Chemotherapeutic Potential of Intracellular Chit S6 against *Aspergillus niger*

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Abstract:

Microorganisms have always been regarded as a treasure source of useful enzymes. During the last twenty years, biochemical reactions performed by microorganisms or catalyzed by microbial enzymes have been extensively evaluated. This investigation aims at selection of chtinase producing bacterial sp. on colloidal chitin agar medium from soils and isolation of intracellular enzyme for therapeutic purpose. Total 9 isolates was recovered, purified and after screening the bacterial isolate chit S6 was selected and Ammonium sulfate crystallized, dialysed intracellular enzyme chitS6 used for chemotherapeutic activity against pathogenic fungi *Aspergillus niger*..Chitin consists of β-1, 4-linked *N*-acetyl glucosamine residues that are arranged in antiparallel (α), parallel (β), or mixed (γ) strands, with the α-configuration being the most abundant. The degree of deacetylation also varies from 0 to 100% (chitosan). Except for the β -chitin of diatoms (chitan), chitin is always found crosslinked to other structural components, such as proteins and glucans. The selected and purified strain of the isolate chit S6 were further diluted to 10^{-1} , 10^{-3} ,10^{-5} and 0.1 ml of these dilutions are plated by spread plate technique on NA medium. These plates were incubated at 27^{0} C .The Visual Examination Was Carried Out at intervals of seven days Up to 38^{th} day. The work shows potential use of chitinase producing bacterial sp. Such as chit S6 as an antifungal agent.

Key words: Colloidal Chitin Agar, Chit S6, Chemotherapeutic, *Aspergillus niger*

Introduction:

Fungal infections have become a major disease concern over the last three decades, in particular for recipients of solid organs and hematopoietic stem cells, AIDS patients, and burn victims, all of whom are usually immunosuppressed for extended periods of time their prolonged immunosuppressed status leads to an increased risk of contracting opportunistic IFIs. IFIs are also on the rise in intensive care settings, likely due to a growing use of procedures with invasive medical devices and long-term use of antibiotics in all cases, the most common etiological agents are *Candida albicans* and *Aspergillus fumigates, Aspergillus niger*

Screening may be one of the most efficient and successful ways of searching for new or suitable microbial enzymes. Because of the inherent rigidity of chitin, fungi need to partially hydrolyse the chitin layer for cell division and morphogenesis, which is carried out by family 18 chitinases.Two subclasses of family 18 chitinases exist: the 'bacterial-type' chitinases are found in bacteria, fungi and mammals; the 'plant-type' chitinases are found exclusively in plants and fungi. Whereas the 'bacterial-type' enzymes are invariably secreted and mostly possess exochitinase activity, the 'plant-type' chitinases are frequently cell wall associated and possess endochitinase activity. Several studies have shown that these enzymes are involved in yeast mother–daughter cell separation. Because these enzymes are not intracellular, it is possible to explore a wider area of chemical space for inhibitors, as these would not be required to cross membranes. Whilst humans possess two active chitinases.

Chitin is the most plentiful source of a natural organic compound after cellulose .This long chain biopolymer containing N-acetyl-Dglucosamine (GLcNAc) monomers form covalent β- 1, 4 linkages . Chitin is widely dispersed in the structural component of many organisms that include crustacean and mollusk shells, arthropod exoskeletons and fungal cell walls. Chitinases play an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. Production of chitinase is widespread in a variety of organisms such as bacteria, fungi, actinomycetes, yeasts, plants, protozoans, coelenterates, nematodes, arthropods and humans. Chitinases have received increasing attention because of their broad applications in the fields of medicine, agriculture, biotechnology, waste management and industrial applications, which include antifungal, hypocholesterolemic, antihypertensive activities and food quality enhancers.

Chitinase is widely distributed among plants, microorganisms, marine invertebrates, fish and insects. It plays a variety of important roles in these organisms ranging from nutrition to defence and control of ecdysis in insects. All these roles involve the degradation of chitin [b-(1,4)-linked homopolymer of N-acetyl-d-glucosamine units], which is mainly used as a structural polymer by these organisms. The degradation of the chitin molecule is achieved by a variety of chitinolytic enzymes that contribute to the degradation process indifferent ways. Exochitinases release short oligosaccharides(most often chitobiose) from the ends of the chitin chains. b-N-Acetyl glucosaminidases release N-acetylglucosamine monomers whereas endochitinases cleave randomly within the chitin chain. The importance of chitinases in many biological processes makes their inhibitors important targets for potential antifungal and insecticidal agents as well as antimalarial agents. This study primarily aims at the isolation and identification of native chitinolytic bacterial strains producing anti-fungal activity.

