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Isolation and Characterization of Chitin from *Alternaria solani* and Assay of its Antibacterial Property

Mayukh Das[1], Subhadip Brahmachari[2], Paulami Koley[2], Amitava Saha[2], Surekha Kundu[1]*

[1] Research Scholar, Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India

[2] Research Scholar, Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road,Kolkata 700019, India

[1*] Assistant Professor, Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India

Abstract

Chitin is the second most abundant polysaccharide in the world and is commonly found in the cell walls of fungi. Chitin has a wide range of biomedical applications and is being extensively studied for their antimicrobial properties. In the present study, chitin was extracted from the phytopathogen *Alternaria solani* (Ellis & Martin) Sorauer. The chitin was extracted by alkali-acid treatment method. The isolated chitin was analyzed by UV-Vis spectroscopy after dissolving in different solvents like methanol, hexane-sulfonate methanol solution and HCl of different concentrations. Different concentrations of the extracted chitin (1mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 50mg/ml) were used for the anti-microbial assay against the gram negative bacterium *E. coli* DH5α. *A. solani* chitin showed antibacterial activity even at the lowest concentration tested which was 1mg/ml. The inhibition zones were found to increase with increase in concentration of chitin and at 50mg/ml concentration of chitin the inhibition zone was 2.15cm in diameter. Therefore this study shows an easy method of extraction of chitin, its characterization and that the chitin extracted from *A. solani* has significant antibacterial activity.

Keywords: *Chitin, antimicrobial properties, Alternaria solani, inhibition zone*

1. Introduction

Chitin is the second most abundant polysaccharide found in nature after cellulose. Chitin is a biopolymer found in various organisms such as crustaceans, insects, annelids, mollusks, coelenterates and is a common material of fungal cell walls (Andrade et al., 2003, Krishnaveni et al., 2015). The name 'chitin' was derived from the Greek word 'chiton', that means "a coat of mail" and was first used by Bradconnot in 1811 (Eagappan et al., 2017). It is a polysaccharide, made up of N-acetyl-D-glucosamine unit which is β 1, 4-linked (Fernandes et al., 2014). The chemical structure of chitin is more or less similar to the cellulose having 2-acetamido-2-deoxy-b-d-glucose (NAG) monomers attached β (1-4) linkages (Shahidi et al., 1999).

Chitin is widely distributed in different classes of fungi such as Basidiomycetes, Ascomycetes, Zygomycetes and Phycomycetes, where it is one of major component of the cell walls of mycelia, stalks and spores. Cell walls of fungal mycelia is composed mostly of chitin in the form of fibrillar polymer and is often associated with glucan molecules in the form of microfibril, which are embedded in an amorphous matrix making up the framework in fungal cell wall (Wu et al., 2004). Fungal cell walls may contain up to 25-30 percent of the dry weight of the cell wall (Latge et al., 2006).

Chitin is known to have antibacterial, antifungal and antiviral properties (Krishnaveni et al., 2015). It is a non-toxic, biocompatible, biodegradable, grease absorbent compound. Chitin is also known as contaminating metal absorbent compound which has

wide-ranging ranging application in various fields of study (Eagappan et al., 2017). Active bio-molecule such as chitin has an important role in food product. It has great commercial importance due their antimicrobial activity, non-toxicity and versatile physical and chemical properties (Dutta et al., 2009). Chitin received much research interests due to having various application such as in agriculture, food industry, biomedicine and textile industry (Ospina et al., 2015).

The are some reports regarding the extraction of chitin from different fungi like *Aspergillus niger*, *Absidia glauca*, *Mucor rouxii*, *Phycomyces blakesleeanus*, *Trichoderma reesei*, *Lentinus edodes*, *Gongronella butleri*. Most commonly utilized fungi is *M. rouxii* in which the quantity of chitin in its mycelium can reach 35% dry weight of the cell wall (Chatterjee et al., 2005, Eagappan et al., 2017).

Nowadays, chitin is also extracted from exoskeleton of different crustaceans though the chemical extraction procedure involves production of harmful byproducts resulting in the environmental pollution. To overcome this problem, the researchers are interested in the fermentation technology to extract the chitin from various fungi like *Agaricus* sp., *Pleurotus* sp., *Ganoderma* sp. and *Fusarium* sp. (Kannan et al., 2010).

