.J.; Piao, z., Yun, Y.C. and Shin, Y.C. (2004).** Purification and characterization of chitosanase from *Bacillus*sp strain KCTC0377BP and its application for the production of chitosanase oligosaccharides . Appl. and Environm. Microbiol. 70 (8):4522-4531.

[13] **Cui, Y.Q.; Vander Lans, R.G.J. M. and Luyben, K.C.A. (1997):** Effect of agitation intensities on fungal morphology of submerged fermentation. Biotechnol. Bioeng., 55: 715-726.

[14] **Davis, B. and Eveleigh, D. E. (1984).**Chitosanases: Occurance, production and immobilization. In, chitin, chitosan and related enzymes, ed. J. P. Zikzkiz. Acad. Press, F. L. pp 161-179.

[15] **Domsch, K.H.; Gams, W. and Anderson, T. (1980):** Compendium of soil fungi. Academic Press.

[16] Ellis, M.B. (1971): Dematiaceous Hyphomycetes. C.M.I., Kew, Surrey, England.

[17] **Eom, T. K., and Lee, K. M. (2003).** Characteristics of chitosanases from *Aspergillus fumigatus* KB-1. Arch. Pharm. Res. 26**:**1036–1041.

[18] **Fen, L. L., Illias, R, M.Kamaruddin, k, Maskat, M.Y and Hassan, O. (2006)**. Development of rapid screening method for low-yielding chitosanase activity using Remazol Brilliant Blue-chitosan as substrate. Enzy. Microbiol. Technol. 38:215-219.

[19] **Fenton, D.M. and Eveleigh, D.E. (1981).** Purification and mode of action of a chitosanase from *Penicillium islandicum*. J. Gen. Microbiol. 126, 151–165.

[20] **Gottleib, D. and van Etten, J.L. (1965).** Changes in fungi with age. J. Bacteriol. 91: 161-168.

[21] **Harper, H. A, Rodwel, V. W and Mayes, P. A. (1977).** Review of physiological chemistry 16th ed Lange Medical Publ. p 66.

[22] Hejazi, R. and M. Amiji, (2003). Chitosan-based gastrointestinal delivery systems, J. Control. Release. 89: 151–165.

[23] **Jennings, D. H. (1995).** The physiology of fungal nutrition. 1, est ed. CambridgeUniv. Press PP 251.

[24] **Johnson, L.E.; Curl, E.A.; Bond, J.H. and Fribourg, H.A. (1959):** Methods for studying soil microflora-plant diseases relationship. Burgess Publ. Co. Minn, U.S.A.

[25] **Kim P.I, Kang, T. H. Chung, K. J. Kim, I.S. Chung, K.C. (2004).** Purification of a constitutive chitosanase produced by *Bacillus* sp. MET 1299 with cloning and expression of the gene.FEMS Microbiol. Lett. 240: 31–39.

[26] **Kim, S.Y., Shon, D. H.and Lee, K. H. (1998).** Purification and characteristics of two types of chitosanase from *Aspergillus fumigatus* KH-94. J. Microbiol. Biotechnol. 8: 568-574.

[27] Liang, D. C, Z., Ai-J, Zhang, G. and Jing. Y. **(2005)**. Cloning and expression of an *Aspergillus fumigatus*chitosanase gene. Acta MicrobiologicaSinica 45 (4): 539-542

[28] **Liu, B.L.; Kao, P.M.; Tzeng, Y. M. and Feng, K.C. (2003):** Production of chitinase from *Verticillium lecanii FO91* using submerged fermentation. Enzyme and Microbial Technology, 33: 410-415.

[29] **Liu, J. and Xia, W. (2006).** Purification and characterization of bifunctional enzyme with chitosanase and cellulose activity from commercial cellulase. Biochem. Eng. J. 30: 82-87.

[30] Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 269-275**.**

[31] Mitsutomi, M., Isono, M., Uchiyama, A., Nakido, N., Ikegami, T. and Watanabe, T. (1998). A chitosanase activity of the enzyme previously reported as β-1, 3- 1, 4-glucanase from *Bacillus circulans* WL-12. Biosci. Bitechnol. Biochem. 62: 2107-2114.

[32] **Nelson, E.P., Toussoun, T.A. and Marasas, W.F.O. (1983).***Fusarium* species, an illustrated manual for identification. The Pennsylvania state univ. U.S .A.

[33] **Nelson, W. (1944).** A photometric adaptation of the Somogyi method for determination of the glucose . J. Biol. Chem. 153:375-380.

[34] **Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H. and Morikawa, Y. (1998)** Purification and characterization of exo-*β*-D-glucosaminidase from a cellulolytic fungus, *Trichoderma reesei*PC-3-7.Appl. Environ. Microbiol. 64: 890–895.

[35] **Perdith, A. and Lestan, D. (1993).** The role of surfactants in enhancements of ligninolytic activity in *Phanerocheatachrysosporium*. Acta chem.. Solv. 40: 29-35.

[36] **Pitt, J.L. (1979):** The genus *Penicillium* and its teleomorphic states *Eupenicilliumand Talaromyces.* Academic Press, Inc. (London) LTD.

[37] **Qin, C.Q. Zhou, B. and Zeng, L.T. (2004)**. The physicochemical properties and antitumor activity of cellulose-treated chitosan, Food. Chem. 84: 107–115.

[38] **Raper, K.B. and Fennell, D.I. (1977):** The genus *Aspergillus.* The Williams and Wilkins company. Baltimore. USA.

[39] **Reid, I. (1983):** Effect of nitrogen supplements on degradation of aspen wood lignin and carbohydrate components by *Phanerochaetechrysosporium*. Appl. and Environm. Microbiol., 45: 830-837.

[40] **Sabry, S. A., Ghanem, K. M and Ghanem, N. B. (1992).** Some factors influencing chitosanase production by *Bacillus amyloliquefacienes.* Bull. Fac. Sci. Alex. Univ. 32(A):240-248.

[41] **Shimosaka, M., Nogawa, M., Ohno, Y. and Okazaki, M. (1993) .**Chitosanase from the plant pathogenic fungus, *Fusarium solani* f. sp. *phaseoli*: purification and some properties. Biosci. Biotechnol. Biochem. 57, 231–235.

[42] **Shindia, A.A.; Abou-Hawa, M.I. and Shalaby, K.S.M. (2001):** Fungal degradation of shrimp shell chitin waste 1-effect of culture conditions on the production of chitinase by a potent *Trichoderma viride*. Egyptian J. of Microbiol., 36 (2): 119-34.

[43] Shindia, A.A. (1997). Mevinolin production by some fungi . Folia Microbiol. 42(5):477-480**.**

[44] **Smith, N.R. and Dawson, V.T. (1944):** The bacteriostatic action of rosebengal in media used for plate counts of soil fungi. Soil. Sci., 58: 467-471.

[45] **Somashekar, D. and Joseph, R. (1996).** Chitosanases-properties and applications. Bioresource Technol. 55, 35–45.

[46] **Somashekar, D. and Joseph, R. (1992).** Partial purification and properties of novel chitosanase secreted by*Rhodotorulagracilis*. Lett .app .microbiol, 14: 1-4.

[47] **Tanabe, T., Moringa, K., Fukamizo, T. and Mitsutomi, M. (2003)**. Novel chitosanase from *Streptomyces giseus* Hut 6037 with transglycosylation activity. Biosci. Biotechnol. Biochem. 67(2):354-364.

[48] Uchida Y and Ohtakara A. (1988). Chitosanase from *Bacillus* species. In: WoodWA, Kellogg ST, eds. Methods in enzymology, Vol. 161. New York: Academic Press;. p. 501–505.

[49] Yoon, H.-G., Kim, H.-Y., Lim, Y.-H., Kim, H.-K., Shin, D.-H., Hong, B.-S. and Cho, H.-Y. (2001). Identification of essential amino acid residues for catalytic activityand thermostability of novel chitosanase by site-directed mutagenesisAppl Microbiol Biotechnol 56: 173–180**.**

[50] **Yun, C. S., Amakata, D., Matsuo, Y., Matsuda, H. and Kawamukai, M. (2005).** New chitosan-degrading strains that produce chitosanase similar to ChoA of *Mitsuariachitosanitabdia*. Appl. Environ. Microbiol. 71: 5138-5144.

[51] Zhang, X.Y., Dai, A.L., Zhang, X.K., Kuroiwa, K. Kodaira, R., Shimosaka, M. and Okazaki, M. (2000). Purification and characterization of chitosanase and Exo-*β*-D-glucoseaminidase from a koji mold, *Aspergillus oryza*IAM2660. Biosci.Biotechnol.Biochem.64 (9): 1896-1902**.**

[52] Zhang, X.Y., Dai, A.L., Zhang, X.K., Kuroiwa, K. Kodaira, R., Shimosaka, M. and Okazaki, M. (2001). Cloning and characterization of a chitosanase gene from the koji mold *Aspergillus oryzae*Strain IAM2660. Biosci.Biotechnol.Biochem.65 (4): 977-981**.**

[53] **Zheng, L.Y. and Xiao, Y. L. (2004).***Penicillium* sp ZD-ZI chitosanase immobilized on DEAE cellulose by cross-linking reaction. Korean J. Chem. Eng. 21 (1): 201-205.

[54] **Zhu, X. F., Wu, X.-Y and Dai, Y. (2003)**. Fermentation conditions and properties of chitosanase from *Acinetobacter* sp. C-17. Biosci. Biotechnol. Biochem. 67 (2):284-290.

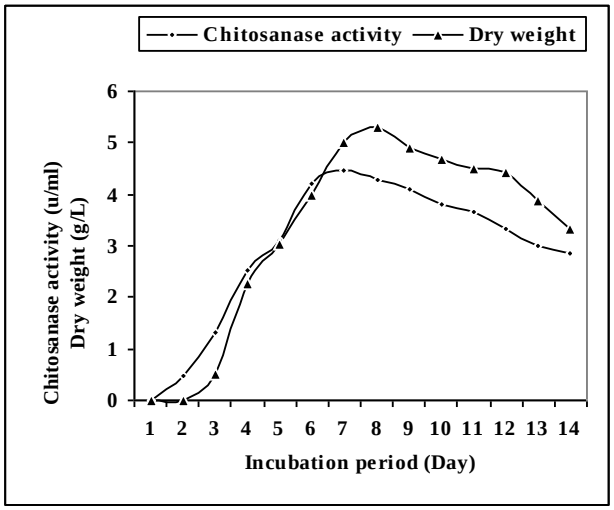

Fig.1:Time course of growth and chitosanase productivity of A. ornatus:

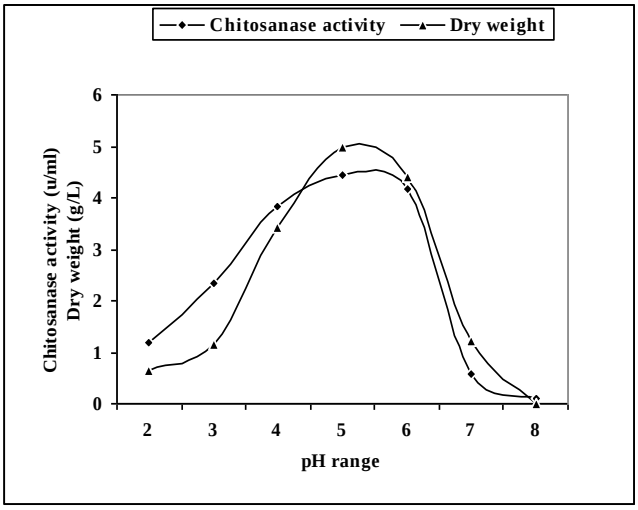

Fig.2: Effect of initial pH-value on growth and chitosanase production by A. ornatus.

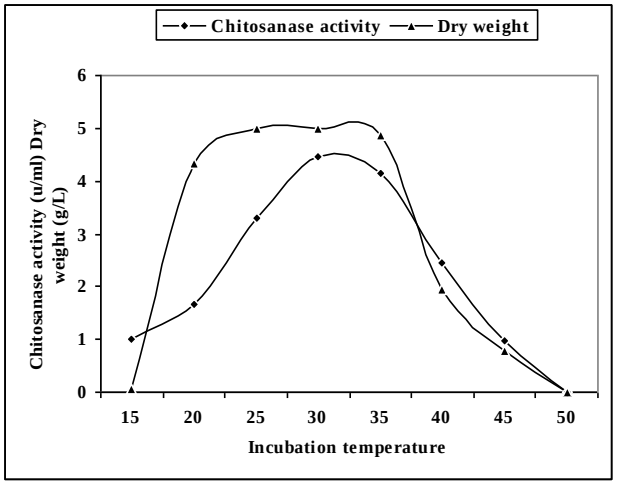

Fig.3: Effect of incubation temperature on growth and chitosanase production by A. ornatus.