Aspargillus niger fungal strains were selected for this study as these strains are well known for their pathogenesis.

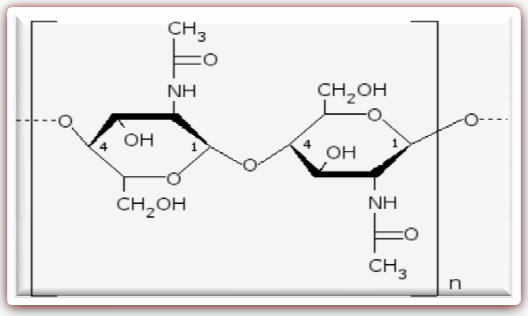


Figure 1.0 Structure of the chitin molecule

Structure of the chitin molecule in **Figure 1.0** shows two of the N-acetylglucosamine units that repeat to form long linear chains in beta-1, 4 linkages. Chitin consists of β-1, 4-linked *N*-acetylglucosamine residues that are arranged in antiparallel (α), parallel (β), or mixed (γ) strands, with the α-configuration being the most abundant. The degree of deacetylation also varies from 0 to 100% (chitosan). Except for the β -chitin of diatoms (chitan), chitin is always found crosslinked to other structural components, such as proteins and glucans Chitin can vary by the arrangement of N-acetylglucosamine strands, degree of deacetylation and presence of cross-linked structural components, such as protein and glucans. Given the great diversity of possible chitin structures, perhaps it is not surprising that actinomycetes typically produce more than one type of chitinase. Not surprisingly, cells grown on α- and β-chitin excreted different chitinases. The composition of excreted chitinases also varied when the cells were grown on colloidal, regenerated, and glycol chitins, even though all originated from α-chitin. These chitins still vary, however, in strand opening, degree of deacetylation, solubility, and probably the presence of covalently linked components other than *N*-acetyl-glucosamine.

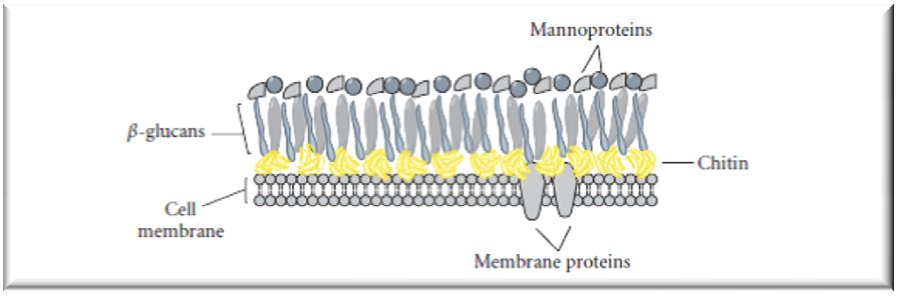


Fig 2.0 Fungal cell wall components. The fungal cell wall contains a cell membrane with various membrane proteins, a protective layer of chitin (yellow) as well as glucans (mostly beta), and mannoproteins on its surface. (Different fungal cell walls contain different glucans.)

Purification of Chitinase:

Purification of protein or enzyme is depending on its sole structure and it will determine both of its biological function and of properties in solution. Purification process is to break up the protein from a complex mixture of protein while maintain its biological function. All purification method conducted based on its electrostatic properties, solubility, ionic strength, stability toward pH and temperature and the molecular size of the protein. The ideal purification were strives to obtain maximum recovery of the desired protein, with least loss of activity and combined with the maximum removal of other contaminants such as proteins and nucleic acid besides trying to retain the biological activity of interest enzyme. The purification study was conducted to identify the certain action in a cell such reaction goes by the enzyme, the metabolic pathway and the regulation of pure enzyme or protein.

Ammonium Sulfate Precipitation

Ammonium sulfate is the salt of choice in chitinase purification since it be full of useful features such as pH versatility, effectiveness in salting out, high solubility and also economical. It is a convenient and non-denaturing method in separate and concentrates the protein. This technique is based on the ionic strength of the protein interest. Thus, when different concentration of salt is added based on standard table, different ionic strength will be obtained until protein molecule tends to interact to one another compared to water molecule and precipitate out. This process is known as salting out. To retain its native conformation, the precipitate can be dissolved again the suitable buffer.