One of the major problems in working with chitin is the insolubility of chitin in water (Sugimoto et al., 1998, Novikov 2004). Making of water soluble derivative of chitin through chemical modifications have been reported (Roy et al., 2017). But chemical modifications change the fundamental skeleton of chitin and the modified chitin lose the original physicochemical and biochemical activities (Tanigaya et al., 1992, Novikov 2004, Roy et al., 2017). On the other hand, modification of chitin with a polymer has advantages like the hydrophilic polymer being modified into hydrophilic chitin without affecting the fundamental skeleton (Sugimoto et al., 1998, Roy et al., 2017). Therefore, the solubility of chitin can be increased by deacetylation and hydrolysis (Roy et al., 2017). In the present study the chitin was analyzed by UV-Vis spectroscopy after dissolving in different solvents like methanol, hexane-sulfonate methanol solution and HCl of different concentrations.
There are limited reports regarding the process of easy extraction of chitin especially from fungal sources and its biological applications. The present work deals with the extraction of chitin from the phytopathogen *Alternaria solani*, its characterization and assay of its antimicrobial activity against a common bacterium.

3. Materials and Methods

A. Chemicals

All the used chemicals and reagents were purchased from Hi-Media (Mumbai).

B. Fungal material

The fungi causing early blight disease of tomato, Alternaria solani (Ellis and Martin) Sorauer, Indian Type Culture Collection, Indian Agricultural Research Institute (ITCC No. 4632) was maintained as pure culture on PDB medium as published in our earlier report (Ray et al., 2015).

C. Collection of mycelium for extraction of chitin

The fungal inoculum was taken from slant culture and inoculated in Potato dextrose broth (PDB) medium. Within five days the mycelium started to grow in the broth. Then fungal mycelium from 3 week old culture was taken from PDB culture and washed with sterilized distilled water. The mycelium was then wrapped in aluminum foil and kept in -20^{o}C for 2-3 days.

D. Extraction of chitin

1gm of mycelium was crushed in a mortar pastel with the help of liquid nitrogen. The powder was suspended in 5ml sterilized distilled water. The mycelial suspension was given a short spin in a falcon tube to get rid of larger debris. To the suspension equal volume of 4% NaOH was added such that final concentration of NaOH is 2%. The falcon tube was kept in 90^{o}C for 2 hours. After 2 hours the falcon tube was centrifuged at 4000 rpm for 15 minutes at 4oC and pellet was taken. 10% Acetic acid was added to the pellet in 40:1 ratio (v/w) and incubated in 60^{o}C for 6 hours. After incubation, it was centrifuged at 4000xg rpm for 15 minutes at 4^{o}C. The pellet was collected as chitin. The chitin was kept for 2 to 3 days in room temperature to dry (Krishnaveni et al., 2015).

E. Preparation of different chitin solutions

Different solvents such as water (sterilized double distilled), hexanesulfonate-methanol solution (9:1 and 1:1), methanol (50% and 100%), ethanol (50% and 100%) and HCl (35% and 70%) were used to dissolve chitin followed by spectroscopic analysis. The solvent HCl (35% and 70%) was used according to Novikov, 2004. The extracted dried chitin was mixed at a concentration of 10mg/ml in of each solvent i.e. in sterilized distilled water, hexanesulfonate-methanol solution (9:1 and 1:1), methanol (50% and 100%), ethanol (50% and 100%) and HCl (35% and 70%). The hexanesulfonate-methanol solution was prepared by adding 1.1gm of the sodium salt of hexanesulfonate in 1000 ml Milli-Q water. The

pH was adjusted to 2.1 by adding ortho-phosphoric acid.

F. Hydrolysis of chitin

The deacetylated chitin was hydrolyzed with 35% and 70% HCl according to Novikov 2004. The tube was incubated at 50°C-52°C in water bath for 2 hours. Tube was allowed to cool in room temperature. 5ml of water was added and the pH was 2.5.

G. UV-Vis Spectroscopy of Chitin

Each mixture of chitin in different solvents were filtered using filter paper having pore size of 10µm to filter out the undissolved suspended particles of chitin. The filtered sample was used for spectrophotometric scan in different wavelength light according to Kumirska et al., 2010.

H. Antimicrobial assays

Antimicrobial assay of A. solani chitin was done using E. coli by disc diffusion method. Luria Bertani (LB) broth/agar was used to culture the bacteria. 100µl of fresh overnight cultures of bacteria was spread uniformly on LB Agar plates. Sterilized paper disc of 5mm diameter with 50µl of increasing concentration of chitin in each disc such as 1mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 50mg/ml was placed on the surface of media for the assay. Plates were incubated overnight in 37oC. The inhibition zones from three experimental plates were measured and the average inhibition zone for each concentration was plotted on a graph.