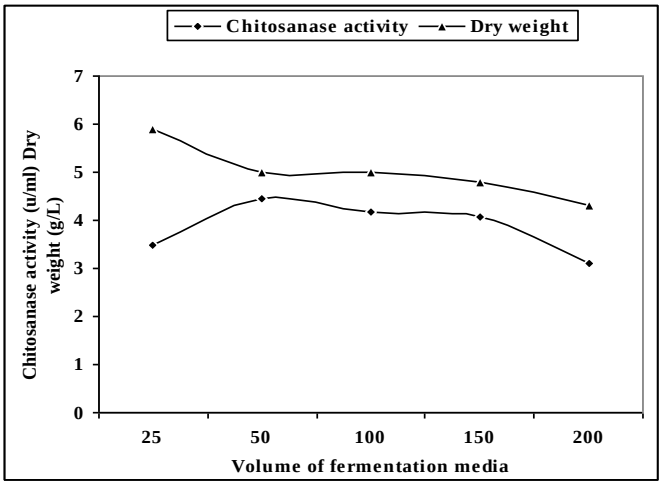

Fig.4: Effect of aeration rate on growth and chitosanase production by A. ornatus

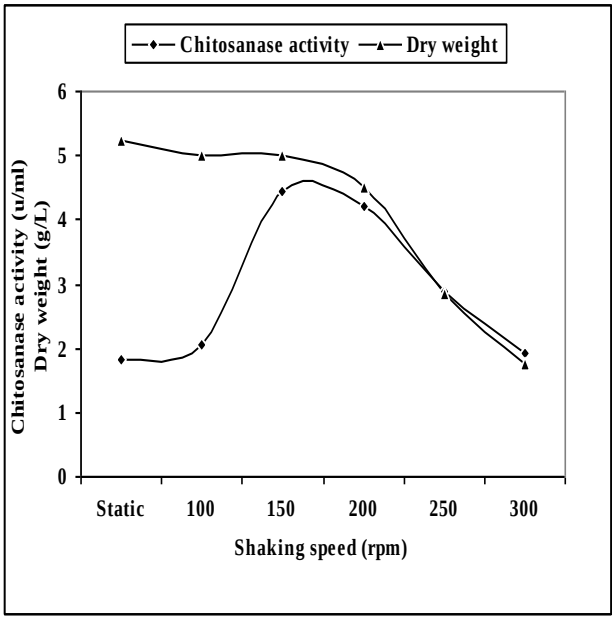

Fig.5: Effect of agitation rate on growth and chitosanase production by A. ornatus.

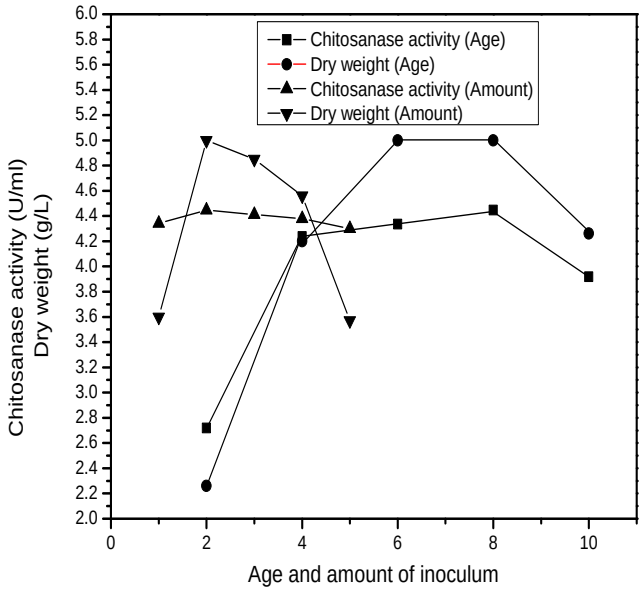

Fig.6: Effect of the age and amount of inoculums on growth and chitosanase production by A. ornatus

