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Purification and Characterization of Chitinase from Novel Isolate *Streptomyces Rubiginosus* SP24.

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Abstract

In the present investigation, chitinase isolated from novel strain *Streptomyces rubiginosus* SP24 was partially purified by addition of ammonium sulfate to crude broth in stepwise protocol till 80% saturation. After that pellets obtained by centrifugation was dialyzed against buffer till complete removal of the salts takes place. Further purification of enzyme was done by use of protein concentrators (MWCO10kD) which helped in concentrating protein by 2 fold and then chromatographic separation of the pure protein by sephadex G-50 gel filtration technique. The 9th fraction collected after chromatographic separation was further used for characterization. The molecular mass of purified chitinase was approx. 33 kD determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Purified product was characterized for HPLC analysis to detect ability of chitinase to breakdown chitin. Results of HPLC revealed 2.781 RT very close to the RT of standard (NAG). FTIR analysis showed strong signals at 3262.85 and 1637.30 respectively. Hence,pure enzyme thus obtained can be used in various industrial as well as medical applications.

Keywords:Chitinase,Sephadex G-50 gel permeation chromatography, *Streptomyces rubiginosus* SP24

Introduction

Enzymes are well-known bio-catalysts and playing an important part in all stages of metabolic reactionsand few of them are considered of special interest and are utilized as organic catalysts in numerous processes on an industrial scale (Nigam PS, 2013). *Actinomycetal* chitinase are known for applications in industries as well as in agricultural fields as biocontrol agent on commercial scales. *Streptomyces* species are well-known for its ability to decompose chitin completely or partially as it posseses aerial hyphae which can penetrate in substrate (Bai et. al. 2016). Special characteristics of chitinase include their capability and appreciable activity under abnormal conditions, mainly of temperature and pH.For complete hydrolysis of chitin (substrate) present in most phytopathogenic fungi, insects's cuticles and peritrophic membranes that protect intestines of insects,

chitinase is required having higher activity as well as stablity for longer period of time.Most of Pathogenic bacteria that infect intestines are required to pass this chitin-rich barrier first. (Tanaka T et. al. 2003, Shen Z et. al. 1997, Sampson MN et. al. 1998)

Chitinase (EC 3.2.1.14) belongs to glycosyl hydrolase family that catalyzes the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine. It is divided into 3 categories: Exochitinases, representing activity only for the non-reducing end of the chitin chain; Endochitinases, which cleaves internal *β*-1,4- glycosidic bond; *β*-*N*-acetylglucosaminidase, which cleaves sequentiall GlcNAc units from non-reducing end of the substrate (Jha S et. al. 2014).To purify enzyme and for its increased production from *Actinomycetes* is an ongoing research in various biotechnological traits. Removal of impurities present in protein is one of the main undertaking task in three level purification strategy.

The main aim of the study was to purify chitinase from *Actinomycetal* strain and study few characteristics.

Methodology

Sample collection and Selection of *Actinomycetal* strain

On the basis of screening and optimization studies, *Streptomyces rubiginosus SP24* (KT198721) were studied further with respect to the chitinase purification and its characterization (Jha S et. al. 2016).

Inoculum preparation and enzyme production

Culture medium composed of (colloidal chitin-1%, $NaNO_3$ - 0.2 g, K_2HPO_4 - 0.1 g, $MgSO_4$ - 0.1 g, $CaCO_3$ - 0.1 g, $FeSO_4 \cdot 7H2O$–0.001 g, KCL-0.05 g). Medium fermentation was carried out for 72 hrs in 250 ml Erlenmeyer flasks having 100 ml of culture medium. 10 ml of activated starter culture was added to each 100 ml medium. After completion of incubation period, medium was centrifuged at 10,000 rpm for 10 min at 4°C. Cell-free supernatant thus obtained was used as crude chitinase enzyme.

Purification strategies for chitinase:

Enzyme precipitation by Ammonium Sulphate Fractionation

Chitinase enzyme was partially purified using ammonium sulphate precipitation technique. For this, 2000 ml of crude chitinase enzyme was centrifuged at 10,000 rpm for 10 min and supernatant thus obtained was saturated to 80% with the use of ammonium sulfate. Precipitates were collected after centrifugation at 10,000 rpm for 10 mins, 4°C which was then again dissolved in minimal volume of potassium phosphate buffer (0.5M, pH -7) and chitinase activity (Mathivanan et al., 1998) and protein concentration (Lowry et. al. 1951) was measured respectively.

Salting out using Dialysis membrane and concentrating protein with use of Protein concentrators

The precipitate obtained after ammonium sulfate fractionation was dissolved in minimal volume of potassium phosphate buffer (0.5M, pH -7) and filled in dialysis membrane and dialysed against same buffer overnight. Three times recurrent changes of buffer was done to remove ammonium

sulphate. Chitinase activity and protein concentration was measured simultaneously.Proteins which were not dissolved after dialysis was removed by centrifugation at 10,000 rpm for 10 mins and supernatant thus obtained was concentrated using protein concentrator (Sigma - 10 kD MWCOF, 15 ml capacity) at 4,000 × g for 20 mins and concentrated sample was collected using 200 microlitre pipette.

Sephadex G-50 gel permeation chromatography:-

Final purification step to achieve purified chitinase enzyme was done with Sephadex G-50 (Sigma). Column was prepared by pouring 1 gm of Sephadex G-50 which was allowed to get swollen overnight in 9ml double distilled water and 2ml methanol. Swollen sephadex was transferred to the column (15 cm × 2.0 cm) and allowed to settle in the column. Pre-equilibration of the column was done with phosphate buffer (0.5 M, pH-7.0) and concentrated sample was passed through the column with flow rate of 1ml/min and eluted using same buffer. The active fractions were pooled and analyzed for chitinase activity and protein content.

SDS-PAGE analysis:

Active fraction showing maximum activity was subjected for analysis of molecular weight and purity of the enzyme with use sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli, (1970). 5% and 10 % stacking and separating gel were prepared respectively for SDS-PAGE and run was carried initially at 80 V and then after increased gently to 100V at the end of experiment. Protein bands were visualized by staining the gel in Coomassie Brilliant Blue R-250 and further stored for characteristic study.

HPLC Analysis:

The purified compound obtained through gradient column chromatography was subjected to HPLC analysis having Shimadzu LC- 2010 HPLC system (Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell. The column used was a C-18 (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Then, a 20-µl of sample was chromatographed using linear gradients of Acetonitrile – Water from 70% to 55% in 30 minutes at a flow rate of 1 ml/minute. The oligosaccharides were monitored at 205 nm with a spectrophotometric detector (Mathur et. al. 2011).

Fourier Transform-Infra red (FT-IR) Spectroscopy

Functional groups in the purified enzyme exhibiting chitinase activity were determined by Fourier transform-infra red (FT-IR) spectra recorded on an FT-IR spectrometer (Bruker) using the test compound (chitinase) and N-acetyl glucosamine (standard).

Results and Discussion

Purification strategy:

Purification of enzyme from fermentation medium is one of the most critical footstep towards isolating protein. Various techniques with modification in traditional methods are now for purification strategy. This holds importance as enzymes have various applications in day-to-day life. Characteristic study of enzyme would help in revealing their property and its use in various fields.

Ammonium sulfate is most selective for protein precipitation as it is highly soluble in water at lower temperature and hence this method was selected. Protein was precipitated out with step wise protocol followed by addition of ammonium sulfate ranging from 0% to 100%. Maximum specific activity was obtained at 80% saturation about 2.468 U/mg and total protein content was found to be at 60.25 mg and then after it decreased. Dialysed enzyme showed increase in specific activity 3.75 U/mg but decrease in total protein content was measured 256 mg as compared to that of crude and ammonium sulfate fractionation. On further concentrating protein with aid of protein concentrators specific activity increased to 4.79 U/mg while total protein content decreased to 132.6 mg showing 3.44 fold increase in recovery of enzyme. Final purified chitinase enzyme was obtained in 9th fraction eluted from sephadex G-50 gel filtration technique and 5.022 % yield of chitinase was obtained (Fig. 1).

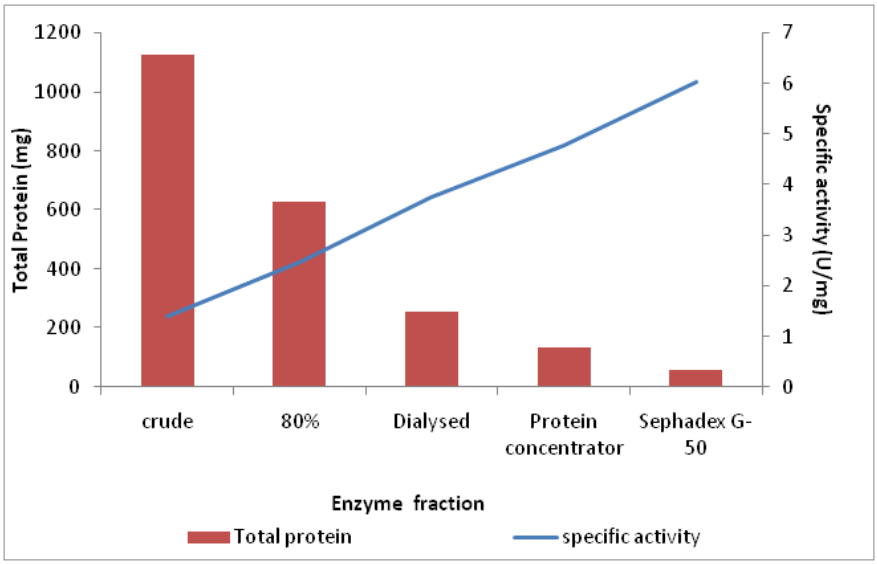


Fig. 1 Purification strategy for chitinase from *Streptomyces rubiginosus SP24*

As reported by Mukherjee and Sen (2006), chitinase from *Streptomyces veezuelae P10* was found to get precipitated maximum at 80% saturation, thereby yielding 16.1% enzyme recovery and about 2.47 fold increase in purification strategy was obtained.

SDS-PAGE Analysis:

Molecular weight of chitinase was found to be approximately 33 kDa from sephadex G-50 gel permeation chromatography. Characteristic study by SDS-PAGE analysis of the purified chitinase revealed one protein bandwith an approximate molecular weight of 33 kDa (Fig. 2).

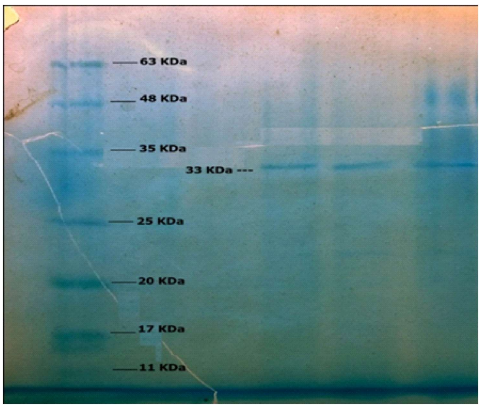


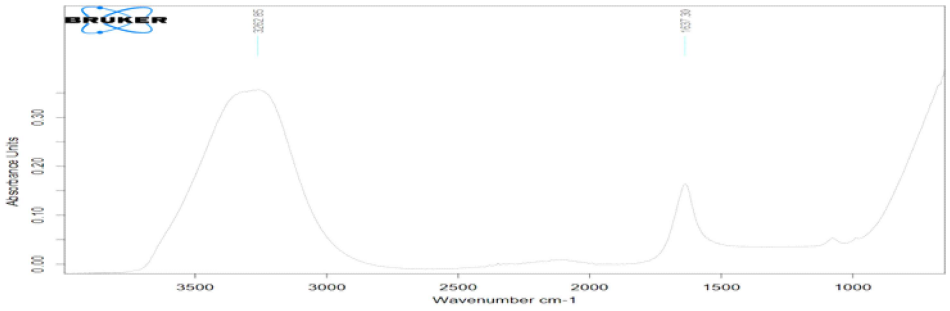
Fig 2 lane-1 Std. protein markers, LANE-2 partially pure enzyme after using protein concentrators (10KD MWCOF) LANE-3 Pure enzyme after Sephadex G-50 gel permeation chromatography LANE-4 Crude enzyme after Ammonium sulfate precipitation