Protein precipitation using ammonium sulfate achieved by dehydration of protein molecule by addition of salt in protein containing solution to increase the protein ionic strength. In solution of protein, a large number of water molecule around the protein strongly bound to the sulfate ion (SO_4^{2-}), and reducing the amount of water availabilities to interact with protein molecules. At a particular concentration of salt, an insufficient quantity of unbound water will remain to keep a given protein in solution and resulted precipitation of that protein. Ammonium sulfate precipitation is compromise between recovery and purity. The temperature condition should be kept at low due to maintain its stability and decrease solubility). High salt concentration will stabilize the enzymes and inhibit protease.

Dialysis

Dialysis method is typically used for desalting and concentrating the protein. Dialysis is a separation process that takes advantage of osmotic forces between two liquids or a liquid and solid. It is not used in separation of enzyme from each other, but it is used to remove residual ammonium sulfate, organic solvent or low molecular weight inhibitor such as sugar or organic acid and at the same time equilibrating the sample in new buffer which means of concentrating a dilute solution. The protein solution was contained within dialysis bag with certain molecular weight cut off which prevents the protein from escaping to dialysis buffer. This process separates two phases containing different molecular weight molecules through the semi-permeable membrane according to the pore size. Only molecules and ions smaller than the molecular weight cut off (MWCO) of the membrane will move out of the dialysis bag. Although this conventional method is time consuming, it is inexpensive and effective in removing all small molecules from protein solution.

Affinity Chromatography and Gel filtration

Affinity chromatography is a procedure that involves a specific interaction between the protein of interest and the affinity matrix. It separates protein based on biospecific but reversible interaction between protein of interest and a specific ligand that covalently attached to chromatography matrix. The ligand must retain its specific affinity for target protein and, after washing unbound material the binding between the ligand and target protein must reversible to allow the target protein to remove in an active form. Affinity chromatography purification is a time-saving procedure and the concentrating effect enable large volumes to be processed.

Gel filtration chromatography is also known as molecular sieving or gel permeation chromatography. The primary objective of gel filtration is to achieve rapid separation of molecules based on size . The separation sizes are made based on their ability to enter the pores within the beads of beaded gel. The gel, which is in bead form, consist of open, cross linked three dimensional molecular network of pores into which molecules of less than maximum pore size may penetrate. The high molecular weight protein molecules are not accessible to penetrate into the porous bead and thus it flow faster out of the gel compared to low molecular weight of protein. Gel filtration also used to determine protein molecular size.

Materials and Methods

Aspergillus niger NCIM 620 strain was obtained from National collection of industrial microorganisms, NCL, Pune, India, chitin crab shell purchased from Himedia, Mumbai, India
Isolation of Chitinolytic Microbes from Soil a number of local strains were isolated from the different soil samples collected from eastern part of Maharashtra, India. The isolated strains were purified according to the standard microbiological techniques. Strains were identified as described .The purified isolates were maintained in the nutrient agar slants at 4^0 c.

Preparation of Colloidal Chitin:

Colloidal chitin was prepared according to the method described by Robert and selitrennikoff (1988) with slight modifications , 10 g of chitin from crab shell (*Himedia, Mumbai*) was added into 150 ml concentrated Hcl and stirred overnight at 4^0 c.The mixture was added slowly to 1000 ml ice cold ethanol (96%) and kept overnight at room temperature with vigorous stirring the precipitate was collected by centrifugation at 5000 rpm for 20 min, washed three times with 0.1 M Sodium phosphate buffer of P^H 7.0 and stored at 4^0 c.

Screening for Potent Strains: Purified strains were plated on the chitin plates for the determination of their chitinase activity. Chitin plates were made with use of chitin agar medium containing 2% colloidal chitin (crab shell), 0.5% yeast extract, 0.05% MgSO4, 0.2% sodium nitrate, 0.05% KCl, FeSO4 pinch, 0.1% K2HPO4 and 2% agar (w/v), adjusted to pH 6.0 using 1 N NaOH/HCl. Colloidal chitin was processed using crab shell . The medium was autoclaved at 121_C for 15–20 min. The purified isolates were inoculated onto chitin plates and incubated for 3–5 days at 30 ± 2^0 C. The potent strain was selected on basis of the formation clear zones on the plates.