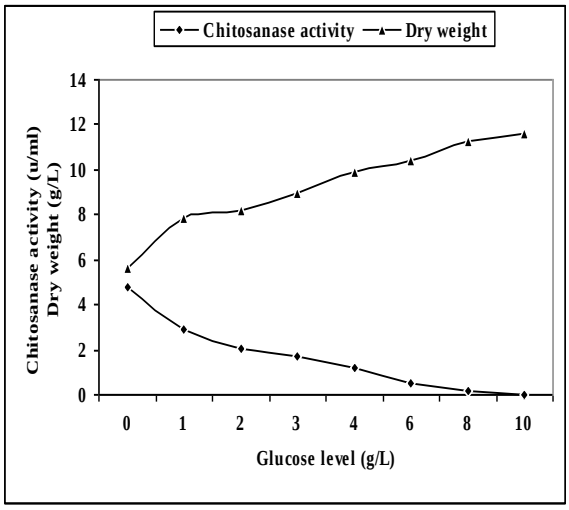

Fig.7: Effect of glucose levels on growth and chitosanase production by A. ornatus.

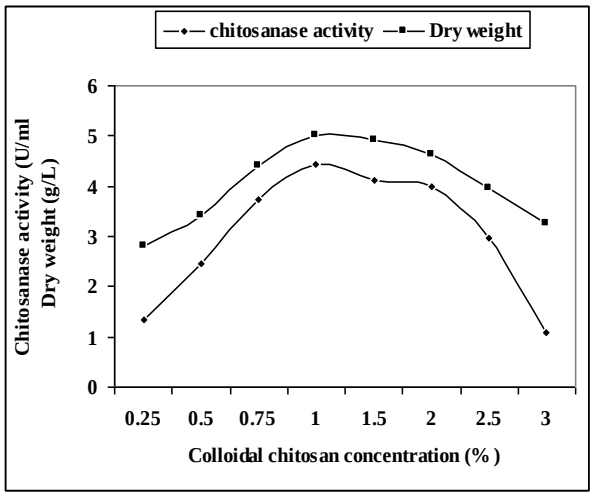

Fig.8: Effect of colloidal chitosan concentration on growth and enzyme production by A. ornatus

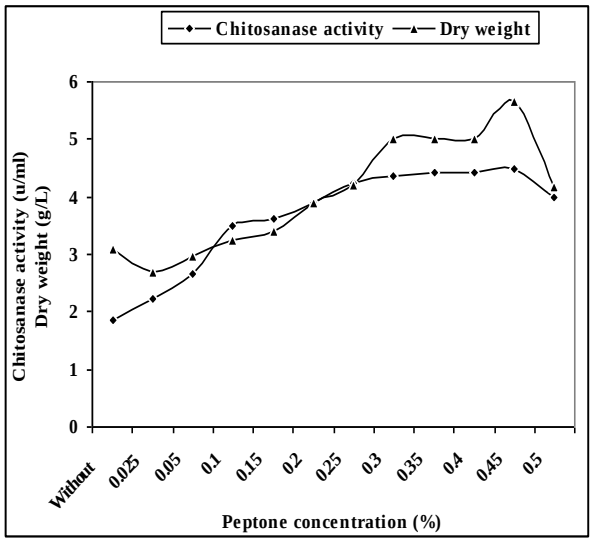

Fig.9: Effect of different concentrations of peptone on growth and enzyme production by A. ornatus.

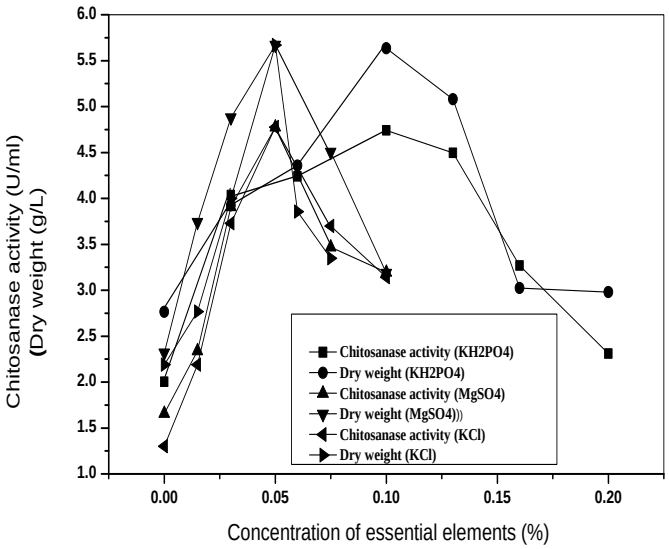

Fig.10: Effect of essential elements on growth and chitosanase production by A. ornatus.

