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Isolation, Screening, Optimization of Cultural Conditions of Submerged Fermentation and Partial Purification of Alkaline Chitinase Enzyme from *Fusarium sp.*

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A B S T R A C T

The importance of microbial chitinase has gained much interest because on the one hand, it reduces environmental hazards and on the other hand it increases production of industrially important value-added products. Hence the present study was conducted with the objective to isolate, screen and characterize chitinase producing fungi from soil samples. In the present study ten fungal isolates have been evaluated for the production of extracellular chitinase and selected one potent isolate based on maximum chitin hydrolysis and identified as *Fusarium sp.* SS06. *Fusarium sp.* KS06 has been used for the production of chitinase under submerged fermentation and partially purified and characterized. *Fusarium sp.* showed maximum enzyme production of at 40°C; pH 8.0 with 1.0 ml of inoculum size. Chitinase enzyme produced from *Fusarium sp*. was partially purified by 80% ammonium sulphate saturated and molecular weight was determined as ~38 kDa. The partially purified enzyme's maximum activity at 25°C and pH 9.0. The partially purified chitinase was able to hydrolyse 100% of its natural substrate colloidal chitin. Whereas with regards to starch and casein only chitin hydrolysed only 30% and 21% respectively. In conclusion the extracellular chitinase producing fungi *Fusarium sp.* was isolated from soil sample and cultural conditions for the production of chitinase under submerged fermentation were optimized. The partially purified alkaline chitinase from *Fusarium sp*. can be a used as potential biocontrol agent.

Introduction

Chitin a nitrogen containing linear polymer of β-1,4 linked N-acetyl glucosamine residues is an important and abundantly available natural polymer next to cellulose. Chitin is widely distributed in the cell wall of higher fungi, exoskeletons of insect and shells of crustaceans (Rustiguel *et al.,* 2012; Thanku *et al.,* 2019). Microorganisms has the ability to destroy fungal diseases that are problem for global agricultural production as the enzymes produced from the microorganisms are able to hydrolyze the cell wall of fungi. The enzymatic method of eradication of diseases has been attracted interest as it is ecofriendly and cost-effective method.

However extensive use of chemical fungicides, which has tripled over the last four decades, has accelerated environmental pollution and bioaccumulation. Moreover, chemical fungicides may be lethal to beneficial insects and microorganisms populating the soil and may enter the food chain. Despite their high effectiveness and ease of use, chemical fungicides have many disadvantages. Hence biological control, the use microorganisms to control plant diseases offers an alternative and at this juncture many microbial enzymes were reported as biocontrol agents (Divatar *et al.,* 2016).

Chitinases (EC 3.2.1.14) are the enzymes that are produced by several bacteria, actinomycetes, fungi and also by higher plants (Shanmugaiah *et al.,* 2008; Sharma and Salwan 2015; Akagi *et al.,* 2006; Matsushima *et al.,* 2006; Viterbo *et al.,* 2001).

The presence of chitinolytic microbes indicates the availability of chitin in the soil. Chitinases also play a major role in many areas such as the production of single cell protein, growth factors (Mizani *et al.,* 2005; Felse and Panda, 2000), mosquito control, a biocontrol agent of fungal pathogens and isolation of fungal protoplasts (Prabavathy *et al.,* 2006; Chang *et al.,* 2007).

The importance of microbial chitinase production has increases because on the one hand, it reduces environmental hazards and on the other hand increases production of industrially important value-added products. With this viewpoint, the present study was undertaken on isolation, screening and characterization of chitinase producing fungi from soil samples collected from Karnataka.

Materials and Methods

Chemicals

The materials, media, reagents used for this study were procured from Sigma-Aldrich, SRL and Hi-Media, India.

Collection of soil samples

Four different soil samples were collected from different regions (*i.e.*, gardens, nearby coconut trees, Bangalore university campus and cultivated soil) for isolation of fungi able to produce extracellular chitinase. Soil samples were stored at 4^0C till further processing.