The molecular weights of chitinases from *Actinomycetal* isolates were reported by various researchers such as from *Streptomyces sp*. NK 1057 43 and 45 kD, from *Streptomycesalbovinaceus* S -22 49 kDa, from *Streptomycessp.* TH-11 29 kDa, from *Streptomycesgriseus HUT 6037* 44 kDa, from *Streptomycesanulatus* 38 kDa (El-Sayed et. al. 2000; Nawani and Kapadnis, 2004; Hoang et. al. 2011; Tanabe et. al. 2000; Bhattacharya et. al. 2007; Narayana and Vijaylakshami 2009; Mander et. al. 2016).

FT-IR analysis of purified chitinase:

Partial characterization by FTIR of pure enzyme obtained from *Streptomyces rubiginosus SP24* showed the presence of N-H bond strectching at 3262.85 and presence of carbonyl group at 1637.30 wavenumber cm^{-1} (Fig. 3 [A] and [B])

[A]



[B]

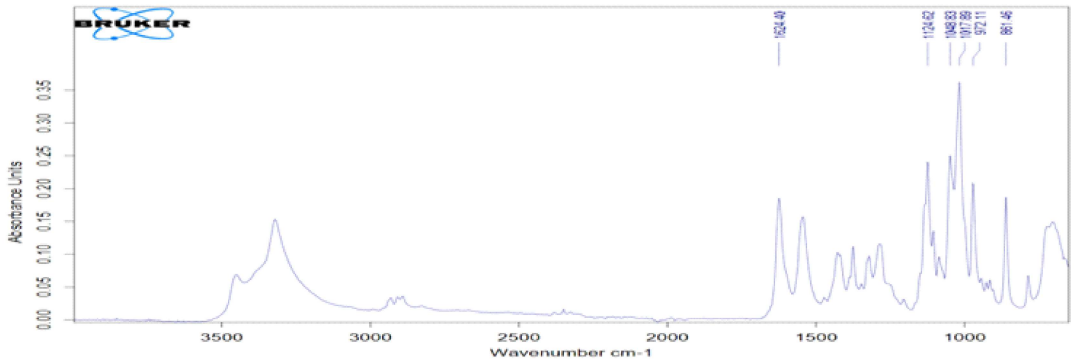


Fig 3 [A] and 3 [B] FT-IR analysis of Standard (N- acetyl glucosamine) and purified chitinase

HPLC analysis of purified enzyme:

Degradation of colloidal chitin with purified chitinase formed product which showed 2.781 Retention Time of 2.781 very close to the Retention Time of standard (N-acetyl glucoamine) 2.871 (Table1).

Table 1. HPLC chromatogram report for purified chitinase

Sr.No	R.T	Area	Height	Area (%)
Standard	2.906	152238.9688	2735.1824	100.0000
Sample	2.871	4705.0044	230.9068	100.0000

Conclusion

Partial purification of chitinase was carried out by ammonium sulfate precipitation (80%) technique and dialysis for the same to remove salts, further using protein concentrators (MWCOF-10KDa) to concentrate maximum amount of protein.Sephadex G-50 gel permeation chromatography was done as a final step towards purification of protein.SDS-PAGE results revealed that molecular weight of protein near 30-35 KD (33 KD). Results of HPLC revealed retention time of chitinase very close to the retention time of standard (NAG). FTIR analysis showed strong signals depicting functional groups N-H bond as well as presence of carbonyl group in purified chitinase.

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References

Bai Y, Eijsink VGH, Kielak AM (2016). Genomic comparison of chitinolytic enzyme systems from terrestrial and aquatic bacteria. *Environ Microbiol* 18: 38–49.

Bhattacharya D, Nagpure A, Gupta RK (2007). Bacterial chitinases: Properties and potential. *Crit. Rev. Biotechnol.* 27:21-28.