Plate examination for fungal Production:

The selected and purified strain of the isolate chit S6 were further diluted to 10^{-1} ,10^{-3} ,10^{-5} and 0.1 ml of these dilutions are plated by spread plate technique on NA medium. And these plate were incubated at 27^0 C .The Visual Examination Was Carried Out at intervals of seven days Up to 38^{th} day.

Extraction and Purification of Enzyme:

The selected and purified strain of the isolate chit S6 was further cultured in colloidal chitin broth of 1000 ml.Incubated viable culture was centrifuged at 7000x .Supernatant was treated with 60% Ammonium sulfate at 4^0 c then crystallized enzyme dialyzed overnight at 4^0 in glycine Further purification carried out by column chromatography using Sepharose G 25 .Purifed enzyme soded as Chit S6. Polyacrylamide gel electrophoresis was carried out on Chit S6 for further characterization of proteins with small modifications.

Chemotherapeutic Activity of Chit S6:

Chit S6 were prepared in different concentrations as 10 µg/ml to 50 µg/ml cup plate method used for efficacy testing against *Aspergillus niger NCIM 620* which further grown on Potato dextrose Medium .Activity confirmed microscopically by observing fungal morphology.

Result and Discussion:

Screening for Potent Strains

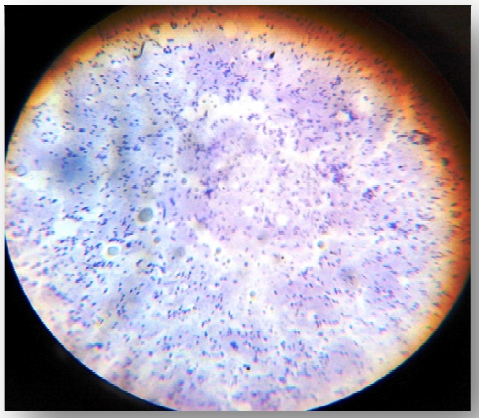
Total 9 isolates was recovered based on clearing Zones around the colonies, the bacterial isolate chit S6 was selected based on observations (Table 1). Chit S6 characterized by different biochemical tests (Table 2). It was found that the Chit S6 was Gram positive Organism, small rod shaped. (Fig 3.0)

Table 1: Screening For Selection of the Strain

Soil Sample No.	Soil Characteristics		Growth On Colloidal Chitin Agar	No. of isolates based on clearing Zones	Isolated Strain Code
	Color	P^H			
1	Brown	6.5	+	1	Chit S1
2	Black	6.0	+	-	Chit S2
3	Red	6.6	++	1	Chit S3
4	Brown	7.0	++	1	Chit S4
5	Black	7.0	++	1	Chit S5
6	**Black**	**7.4**	**++++**	**2**	**Chit S6**
7	Red	7.0	+	1	Chit S7
8	Brown	6.5	+	1	Chit S8
9	Red	6.5	+	-	Chit S9
10	Brown	6.0	+	-	Chit S10
11	Black	7.4	++	1	Chit S11
Total No.of Isolates				9	

Table 2: Characteristics of isolate Chit S6

Characteristics	Observation
Straw-colored colonies	+
Oxidase production	-
Catalase production	+
Hydrolysis of gelatin	+
Urease activity	-
Argentine dihydrolase activity	+
Indole production	-
Growth on Nutrient Agar	+
Growth on Potato Dexrose Agar	-
Growth on MacConkey's Agar	-



+ Positive; - Negative

Fig: 3.0 Gram Positive Chit S6

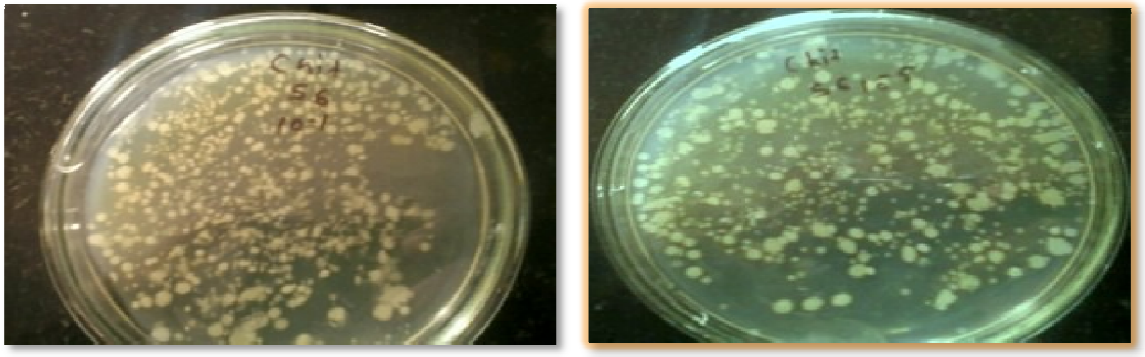
Plate examination for fungal Production:
The Visual Examination Was Carried Out at intervals of seven days Up to 38th day. There was no fungal growth observed when compared with control up to 38 days whish indicates ChitS6 as potential chemotherapeutic agent against fungal sp. (Table 3, Fig4.0)

Table 3: Isolate ChitS6 Examination at 27^{0}C.

Plate	Time of Incubation in days						
	0 day	7 day	14 day	21 day	28 day	32 day	38 day
10^{-1}	-	-	-	-	-	-	-
10^{-3}	-	-	-	-	-	-	-
10^{-5}	-	-	-	-	-	-	-
Control	-	+	++	+++	++++	+++++	++++++

- = No fungal growth observed , + = fungal growth observed

Fig 4.0 Plate Chit S6 10^{-1} and Chit S6 10^{-5} at end of 38 day of incubation.



Extraction and Purification of Enzyme:
The extracted and purified enzyme Chit S6 further characterized by PAGE three fractions of proteins compared with std protein marker it was observed that First fraction have MW of 55KDa resembling heavy chains in proteins .Second fraction of proteins were not giving clear MW for heavy chains. Third fraction of protein has MW 55 KDa but are slightly less heavy than first fraction. It confirmed that Chit S6 a high MW protein with average size of 55KDa.(Fig5.0)

Chemotherapeutic Activity of Chit S6:
The efficacy of ChitS6 was tested against *Aspergillus niger NCIM 620* it was observed that the sample shows good anti fungal activity as concentration increased to50 µg/ml. (Table 4). When 0.1 ml of 50 µg/ml of sample treated with *Aspergillus niger NCIM 620* on slide microscopically it was observed that the hyphae of fungi were degraded by ChitS6. (fIg 6.0)

Table: 4 Efficacy test on Chit S6

Sr.No.	Concentration of Chit S6	Zone of inhibition in mm*
1	10 µg/ml	8
2	20 µg/ml	10
3	30 µg/ml	12
4	40 µg/ml	14
5	50 µg/ml	15
*Including diameter of Cup 5mm		

Fig5.0 PAGE for Chit S6

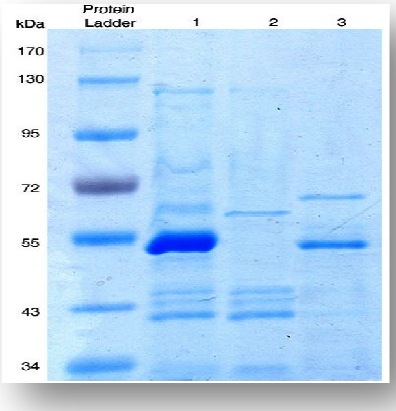
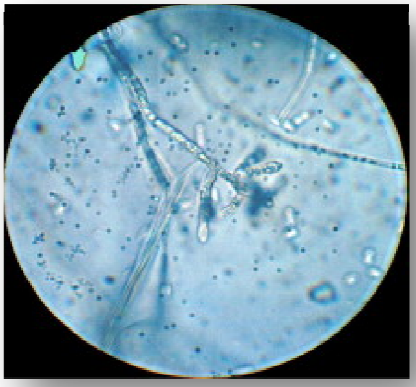


Fig 6.0 Hyphae degradation by ChitS6.



Conclusion:

The natural sources such as soils are playing important role for identifying potential antimicrobial chemotherapeutic agents. This work is the initial findings and target identification, mechanism of action is in process. The future of such enzymes as cytotoxic agents will resemble a suitable anticaner agent. The further study on Chit S6 on Breast cancer cell lines are in progress.

Acknowledgement:

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