Preparation of colloidal chitin

Extrapure chitin powder (40g) from Hi-Media was dissolved in 600ml of conc. HCl and stirred continuously for 60 mins at 4°C and kept for overnight. The suspension was added to cold 50% ethanol with vigorous stirring at incubated at 25°C for overnight. The precipitate was collected by centrifugation at 10,000 rpm for 20 min and washed with sterile distilled water several times until the colloidal chitin became neutral (pH 7.0). It was freeze and dried to powder and stored at 4°C until further use (Mathivanan *et al.,* 1998).

Isolation of fungi from soil samples

Media preparation

The fungi were isolated from the soil sample on Czapek Dox agar (CZA) medium. CZA medium was prepared by dissolving Glucose-30.00g; $NaNO_3$-2.00g; K_2HPO_4-1.00g; $MgSO_4$- 0.50g; KCl-0.50g; $FeSO_4$-0.01gin 800 ml of distilled water and pH of the solution was adjusted to 6.50±0.20 and then Agar-15.00g was added. The final volume was made up to 1 liter and the media was sterilized by autoclaving at 121°C, 15lbs of pressure for 15 minutes and then CZA agar plates were prepared for the inoculation of the soil sample.

Serial dilution

Fungal strains were isolated from soil samples through serial dilution plate method(Waksman 1927).1g of soil samples collected from various locations was taken into a sterile test tube containing 10ml of distilled water and stirred well till the homogenous soil suspension was obtained (10-1 dilution). 1ml soil suspension from 10-1 dilution tube was added in to another test tube containing 9 ml of distilled water and stirred well (10-2 dilution).

This procedure continued for 10-3 and 10-4 dilutions. The samples of 10-2 and 10-3 dilutions were inoculated on CZA plates by spread plate method (Cappuccino and Sherman 2005). The inoculated plates were incubated at 300C for 3 to 5 days and observed for the growth of fungi at regular intervals.

Sub-culturing and maintenance

The isolated strains were tentatively identified in the laboratory as defined by Raper and Fennell (Raper and Fennell 1965). The isolated strains were sub-cultured. The stock cultures of the isolates were maintained on Czapek Dox Agar slants which were incubated for 120 h to 168 h and were stored at 40C.

Screening of chitinase producing organism by plate assay

The fungal cultures were spotted on the selected colloidal chitin agar media(Colloidal chitin-5.00g/l, KH_2PO_4-2.00g/l, $MgSO_4$. $7H_2O$-0.30g/l, $(NH_4)_2SO_4$-1.40g/l, $CaCl_2$. $2H_2O$-0.50 g/l, Bactopeptone-0.5g/l, Urea-0.30g/l, $FeSO_4.7H_2O$-0.005g/l, $MnSO_4.7H_2O$-0.0016g/l, $ZnSO_4.7H_2O$-0.0014g/l, $CoCl_2$. $2H_2O$-0.002g/l, Agar: 15g/l, pH: 6.00) and the plates were incubated at 28ºC for 5 days. Development of halo zone around the colony was considered as positive for chitinase enzyme production.

Rapid Confirmation of Chitinase Producing Fusarium Sp. by TLC

TLC plates were prepared with silica gel G homogenous slurry solvent system consists of water and phenol in a ratio of 1:1 was used as mobile phase. the catalyzed chitin samples were subjected to TLC for rapid confirmation. The chromatogram was developed in the iodine chamber for the appearance of the spots and calculation of Rf values.

Fermentation medium for alkaline chitinase production

Inoculum preparation

According to Lingappa K and Babu CV, the fungal inoculum was prepared by adding 10 ml of 0.01% Tween 80 solution to 168 h freshly cultured slant and scrapped gently on the slants with the help of sterile loop to attain homogeneous spore suspension. Each 1 ml of spore suspension of inoculum has a final concentration of 1x 107 spores/ml (Lingappa and Babu, 2005).