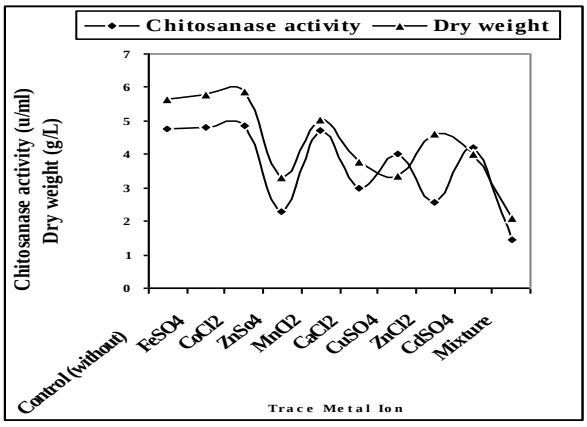

Fig.11: Effect of heavy metal ions on growth and chitosanase production by A. ornatus.

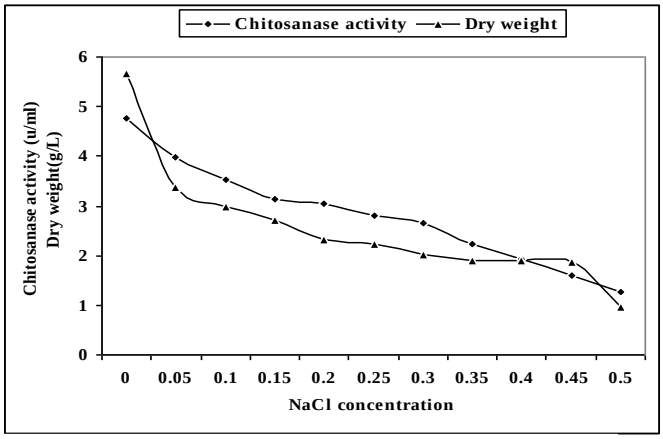

Fig.12: Effect of different concentrations of NaCl on growth and chitosanase production by A. ornatus.

Table.4: Effect of different vitamins on growth and chitosanase production by Aspergillus ornatus.

Vitamins	Concentration. (Mg\L)	Chitosanase activity (U/ml)	Dry wt. (g/L)
control	0.0	4.746± 0.2558	5.67
Thiamine	10	4.57± 0.01155	4.914
	50	4.59± 0.94516	4.51
Riboflavin	10	4.66± 0.87757	4.96
	50	4.718± 1.79347	4.774
Pyridoxin	10	4.452± 1.67288	4.36
	50	4.498± 1.02842	4.308
Cyanocoblamine	10	4.126± 4.25475	4.852
	50	4.116± 2.40308	4.042

Table.5: Effect of some surfactants on growth and chitosanases production by Aspergillus ornatus.

Emulsifying agent	Conc.%	Chitosanase activity (U/ml)	Dry wt. (g/L)
Control	0	4.746	5.76
Tween 80	0.5	4.54	5.0
	1.0	4.68	5.1
	2.0	4.72	5.2
Tween 40	0.5	4.246	4.6
	1.0	4.32	4.9
	2.0	4.47	5.0

Table.6: Effect of different natural additive on growth and Chitosanase production by Aspergillus ornatus.

Source	Chitosanase activity (U/ml)	Dry wt. (g/L)
Control	4.746±0.2558	5.67±0.068369
Sand extract	4.692±0.55426	5.462±0.05175
Soil extract	4.582±1.0356	5.22±0.04933
Malt extract	2.16±1.03546	1.442±0.163
Beef extract	3.35±0.14769	3.24±0.22259
Wheat bran	4.343±0.00667	4.872±0.11547