El-Sayed ESA, Ezzat SM, Ghaly MF, Mansour M, El-Bohey MA (2000). Purification and characterization of two chitinases from *Streptomyces albovinaceus* S-22. *World J. Microbiol. Biotechnol.* 16:87-89.

Hoang KC, Lai TH, Lin CS, Chen YT, Liau CY (2011). The chitinolytic activities of *Streptomyces sp.* TH-11. *Int. J. Mol. Sci.* 12:56-65.

Jha S, Jha CK, Modi HA, Saraf M (2014). Microbial chitinases: manifestation and prospective. Inustrial Microbiology: Microbes in Process. (eds.) Dr. Neelam & Dr. Abhinav Aeron. *Nova Science Publishers,* pg. 147 – 162.

Jha S, Modi HA and Jha CK. Characterization of extracellular chitinase produced from *Streptomyces rubiginosus* isolated from rhizosphere of *Gossypium sp*., *Cogent Food & Agriculture*, 2016, 2: 1198225.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,*227: 680-685.

Lowry OH, Rosebrought NJ, Farr AL,and Randall R J (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem*., 193, 265–275.

Mander P, Cho SS, Choi YH, Panthi S, Choi YS, Kim HM, Yoo JC (2016). Purification and characterization of chitinase showing antifungal and biodegradation properties obtained from *Streptomyces anulatus* CS242. Arch. Pharm. Res. 39(7):878-886.

Mathivanan N, Kabilan V and Murugesan K (1998). Purification, characterization, and antifungal activity of chitinase from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, *Pucciniaarachidis.Canadian Journal of Microbiology*, 44: 646–651.

Mathur A, Rawat A, Bhatt G, Baweja S, Ahmad F, Grover A, Madhav K, Dhand M, Mathur D, Verma SK, Singh SK, Dua VK (2011). Isolation of Bacillus producing Chitinase from Soil: Production and Purification of Chito-oligosaccharides from Chitin Extracted from Fresh Water Crustaceans and Antimicrobial Activity of Chitinase. *Recent Research in Science and Technology*, 3(11): 01-06.

Mukherjee G, Sen SK (2006). Purification, Characterization, and Antifungal Activity of Chitinase from *Streptomyces venezuelae* P10. *Current Microbiol*, 53(4):265-9

Narayana KJP, Vijayalakshmi M (2009). Chitinase production by *Streptomyces sp*. ANU 6277. Braz. J. Microbiol. 40:725-733.

Nawani NN, Kapadnis BP (2004). Production dynamics and characterization of chitinolytic system of *Streptomyces sp.* NK 1057, a well-equipped chitin degrader. *World J. Microbiol. Biotechnol.* 20:487-494.

Nigam PS (2013). Microbial Enzymes with Special Characteristics for Biotechnological Applications. *Biomolecules*, *3*(3), 597–611. http://doi.org/10.3390/biom3030597

Reissig JL, Strominger JL, and Leloir LF (1955). A modification colorimetric method for the estimation of N-acetylamino sugars. *The Journal of Biological Chemistry*, 27: 959–966

Sampson MN, Gooday GW (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiol*, 144:2189–2194. doi: 10.1099/00221287-144-8-2189.

Shen Z, Jacobs-Lorena M (1997). Characterization of a novel gut-specific chitinase gene from the human malaria vector *Anopheles gambiae*. *Biol Chem,* 272(46): 28895–28900. doi: 10.1074/jbc.272.46.28895.

Tanabe T, Kawase T, Watanabe T, Uchida Y, Mitsutomi M (2000). Purification and characterization of a 49-kDa chitinase from *Streptomyces griseus* HUT 6037. *J. Biosci. Bioeng,* 89 (1): 27 -32.

Tanaka T, Fukui T, Atomi H, Imanaka T (2003) Characterization of an exo-â-Dglucosaminidase involved in a novel chitinolytic pathway from the hyperthermophilic *Archaeon thermococcus kodakaraensis* KOD 1. *J Bacteriol*, 185 (17) : 5175–5181. doi: 10.1128/JB.185.17.5175-5181.2003.

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