Fermentation medium composition

The production medium consists of Sucrose-30,00g/l; Sodium nitrate-2.00g/l; 1.0g of K_2HPO_4-1.00g/l, $MgSO_4.7H_2O$-0.50g/l;KCl-0.50g/l and 0.01g of $FeSO_4$-0.01g/l; pH 6.8. The selected *Fusarium sp.*SS 06 was cultured on production medium for 96 -120h. The selected *Fusarium sp.* SS 06 was cultured on production medium was used as inoculum. The production studies were carried out in Czapek dox broth for better yield at pH 5.0, temperature 30^0C and 1.0 ml inoculum was used.

Extraction of chitinase of fermentation medium

The samples were withdrawn periodically at every 24 h in aseptic condition. The extract was filtered through Whatman filter No.1. The filtered extract was centrifuged at 2000-3000 rpm for 15 mins and chitinase activity assay was carried out from the crude extract.

Assay of chitinase activity

Chitinase activity was measured using colloidal chitinas the substrate (1.50 % suspension of each of the colloidal chitin prepared in a phosphate buffer (50 mM, pH 6.0) separately. Enzyme solution (0.50 ml) was added to 1.0 ml of substrate solution, which contained the mixture and it was incubated at 37°C for 15 minutes. After centrifugation, the amount of reducing sugars produced in the supernatant was determined by Dinitrosalicylic acid (DNS) method by using N-acetyl glucosamine as a reference compound (Miller 1959).

One International Unit (IU) of chitinase activity was defined as the amount of the enzyme required to produced 1 µ moles of reducing sugar per minute under standard assay conditions.

Optimization of chitinase production by One Variable At a Time (OVAT)

Optimization of the chitinase production was done by varying different physico-chemical factor one at a time keeping the other factors constant.

Effect of pH

The effect of pH on enzyme production was studied with different pH ranging from 3.0-10.0 with increments of 1.0 and doing the assay using cell free supernatant(Plate-4).

Effect of temperature

The effect of incubation temperature on enzyme production was studied at different temperature ranging from 25-40°C with increments of 5°C(Plate-5).

Effect of inoculum size

The effect of inoculum size was studied at different inoculum levels *i.e.,* 0.25, 0.50, 0.75, 1.00 and 1.25 ml.

Partial purification of chitinase

The crude extract (about 250 ml) was precipitated at 4°C using 80% ammonium sulphate saturation. The protein deposit was obtained by centrifugation (8000 X g for 30 mins), dissolved in a defined volume of 50 mM phosphate buffer (pH 7.2) and dialysed against the same buffer overnight in a refrigerator using the 12 kDa MW cut-off membrane (Sigma,USA). The dialysate was collected and used for characterization of partially purified chitinase.

Molecular weight determination by SDS-PAGE

The active fractions were checked for purity and molecular weight of partially purified

chitinase was determined by SDS–PAGE using Mini Protean II apparatus (Bio-Rad Laboratories Inc., USA) on 12% polyacrylamide gel according to Laemmli UK (Laemmli 1970).

Characterization of chitinase

The partially purified chitinase was characterized with respect to its optimal temperature, pH and substrate specificity for enzymatic activity.

Optimum pH determination

The optimum pH for chitinase activity was measured at different pH values (4.0-11.0) in 0.1 M phosphate buffer by DNS method using colloidal chitin as a substrate(Miller 1959).

Optimum temperature determination

The optimum temperature for chitinase activity was determined by performing enzyme assay at different temperatures ranging from 37-55°C at pH 7 in phosphate buffer (0.1 M) using colloidal chitin as a substrate(Miller 1959).

Substrate specificity

Substrate specificity was investigated by incubating partially purified chitinase with various substrates separately, such as chitin, starch and casein concentration of 0.45% w/v in 0.1 M phosphate buffer (pH 7) using standard assay method(Miller 1959). The relative activity was calculated using colloidal chitin as a control.

Results and Discussion

Isolation of fungal strains for chitinase production

Chitinase producing fungal strains were isolated from the soil samples from different sites in and around Bengaluru (12059′ N latitude and 77035′ E longitude), Karnataka, and India.

Totally, 10 different fungal strains isolated and confirmed as *Fusarium Sp.*by staining technique and labelled as KS 01, KS02, KS03, KS04, KS05, KS06, KS07, KS08, KS09 and KS10 were isolated (Table 1 and Plate 1).

Screening of chitinase producing organisms by plate assay method

In plate assay potential chitinase producing strains were isolated based on the observation of clear zone around the colony.

The *Fusarium Sp.* producing extracellular were segregated as poor (less than 6 mm diameter zone), medium (6-9 mm diameter zone) and good (above 9 mm diameter zone).

Among the 10 isolated fungal isolate KS 06 has maximum diameter of clear zone (i.e., 1.04). Hence *Fusarium Sp.* KS06 was selected for further studies (Plate 2). The results of the present study are in accordance with Vincy *et al.*,(Vincy *et al.*, 2014).

Rapid confirmation of chitinase production from *Fusarium sp.* by TLC

Catalyzed chitin samples subjected to TLC against standard glucose amine sample which has Rf value 0.76. The Rf value of catalyzed chitin sample in the present study was 0.80which almost the same Rf value as compared to standard. The results clearly indicate that *Fusarium sp.*KS06 produces extracellular chitinase (Plate 3 and Table 2).

Optimization of chitinase production by One Variable At a Time (OVAT)

For industrially important enzymes, their hyper-production is an issue of central importance for commercial application.

Table.1 Fungal isolates from environmentally stressed soils

S. No.	Sources	No. of Isolates
1	Garden soil	02
2	Near coconut tree	02
3	Near Bangalore university campus	03
4	Cultivated soil	03

Table.2 Confirmation of alkaline chitinase from *Fusarium sp.*KS 06 by TLC

S.No.	Sample	Rf Value
1	Standard Glucose amine	0.80
2	Chitin hydrolyzed product from chitinase	0.76

Plate.1 *Fusarium sp.*

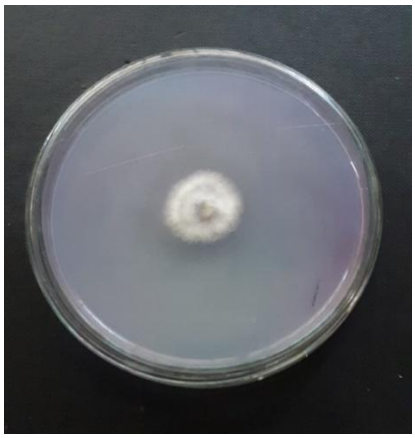


Plate.2 Plate assay

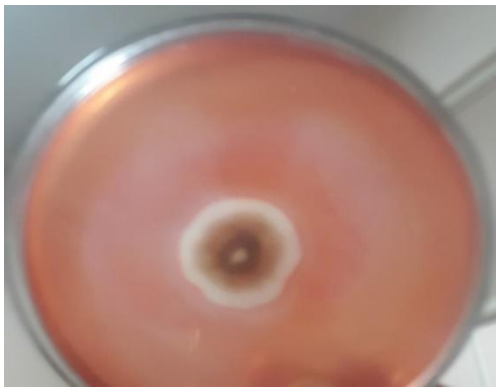


Plate.3 Thin layer chromatography

Lane 1: Sample; Lane 2: Standard Glucose amine

Fig.1 Effect of different pH on chitinase production

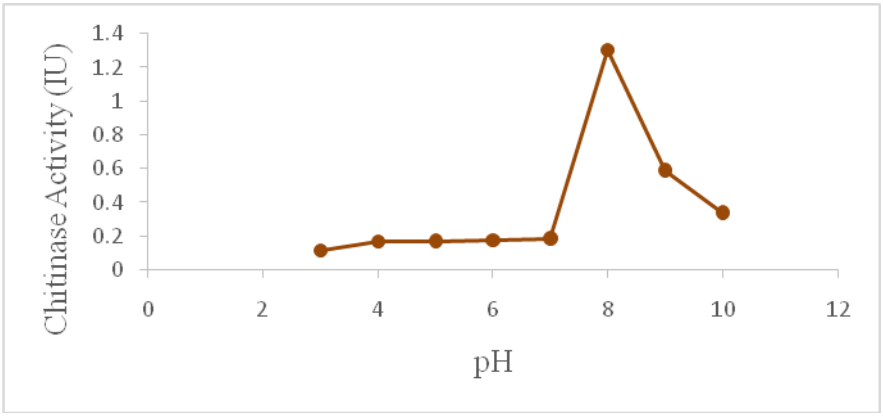


Fig.2 Effect of Temperature on Chitinase Production

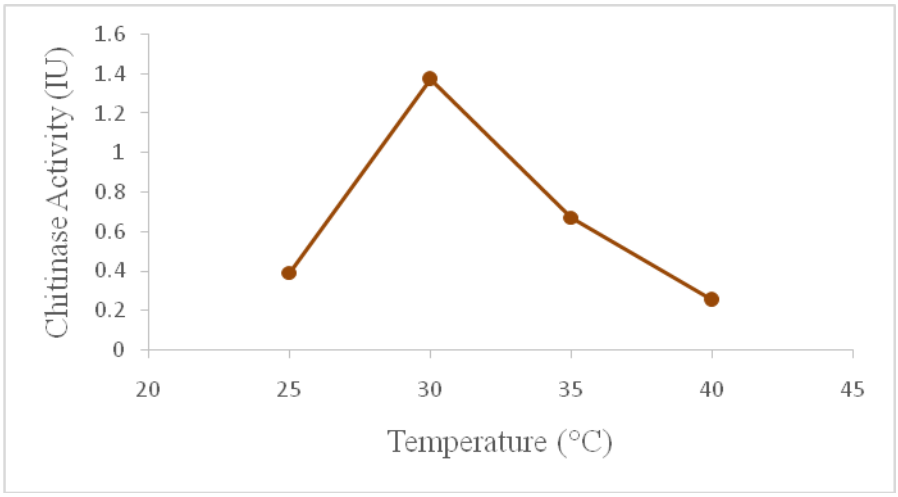


Fig.3 Effect of inoculum size on chitinase production

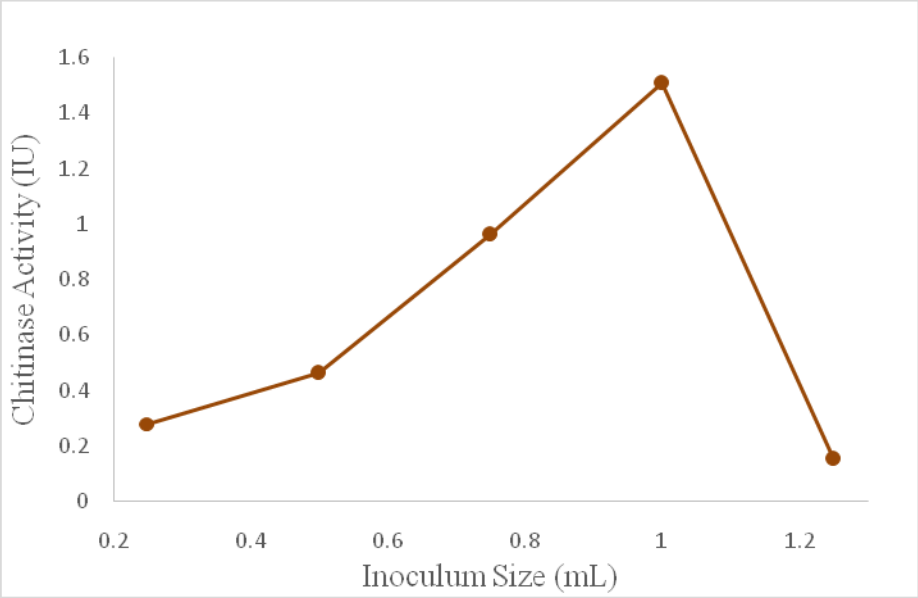


Fig.4 Ammonium salt fractionation of chitinase enzyme

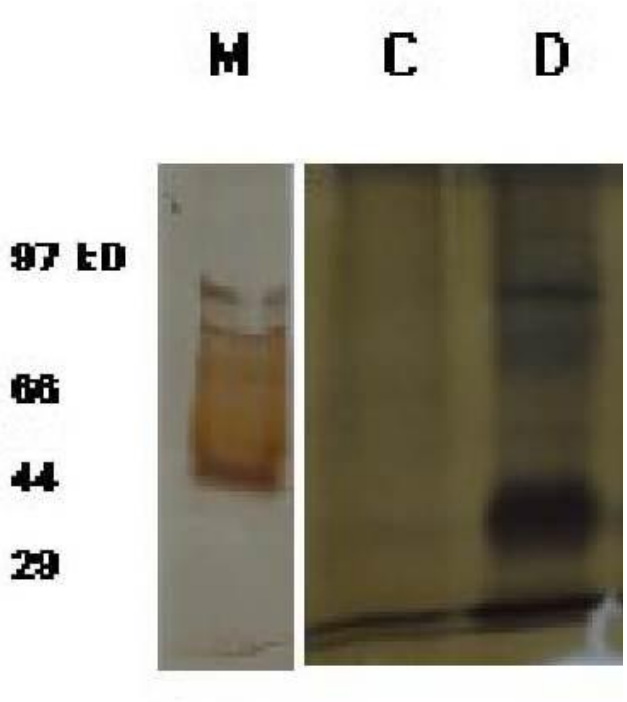


Fig.5 Effect of pH on chitinase activity

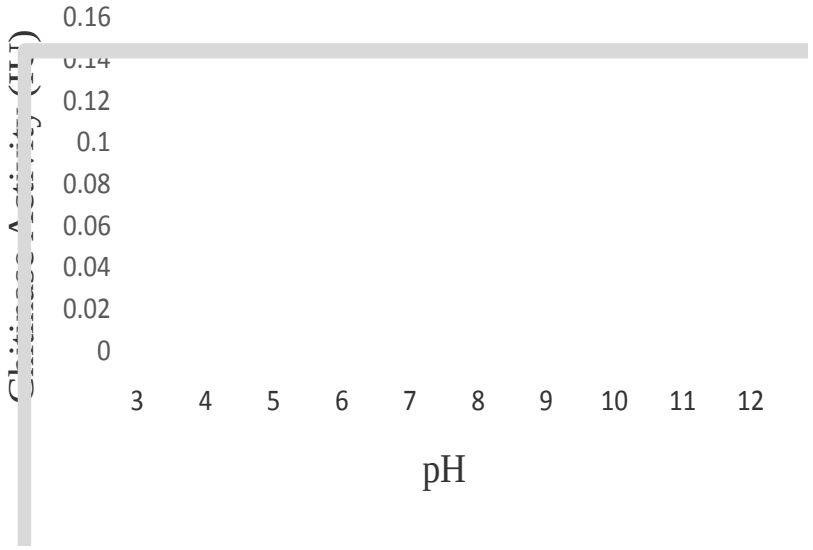


Fig.6 Effect of temperature on chitinase activity

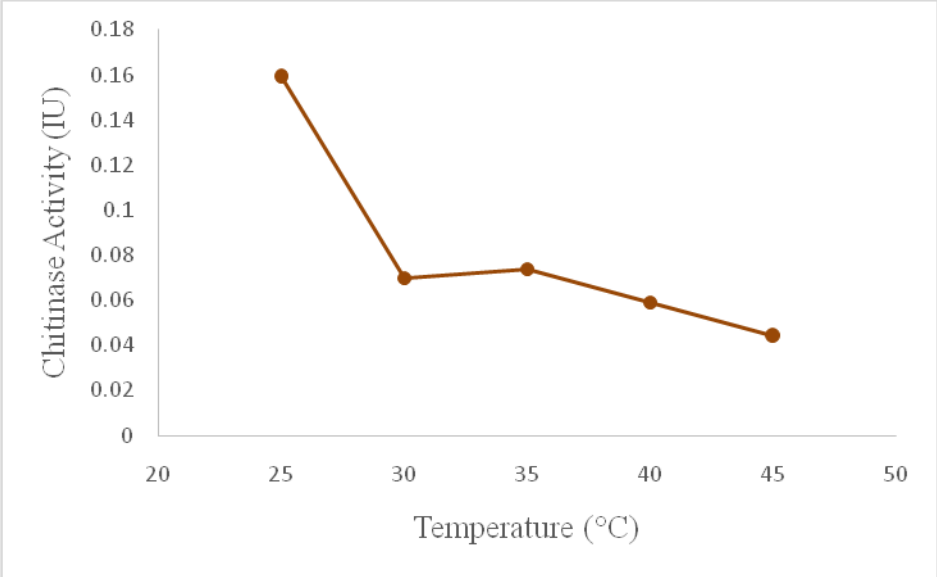
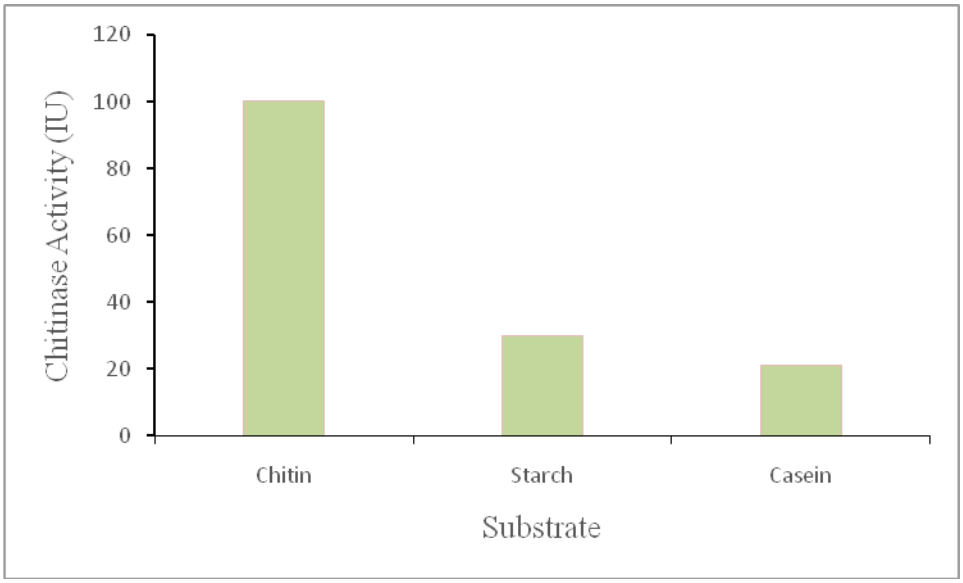


Fig.7 Substrate specificity of chitinase enzyme



Microbial enzyme production is influenced by the physico-chemical factors e.g., temperature, agitation, pH, culture/production medium constituents (Dekker *et al.,* 2007; Chauhan *et al.,* 2014; George *et al.,* 2014).

Optimization of these factors for improving the yield of enzyme is crucial to make the process economical (George *et al.,* 2014).The chitinase production with isolate *Fusarium sp.* KS 06 was optimized with respect to pH, temperature and inoculum size.

Effect of pH

In the present study the enzyme activity for different pH ranging from 3.0-10.0 were determined. The maximum chitinase production was found at pH 8.0. Further increase in the pH decreased the activity of chitinase. Chitinase production by *Fusarium sp.* KS 06 was found to be maximum at pH 8.0 (*i.e.,* 1.302 IU) (Figure 1).