4. Results

A. Extraction of Chitin from *Alternaria solani*

Chitin was extracted from mycelium of 3 week old *A. solani* culture. At the end of extraction procedure, the pellet was collected as creamish white coloured chitin (Fig. 1). The chitin was extracted from *A. solani* at an amount of 144mg/gm of dry mycelium biomass which comprises about 14.4% of dry weight.

B. Characterization of chitin extracted from *A. solani*

UV-Vis spectroscopy was used for the optical characterization of the chitin. The wavelength in the absorption spectrum (λmax) of extracted fungal chitin in each solvent was within 190 to 210nm (Fig. 2). Higher peak of absorbance was found in the highest concentration of every solvent used i.e. 9:1 hexane sulfonate-methanol solution, 100% methanol, 100% ethanol (Fig. 2A, B, C) compared to the lower concentration of the respective solvents used i.e. 1:1 hexane sulfonate-methanol solution, 50% methanol and 50% ethanol. Chitin was least soluble in pure water. But in the case of HCl, the maximum peak of O.D. was found in the case of 35% HCl compare to 70% HCl (Fig. 2D). The comparative data show the presence of higher peak in HCl solution but the peak was lower in the case of other solvents like hexanesulfonate-methanol, methanol, ethanol, the weakest being pure water (Fig. 2E). The absorbance were found to be lower (less than 2) in the other solvents in the 190 nm to 210 nm wave length range whereas the hydrolyzed chitin in HCl solution showed higher (absorbance of 3) in the 190 to 210 nm. The chitin in 35% HCl showed a higher peak at 200 and 210 nm compare to the solution of chitin in 70% HCl.

C. Assay of Antimicrobial activity of chitin extracted from *A. solani*

Antimicrobial activity of chitin extracted from *A. solani* was assayed with different concentrations of chitin on the bacterium *E. coli* DH5α (Fig. 3). The inhibition zones were observed in each applied concentration of chitin (1mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 50mg/ml) (Fig. 3A). In vitro antibacterial screening of the extracted chitin was evaluated and the inhibition zones are given in the graph (Fig. 3B). The inhibition zones were found to increase with increasing concentration of chitin. The highest zone of inhibition was noticed in the applied chitin solution of 50mg/ml. Results of the inhibition zones are given as average of three readings (in cm) in the Table 1.

Table 1: Inhibition zones around the bacterium *E. coli* (DH5α) with different concentrations of chitin extracted from *Alternaria solani*

Chitin concentration (mg/ml)	Diameter of inhibition of zone (cm)
1mg/ml	1.24
5mg/ml	1.45
10mg/ml	1.65
20mg/ml	1.67
50mg/ml	2.15

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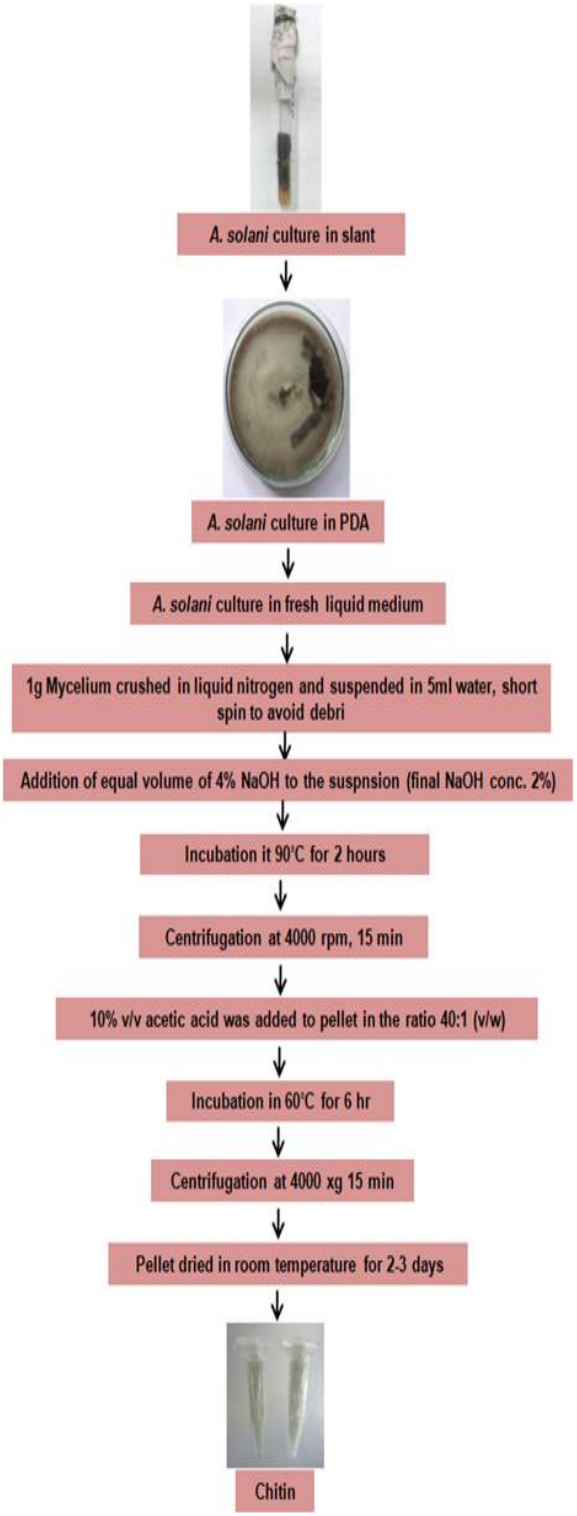

Figure 1: Flow chart of method for extraction of chitin from Alternaria solani

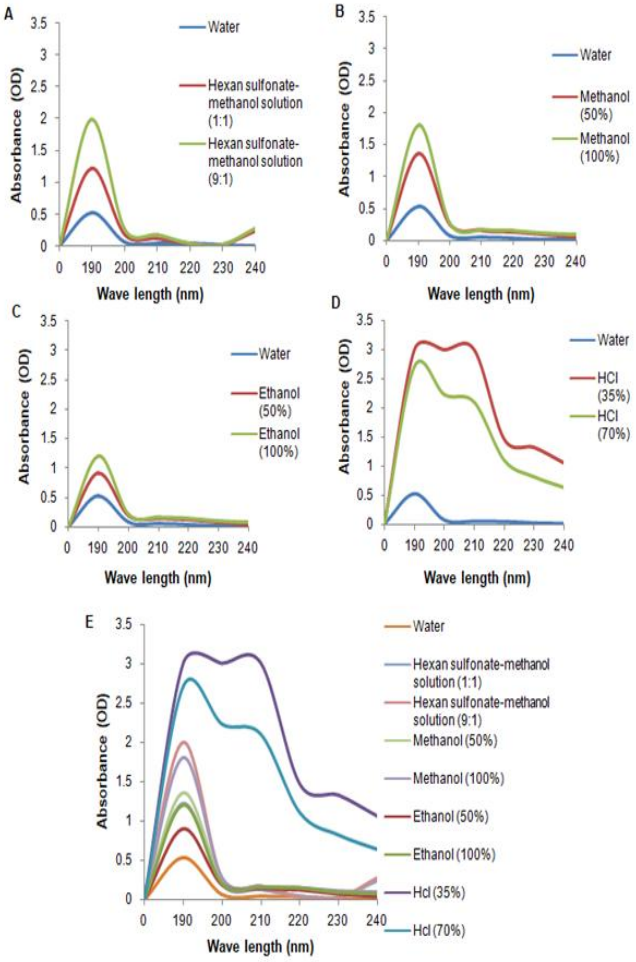

Figure 2: UV-Vis spectroscopy of extracted chitin of Alternaria solani in different concentrations of (A) Hexanesulfonate-methanol solution, (B) Methanol, (C) Ethanol and (D) HCl. (E) Comparative representation of UV-Vis spectroscopy of chitin in different solvents.

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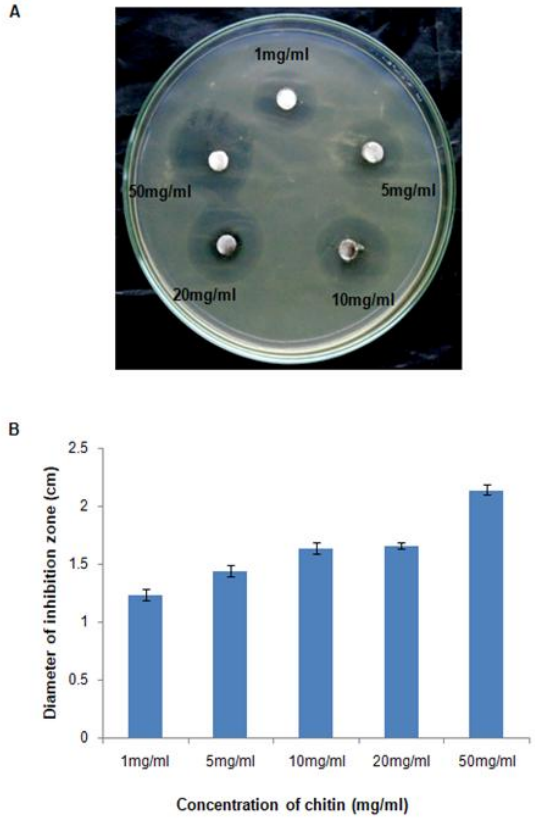

Figure 3: Anti-microbial effect of different concentrations of chitin extracted from A. solani on gram negative bacterium E. coli (DH5α). (A) Inhibition zones formed around on E. coli DH5α in disc diffusion assay using chitin. (B) Graphical representation of comparison of anti-microbial effect of different concentration of chitin.

5. Discussion

In spite of the significance of chitin extraction study, comparative study of chitin characterization in different solvent and its antimicrobial activity background, research in this field is scarce. Insight into the antimicrobial properties of extracted chitin to a common bacterium not only has direct applications, but is of fundamental interest and the knowledge is essential for overall understanding of the mechanism of inhibitory effect. This study characterized chitin chitin in different solvents. In the present study, an attempt has been made to describe the solubility of extracted chitin in different solvents and subsequent spectroscopy. The solubility of chitin from other sources has been reported (Roy et al., 2017). In the present study, chitin was assayed spectroscopically after dissolving in of four solvents solvents of different concentrations. Maximum solubility was obtained in a solution of lower concentration of HCl (35%) rather than the higher concentration (70%). In other solvents (double distilled water, hexanesulfonate-methanol solution, methanol and ethanol), the chitin was not completely soluble due to its chemical structure. There are two monomer units present in the chitin N-acetyl-D-glucosamine and N-amino-D-glucosamine (Eagappan et al., 2017, Roy et al., 2017). Strong hydrogen bonds are present between the acetyl groups of same or adjacent chitin chains (Roy et al., 2017). Moreover the hydrophilic nature of N-amino-D-glucosamine is masked by the hydrophobic N-acetyl-D-glucosamine (Roy et al., 2017). Therefore, the solubility of chitin can be improved by the hydrolysis method (Novikov, 2004). The depolymerization of the chitin always depends on the initial degree of deacetylatio and this deacetylation course depends only on the experimental hydrolysis condition which was done in our experiment. The UV Spectroscopy is subsequently used to measure the concentration of acetylated compound (Novikov, 2004). Nowadays, the antimicrobial activity of chitin against different groups of microorganisms like bactria, fungi and yeast has gained considerable attention (Eagappan et al., 2017). In the present study, significant inhibition zones were obtained around the gram-negative bacterium E. coli DH5α when tested with A. solani chitin. The possible mechanism behind the formation of the inhibition zones around the bacterial colonies can be speculated. According to previous report (Helander et al., 2001), chitin due to having polycationic nature, interacts with anionic groups on the bacterial cell surface. This results in the formation of an impermeable layer around the cell that prevents the transport of essential solutes. Earlier worker (Eagappan et al., 2017) reported antimicrobial activity of chitin extracted from the mushroom *Pleurotus* sp. The present research coincides with these previous observations made on chitin from other sources. Thus chitin extracted from *A. solani* has significant antibacterial activity and may be utilized for wider applications.

6. Conclusions and future aspect

In summary, we can conclude that an easy process of chitin extraction from *A. solani* was developed. The chitin was characterized in different solvents using UV-Vis spectrophotometric procedure. This work also concluded that the best solvent for the extracted chitin amongst the solvents tested, is 35% HCl. In general the poor solubility of chitin in the most common solvents (double distilled water, hexanesulfonate-methanol solution, methanol and ethanol) is a great limitation for utilization of chitin for various purposes. The knowledge generated from this work can be utilized in isolating chitin from the phytopathogen *A. solani* which can be utilized as an elicitor or host defence in laboratory studies. The

antibacterial properties can be utilized in biomedical, agricultural and food industries after further studies.

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