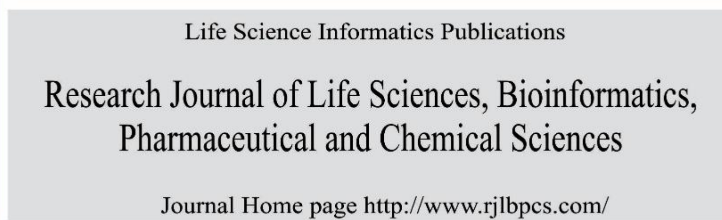

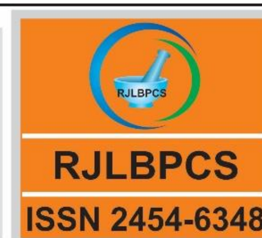

Original Research Article **DOI: 10.26479/2018.0406.48**

ISOLATION AND CHARACTERIZATION OF CHITOSAN FROM *ALTERNARIA SOLANI* AND ASSAY OF ITS ANTIBIOTIC PROPERTY

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ABSTRACT: Chitosan is a natural polymer has wide range of biomedical and agricultural applications specially for its antimicrobial properties. In the present study, chitosan was extracted from the phytopathogenic fungi *Alternaria solani* (Ellis & Martin) Sorauer. The extraction of chitosan was done by a modified alkali-acid method. The *A. solani* chitosan was analyzed by UV-Vis spectroscopy using different pH levels of pH 5, 8 and 11 and with two different temperatures of 25°C and 70°C. A higher peak was observed for pH 8 at room temperature compared to pH 5 a pH 11. Different concentrations of the *A. solani* chitosan (5mg/ml, 10mg/ml, 20mg/ml, 50mg/ml and 100mg/ml) were used for the anti-microbial assays against the gram negative bacterium *E. coli* DH5α. Significant antibacterial activity was observed even in the lowest concentration tested i.e. 5mg/ml. The inhibition zone of 2.25cm diameter was observed in the highest tested concentration of 100mg/ml. The implications of the observations made in the present study are also discussed.

KEYWORDS: Chitosan, Antimicrobial, *Alternaria solani*, Spectroscopic analysis, pH, Temperature.

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1. INTRODUCTION

Chitosan is a natural, biodegradable biopolymer that has received much research interest due to their potential application in different fields such as agriculture, biomedicine, food industry and textile industry [1, 2]. It is a cationic amino polysaccharide composed of β-1, 4 D-glucosamine (GlcNAc)

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linked to N-acetyl-D-glucosamine residues [3, 4, 5]. This polysaccharide is present in a wide range of organisms, such as insects, annelids, crustaceans and cell wall of fungi [6, 5]. Chitosan is soluble in acidic solutions and produced from the deacetylation of chitin [7, 8, 9]. In recent years, several research reports deal with extraction of chitosan but simple methods for characterization and quantitative analysis are still limited. There are several reports regarding the conversion of glucosamine monomer by the hydrolysis of chitosan such as colorimetric, chromatographic and fluorometric techniques [10, 11]. There is a method for determination of chitosan in wood and water sample based on the acid hydrolysis of chitosan to glucosamine by chromatographic separation [12]. A new approach of colorimetric detection of chitosan was developed which can also be used for its quantification [13]. The conditions of this method have been optimized for chitosan analysis in the presence of polyanionic electrolyte dextran sulphate. Most quantitative detection of chitosan involves total hydrolysis to monosaccharide followed by subsequent characterization [14]. Chitosan has glycosidic linkages which are resistant to acid hydrolysis. Such hydrolysis methods are difficult to apply and chitosanases enzyme used for depolymerization, but availability and cost limits the use of this enzyme [11]. Production of chitosan from different organisms has recently received increased attention. Due to its well defined chemical structure, it can be modified chemically and enzymatically [15]. Chitosan has physical and biological significance and finds wide applications. It is not only easily biodegradable; it is biocompatible with many organs, tissues and cells. Therefore much research interest has developed regarding the production of chitosan [11]. Chitosan production from fungal mycelium has received more research interest due to having significant advantages over the sources from other organisms. The seasonal limited supply of the crustacean waste and other source of chitosan by fishing industry is a major limitation [16, 1], whereas fungal mycelium can be obtained easily by fermentation process that does not have seasonal limitations. Moreover lower levels of inorganic materials are present in fungi compared to other sources and this reduces the demineralization techniques required during the processing [17,5]. Fungal chitosan is comparatively more effective in triggering plant immune response and therefore more suitable for agricultural and biomedical applications [18, 1]. There are few reports regarding the extraction of chitosan from different mushrooms like *Lentinus edodes*, *Agaricus sp.*, *Pleurotus sp.*, *Ganoderma sp.* and also there are very few reports of chitosan extraction from filamentous fungi such as *Fusarium sp.*, *Aspergillus niger*, *Mucor rouxii*, *Gongronella butleri* [19]. So, the production of chitosan from filamentous phytopathogenic fungi is even more limited. Nowadays, there is considerable attention in the antimicrobial activity of chitosan against different group of microorganisms such as fungi, yeast, and bacteria [20]. On the whole there are limited reports on the isolation and characterization of chitosan from filamentous fungi. The present study shows a method of isolation of chitosan from the phytopathogenic filamentous fungus *A. solani* and its characterization at different pH levels and temperature using spectrophotometric analysis. Its antimicrobial activity against was also tested

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against a common gram negative bacterium.

2. MATERIALS AND METHODS

2.1. Chemicals

All the used chemicals were analytical reagent grade and purchased from Hi-Media (Mumbai).

2.2. Fungal Material

The fungi causing early blight disease of tomato, *Alternaria solani* (Ellis and Martin) Sorauer, (ITCC No. 4632) was maintained as pure culture on PDB medium as published in our earlier report [21]. Pure cultures were maintained through repeated sub-culture and stored in 4°C.

2.3. Culture and collection of *A. solani* mycelium for extraction of chitosan

The fungal inoculum was obtained from pure culture and inoculated in fresh liquid medium (Potato dextrose broth medium). The mycelium started to grow in the liquid medium within five days. Then fungal mycelium from 3 weeks old culture was collected from liquid culture and washed with sterilized distilled water. The mycelium was then wrapped in aluminum foil and stored in -20°C for 2-3 days.

2.4. Extraction steps of chitosan from *A. solani* mycelium

1gm of mycelium was taken in a mortar pastel and crushed with liquid nitrogen. Then the powder was suspended in 5 ml sterilized distilled water. The mycelial suspension was given a short spin in a falcon tube to avoid the larger debris. Equal volume of 4%NaOH was added to the suspension such that final concentration of NaOH was 2%. The falcon tube was incubated in 90°C for 2 hours. After incubation, centrifugation was done at 4000 rpm for 15 minutes at 4°C and pellet was taken. 10% