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ISOLATION, PURIFICATION AND OPTIMIZATION OF CHITOSANASE PRODUCTION FROM A COMMON MAHABUBNAGAR AGRICULTURAL FIELD FUNGI *ASPERGILLUS FUMIGATUS* OF TELANGANA STATE

P. Shivakumar Singh[1] and G. M. Vidyasagar[2]*

[1]Department of Botany, Palamuru University, Mahabubnagar, Telangana State, India-509001.

[2]Medicinal Plants and Microbiology Research Laboratory, Department of Post Graduate Studies and Research in Botany, Gulbarga University, Gulbarga-585 106, Karnataka. India.

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***Corresponding Author**
G. M. Vidyasagar
Medicinal plants and microbiology Research Laboratory, Department of Post Graduate Studies and Research in Botany, Gulbarga University, Gulbarga-585 106, Karnataka. India.

ABSTRACT

Objective: Isolation, purification, optimization of chitosanase production using a common fungi *Aspergillus fumigatus*. **Methods:** The standard methods followed like Collection of soil sample, Screening of microbes in chitosanase detection broth, Optimization of Physical Parameters, Optimization of Nutritional Parameters and SDS-PAGE were recorded. **Results:** Chitosanases have inward much consideration as of their extensive series of applications. Even though the majority fungal chitosanases use sugar as their chief carbon source, the present investigation represents the chitosanase production using a common fungi *Aspergillus fumigatus*. The isolation, purification methods were standardised. The optimization of physical parameters like pH, temperature, inoculum size, agitation speed and optimization of nutritional parameters like carbon source, nitrogen source were reported. **Conclusion**: This broad range of specificity is advantageous for the production of partially deacetylated chitosan oligosaccharides and for the economical use of chitosan.

KEYWORDS: Isolation, optimization, chitosanase production, fungi of Mahabubnagar District. Telangana State.

INTRODUCTION

Chitosanases (EC 3.2.1.132) are hydrolases acting on chitosan. Chitosan is a linear polysaccharide composed of-1,4-linked D-glucosamine residues. In nature, this polymer is partially acetylated, so that the name chitosan describes a large family of polymers containing various proportions of D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc) residues. Chitosan occurs in the cell walls of a limited number of fungi and in certain green algae, such as Chlorella, and is also produced commercially by alkaline deacetylation of shellfish (usually crab) chitin.[1-2]

Chitosanases are produced by many organisms, including actinomycetes, fungi, plants and bacteria. Bacterial chitosanases have received special attention because they are important for the maintenance of the ecological balance and have been used to determine the mechanism of chitosan hydrolysis at both biochemical and molecular levels. Previously, several microorganisms including bacteria were reported to efficiently produce chitosanases to degrade chitosan to glucosamine oligomers. Furthermore, fungi and actinomycetes also produce chitosan oligomers from chitosan. Chitosanases are also found in vegetative parts and seeds of some higher plants.[3] The same authors reported that *Triticum aestivum* and *Hordeum vulgare* seeds are the best monocotyledons sources of chitosanases while *Pisum sativum* seeds and *Cucumis sativus* fruits are the best dicotyledons sources. Microbial chitosanases are classified into three subclasses based on their cleavage position specificity: subclass I (e.g., *Streptomyces* sp. N174 chitosanase) cleaves GlcN-GlcN and GlcNAc -GlcN linkages; subclass II (e.g., *Bacillus* sp. No.7-M) cleaves only GlcN-GlcN linkages; and subclass III (e.g., *Bacillus circulans* MH-K1 chitosanase) cleaves both GlcN- GlcN and GlcN-GlcNAc linkages. A few plant chitosanases also have been characterized and divided into two classes based on substrate specificity: class I, which includes chitosanases from stressed barley, cucumbers, and tomatoes, can degrade only chitosan, whereas class II, which includes chitosanases from tomato stems, *Citrus sinensis* and pineapple stem, can degrade both chitin and chitosan.

Chitosan and its oligosaccharides, which are known to possess multiple functional properties, have attracted considerable interest due to their biological activities and potential applications in the food, pharmaceutical, agricultural and environmental industries. The biological activities of chitosan and chito oligosaccharides include hypo-cholesterolemic, antimicrobial, immune stimulating, antitumor and anticancer effects, accelerating calcium and iron

absorption, anti-inflammatory, antioxidant and Angiotensin-I-converting enzyme (ACE) inhibitor activities. They effects are correlated with their structures and physicochemical properties.[4] The oligomers of D-glucosamine (GlcN) and of N-acetyl-D-glucosamine (GlcNAc) have interesting biological activities including anti-tumor effects hypo-cholesterolemic effects, anti-microbial activities (San Lang et al., 2008; Souad and disease-resistance responses, and as phyto-alexin elicitors in higher plants. Glucosamine produced by hydrolysis of chitosan has attracted attention due to it s therapeutic activity in osteoarthritis and has been evaluated as food supplement. It also has applications in promoting wound healing, bone regeneration, and antibacterial effect in dentistry. Acetylated form of glucosamine is sweeter in taste and fit for oral administration. Sulfate and hydrochloric salt of glucosamine has already been commercialized. Chitosanases are generally believed to play an important defense role against invading pathogens because of their potential to hydrolyse fungal wall polysaccharides.[3] Also chitosanase show applications in various fields, such as biomedical, pharmaceutical, agricultural, biotechnological and in food industry.[4] Chitosan may be hydrolyzed with acids at high temperatures to form glucosamine but this method is of low yield, high cost, generates a lot of acidic waste which leads to environmental pollution and not fit for human use. Thus, enzymatic method is preferable for production of glucosamine.

The aim of the present study is to isolate potential microbes capable of chitosanase production, enhancement of enzyme production by process optimization, partial purification of the enzyme.

MATERIALS AND METHODS

Collection of soil sample

The soil samples were collected from the agriculture fields of Palamuru district, Telangana state in screw cap bottles and used for the isolation of fungi. 0.1 ml serially diluted samples were plated over Sabouard's Dextrose agar (SDA) and Nutrient agar (NA) for growing fungi and bacteria respectively. The colonies were preserved on agar slants at temperature 4^{o}C for further work.

Screening of microbes in chitosanase detection broth[5]

Screening of Fungi

The fungal colonies were inoculated into Chitosan detection broth having composition, (per L) Chitosan 1g, 0.2g K_2HPO_4, 0.1g KH_2PO_4, 0.07g $MgSO_4.7H_2O$, 0.05g NaCl, 0.05g KCl,

0.01g $CaCl_2$, and 0.05g yeast extract and its initial pH was 6 at 30°C incubate for 7 days on a rotatory shaker at 120rpm.

Identification of fungal strain

The fungal strain which shows highest chitosanase activity was selected and sent to "Agharkar Research Institute, Pune" for the morphological identification process.

Optimization of Physical Parameters[6-14]

Optimization of pH

In first set of experiment, the seed cultures were inoculated in 250ml Erlenmeyer flask containing 50ml of CDB medium and incubated at different pH ranging from 4.0 to 7.0with an interval of 1.0 at an incubation period of 37°C, at 120 rpm for 7 days.

Optimization of Temperature

In another set of experiment, the cultures were inoculated at pH 5.0 of the medium and incubated at different temperature ranging from 30°C to45°C with an interval of 5°C at 120 rpm for 7 days.

Optimization of Inoculums size

In the next set of experiment, the inoculum size was varied from 1% to 4% of spore suspension with an interval of 1% with initial pH 5.0.The flasks were incubated at temperature 40°C, at 120 rpm at an incubation period of 7 days.

Optimization of Agitation Speed

In the next set of experiment, the fermentation was carried out at varying agitation peed. The medium with an initial pH 5.0 at temperature 40°C was inoculated with 2% spore suspension incubated at different shaking speed ranging from 140-200rpm with an interval of 20 rpm for 7 days.

Optimization of Nutritional Parameters

Optimization of Carbon Source

The carbon source of the medium i.e. Chitosan was varied from 1% to 4% (w/v) with an interval of 1% (w/v). The initial pH 5.0 of the medium was inoculated with 2% spore suspension incubated at 40°C, at 180rpm for 7 days.

Optimization of Nitrogen Source

The nitrogen source of the medium i.e., yeast extract was varied from 1% to 4% (w/v) with an interval of 1% (w/v). The medium with initial pH 5.0 was inoculated with 2% spore suspension and 2% of Chitosan as a sole carbon sources kept at an incubation period for 7 days at 40^{o}C, at 180 rpm.

Purification of Chitosanase enzyme

The purification was carried out using crude enzyme extract. The enzyme was purified by the following steps at 0-4^{0}C, unless otherwise mentioned.

Ultra filtration

Ultra filtration was carried out by using a membrane with molecular weight cut off of 10 KDa. The concentrated retentive was used for ammonium sulphate precipitate.

Ammonium sulphate precipitation and Dialysis

Finely powdered ammonium sulphate was added to the crude extract. The Chitosanase activity was associated with the fraction precipitated at 40-60% saturation. The precipitate was collected by centrifugation at 9,000g for 15 min, dissolved in phosphate buffer P^{H} 5 and dialyzed against the same buffer.

SDS-PAGE[15]

SDS-PAGE was performed according to the method of Laemmli, with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R0250 and distained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The following standard proteins were used for molecular weight determination 200kDa (Myosiam) 116kDa (β-Galactosidase) 97kDa (Phosphorylase b), 66kDa (Bovine serum albumin), 55kDa (Glutamic dehydrogenase) 45kDa (Ovalbumin) 36kDa (Glyceraldehyde-3-phosphate dehydrogenase) 29kda (Carbonic anhydrase), 24kDa (Trypsinogen) 20kDa (Trypsin inhibitor) 14.2kDa (α-Lactalbumin) and 6.5kDa (Aprotinin).

RESULTS AND DISCUSSION

Screening of chitosanase producing microorganism

A total of 8 fungal strains were obtained by serial dilution method from the soil sample. All strains were screened for chitosanase in chitosan detection broth as shown in (Table 1)Among all the eight isolates, SK-4 was found to be the best for enzyme production activity

1.392 IU. And hence was selected for further studies. This strain was selected for submerged fermentation for the chitosanase production.

Table: 1 Screening of Chitosanase Producing Microorganism.

Sl. No.	Micro-organism Fungi	Chitosanase Activity (IU)
1	SK-1	0.0143
2	SK-2	0.2366
3	SK-3	0. 9234
4	SK-4	*1.3924*
5	SK-5	0.6573
6	SK-6	0.3354
7	SK-7	0.0957
8	SK-8	0.0786

Identification of SK-4 strain

The fungal strain which was sent for the morphological identification in "Agharkar Research Institute, Pune'' was *Aspergillus fumigatus.*

Optimization of Physical Parameters

Optimization of pH

Optimization of pH for chitosanase production revealed that a pH of 5.0 was found to be best for enzyme production by. By *Aspergillus fumigatus* from pH 4.0 to 5.0 the activity shown to be increasing whereas the activity was decreased after pH 5.0. Therefore, pH 5.0 was found to be best for enzyme production i.e0.073IU. The results are depicted in fig: 01.

The activity of the enzyme. Sutee Wangtueai et al reported[16] that mostly, the production of chitosanases was associated with the bacterial growth, in which the concentrations of enzyme and cells were maximized by using the initial P^H of 6.0. The maximal specific growth rate obtained was 0.060 IU at the initial P^H 6. Higher or lower initial pH's gave less favourable specific growth rates. According to Yeon Jin Choi et al.[17] when the enzyme activity was measured under standard assay conditions for pH was 6.0, the maximum activity was observed ie. 0.053 IU. Below the pH6.0 the activity was found to be minimizing whereas above the P^H of 6.0, the activity of the chitosanase enzyme was found to be gradually decreasing. Hence it shows that our results are better when compared to the other experiments mentioned above. The results shown similarly with A.A Shindia et al., the previous reports.[18]

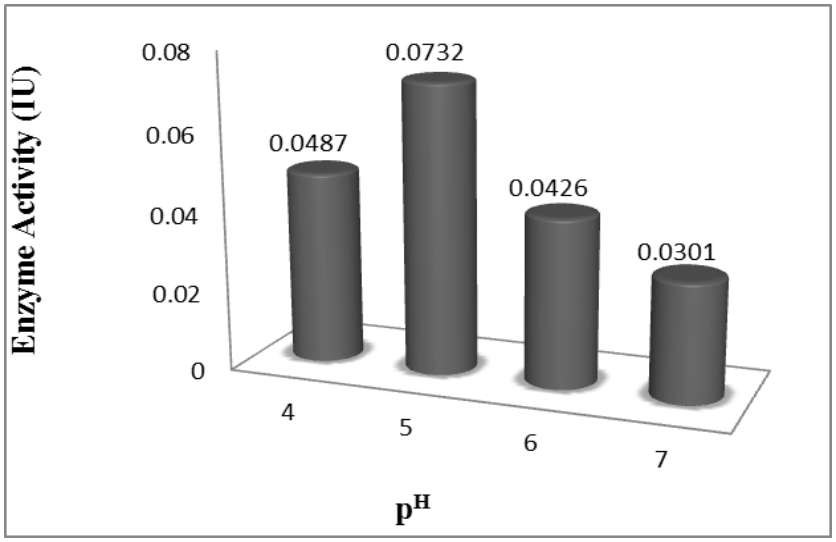

Fig. 1: Optimization of pH.

Optimization of temperature

Optimization of temperature was carried out for chitosanase production at different temperatures ranging from 30^0C to 45^0C with an interval of 5^0C. The results showed that the enzyme production increased with an increase in temperature from 30^0C to 40^0C, thereafter the activity was decreased. Hence a temperature of 40^0C i.e. 0.13 IU. The results were depicted in fig: 02.

It was found to be the best for the enzyme production. Sanna T El-Sayed et al.,[19] demonstrated maximum growth at 50^0C ie.. 0.113.Tzu-Wen Liang et al (2010) reported the optimum temperature for the production of chitosanase was 60^0C showing an activity of 0.099 IU. In another case of study[16] by Sutee Wangtuaei et al.,

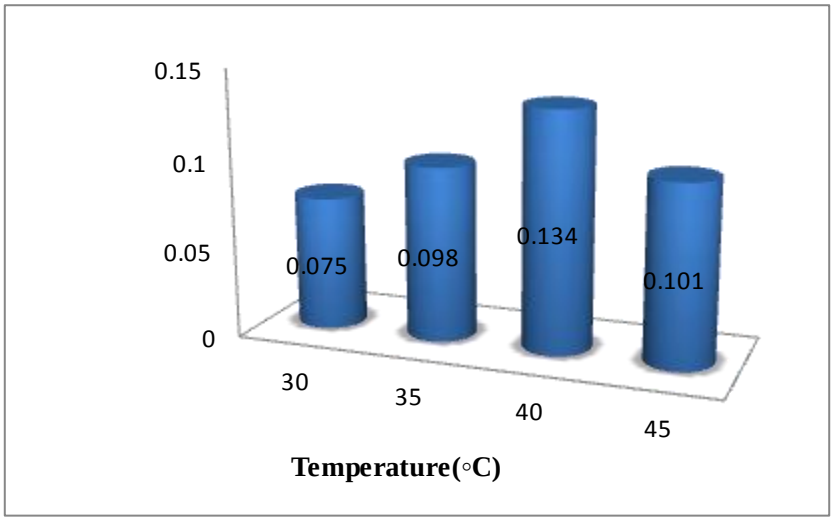

Fig. 2: Optimization of temperature

Optimization of Inoculum size

Optimization of inoculum size for the chitosanase production carried out at different inoculum size varied from 1% to 4% spore suspension with an interval of 1%. The results showed that the enzyme production was increased with an increase in inoculum size from 1% to 2%, thereafter the activity was decreased. Hence an inoculum size of 2% i.e 0.365IU which was higher than 1%, 2%, and 4% inoculums size. (The result are depicted in fig. 3.) The best for the enzyme production. According to A.A. Shindia et al.,[18] The optimum yields of parameter were obtained by using 8 day-old inoculum of *A.ornatus* by the size of inoculum in the range from 2 to 10%. Also, the yield of biomass was not influenced appreciably within the range of 4 to 8% of inoculums 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 inoculums may tend to inhibit the growth of microorganism

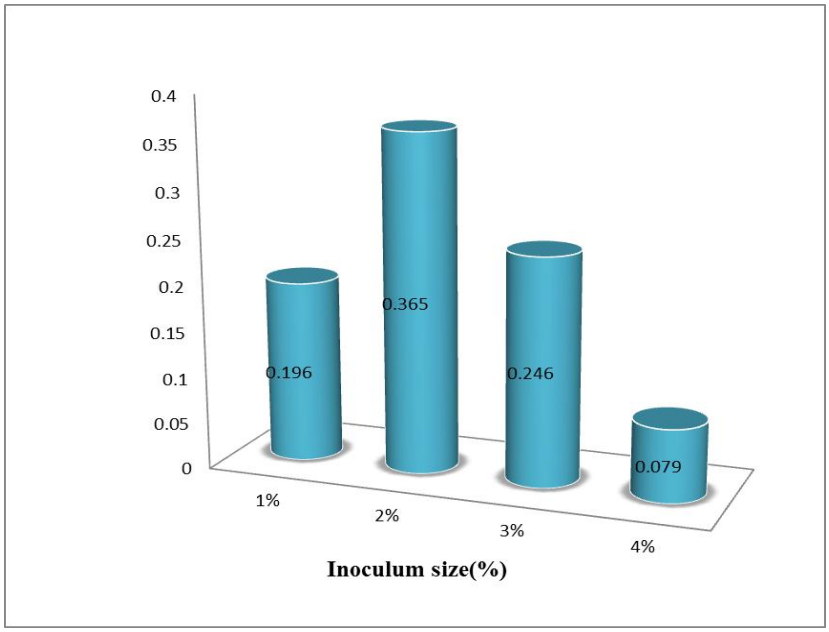

Fig. 3: Optimization of inoculum size.

Optimization of Agitation Speed

Optimization of agitation speed for the chitosanase production was carried out at varying agitation speed from 140 to 200rpm with an interval of 20rpm. The result showed that the chitosanase production was increased with increase in agitation speed from140rpm to 180 rpm, thereafter the enzyme production i.e.0.530IU was decreased. Therefore, 180 rpm found optimum agitation speed for enzyme production. (The results were depicted in fig. 4.) found optimum agitation speed for enzyme production. According to A. A. Shindia et al.,[18] the growth of *A. ornatus* was induced with agitation speed compared to static culture and

maximum biomass at 180r.p.m after this gradually decrease were obtained up to 300 r.p.m. This is comparable with our results. 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 and may have negative effects on morphological states such as rupture of cells etc. Hence our results are in good agreement with those mentioned above.

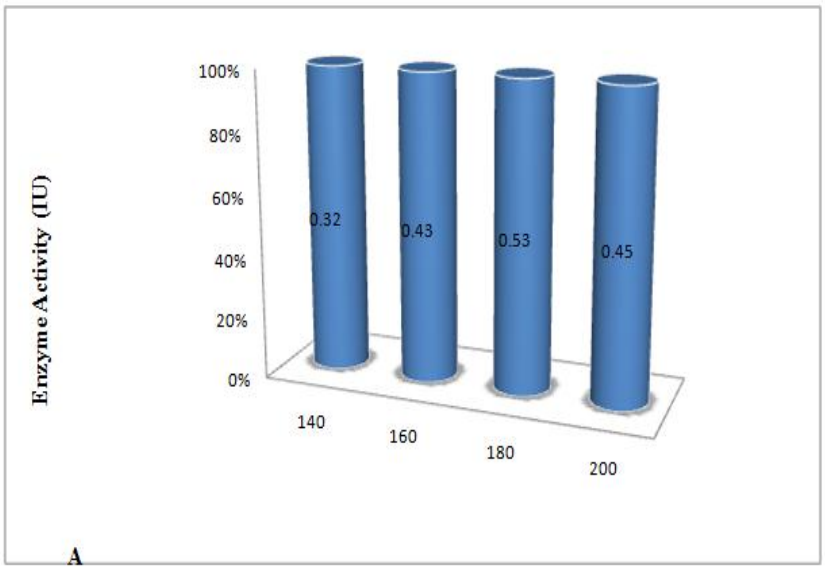

Fig. 4: Optimization of Agitation Speed

Optimization of Nutritional Parameters

Optimization of carbon source

Chitosan as a sole carbon source at 2% showed the highest activity for chitosanase production i.e.0.82IU. It was found that the activity was increasing from 1% to 2% (w/v) and decreased from 2% to 4%. (The results were depicted in fig. 5.) When compared with Sutee Wangtueai et al., the highest concentrations of cells and enzymes were 0.5%. No bacterial growth was found at 1.0 and 2.0% chitosan because high viscosity of the culture medium limited oxygen availability for the bacterial growth. In this study, the Chitosan concentration of 1.0 and 2.0% could not be used as appropriate substrate concentration for the production of chitosanases. Therefore, 0.5% chitosan was finally selected for the optimal growth and chitosanases production from *Bacillus cereus* TP12.24. with an activity of 1.562 IU. Similarly according to A.A.Shindia et al.,[18] it was found that 1.0% of colloidal Chitosan gave the maximum production of enzyme with an activity of 1.6 IU. While the lowest productivity was recorded at 3.0% Chitosan. It was reported that the yield of chitosanase depends on microorganism as well as nature of chitosanase and its levels. For maximum chitosanase productivity, the optimal concentration of colloidal Chitosan was 1.0% for different microorganisms.[20]

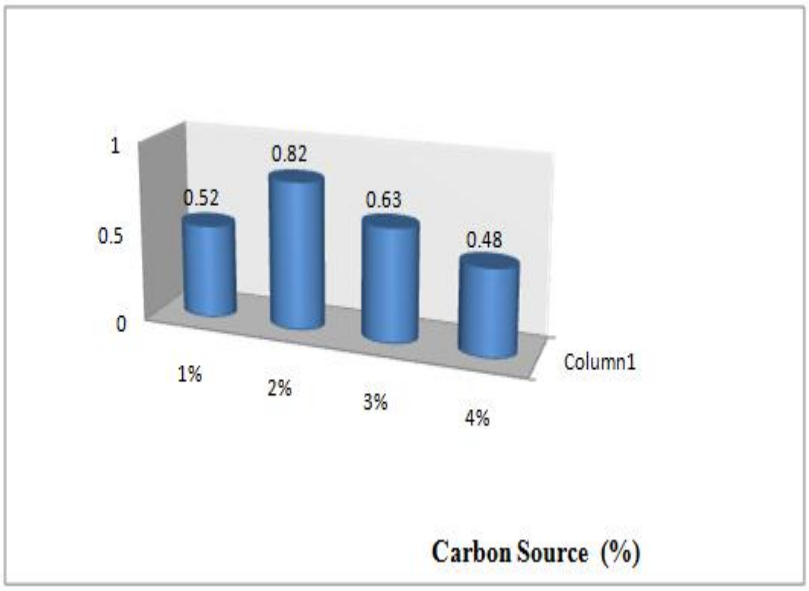

Fig. 5: Optimization of carbon source.

Optimization of Nitrogen Source

Optimization of nitrogen source of the medium revealed that yeast extract at 3% was best for the enzyme production i.e. 0.98IU. The result shows that the enzyme production was increased with increase in yeast extract concentration from 1% (w/v) to 3% (w/v) and thereafter the enzyme production was decreased. The results are depicted in fig. 6.

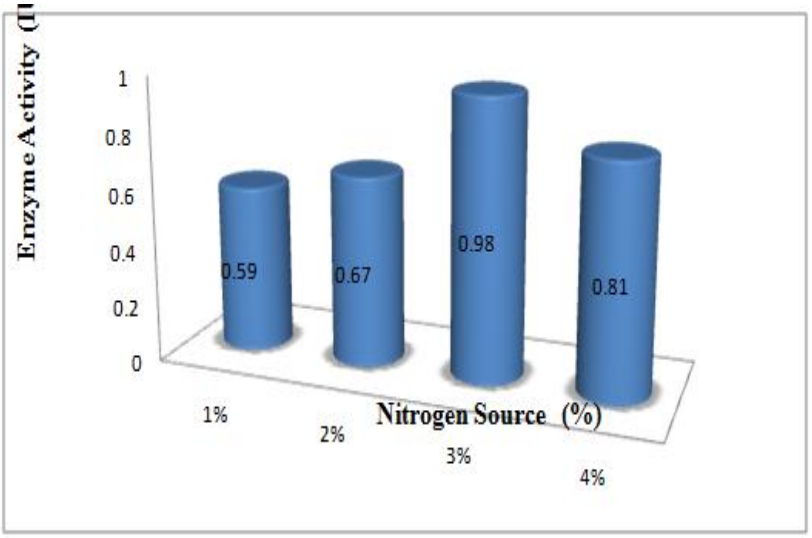

Fig. 6: Optimization of nitrogen source

Purification of Chitosanase enzyme

The protein concentration of the culture supernatant was estimated by Lowry method Lowry's the enzyme was purified by using ultra filtration, Ammonium sulfate precipitation

and Dialysis and The purity was checked by SDS-PAGE and has been found that the molecular weight of the enzyme is kDa.

In order to develop a chitosanase suitable for the applications, a fungi with high chitosanase activity was isolated from soil, and it was designated SK-4. The purification was carried out using crude enzyme extract. The concentrated retentive was used for ammonium sulphate precipitate. The Chitosanase activity was associated with the fraction precipitated at 40-60% saturation. Then SDS page was performed which shows that molecular weight of chitosanase. According to Yeon Jin Choi et al.,[21] The molecular mass of the purified chitosanase (45 kDa) is the similar to those of other chitosanases from *Bacillus* species. However, the enzymatic characteristics of the purified chitosanase, such as substrate specificity and specific activity, were different from those of other chitosanases from *Bacillus* species. The specific activities of other purified chitosanases are in the range of 5.84 to 334 U/mg, but the chitosanase of *Bacillus* sp. strain KCTC 0377BP has the highest specific activity, at 1,700 U/mg. Most of the enzymes show high activity only for the chitosans with a DDA of 70 to 100%. However, *Bacillus* sp. strain KCTC 0377BP chitosanase showed a high relative activity against a broad range of deacetylated chitosan (40 to 100%). This broad range of specificity is advantageous for the production of partially deacetylated chitosan oligosaccharides and for the economical use of chitosan. The merine sediments also isolated and the antagonistic activity also observed by previously also supported to the present work.[22-23]

CONCLUSION

This broad range of specificity is advantageous for the production of partially deacetylated chitosan oligosaccharides and for the economical use of chitosan.

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