The results of the present study are in concurrence with the results of Shivalee *et al.,* and Ahmadi *et al.,* (Shivalee *et al.,* 2018; Al-Ahmadi *et al.,* 2008).

Effect of incubation temperature

In this present work the chitinase activity was studied for the temperature from 35°C - 40°C. It was observed in the present study that the maximum enzyme production from the *Fusarium* Sp. was found to be maximal after 96 hours of time at 30°C (1.373 IU) (Figure 2).

The results of the present study are in concurrence with the findings of Kannan and Ramachandra wherein optimum temperature for chitinase production was found to be 30 °C by *Serratia marcescens* (Paul *et al.,* 2012).

Taechowisan *et al.,* (Taechowisan T *et al.,* 2003) and Shanmugaiah *et al.,*(Shanmugaiah V *et al.,* 2008) reported that the production of chitinase by *Streptomyces aureofaciens* and *B. laterosporus* MML2270 respectively was found to be optimal at 30-40°C.

These results were further supported by findings of Annamalai *et al.,* reported maximum chitinase production at 37°C by *Alcaligenes faecalis* (Annamalai *et al.,* 2010).

Effect of inoculum size

Out of five inoculum size tested (0.25, 0.50, 0.75, 1.0 and 1.25 ml), 1.0 ml of inoculum was found to be the most suitable for highest production of chitinase by *Fusarium sp.* KS 06 in submerged fermentation (*i.e.*,1.507 IU)(Figure 3).

The results of present study are in agreement with Shivalee *et al.,* reported that inoculum size influenced the production of chitinase by Streptomyces pratensis strain KLSL55. There was a gradual increase in enzyme production with increase in inoculum size; the maximum enzyme production (141.20 IU) was recorded with 1.25 ml of inoculum (1X108spores' ml) (Shivalee *et al.,* 2018).

Partial purification of chitinase

The crude extract (about 250 ml) was precipitated at 4°C using 80% ammonium sulphate saturation. The protein deposit was obtained by centrifugation (8000 X g for 30 mins), dissolved in a defined volume of 50 mM phosphate buffer (pH 7.2) and dialysed against the same buffer overnight in a refrigerator using the 12 kDa MW cut-off membrane (Sigma, USA).

The dialysate was collected and used for characterization of partially purified chitinase. Purity of the enzyme was examined by SDS-PAGE and the estimated molecular weight (MW) was found to be ~38 kDa (Figure 4).

Characterization of chitinase

Determination of optimum pH

The effect of pH on chitinase activity was investigated at different pH values (4.0-11.0) (Fig. 4). Chitinase exhibited optimum activity at pH 9.0 (Figure 5). The results are in concurrence with Ben Amar Cheba *et al.,* wherein the optimum pH of chitinase was found to be at pH 7.0 and 8.0 (Cheba *et al.,* 2016).

Determination of optimum temperature

The optimum temperature for chitinase activity was 25°C. However, the enzyme retained about 40% activity at 45°C (Figure 6). Our results are in close agreement with Ben Amar Cheba *et al.,* who reported chitinase showed good activity at between 30-45^{0}C, but the optimum temperature was 40^{0}C (Cheba *et al.,* 2016).

Substrate specificity

The hydrolysis of various substrates with chitinase showed that the partially purified chitinase of *Fusarium* sp. was able to hydrolyze colloidal chitin, starch and casein exhibiting relative chitinase activity 100%, 30% and 21% respectively (Figure 7). The results of substrate specificity indicate that the chitinase enzyme extracted from *Fusarium* sp. KS 06 was very much specific to its natural substrate chitin. Our results are in agreement with Va *et al.,* (2011) and Taechowisan *et al.,* (2003).

Chitin is a versatile and promising biopolymer with numerous industrial, medical and commercial uses. The extracellular chitinase producing fungi *Fusarium* sp. was isolated from soil sample and cultural conditions for the production of chitinase under submerged fermentation were optimized. The partially purified alkaline chitinase from *Fusarium* sp. can be a used as potential biocontrol agent.

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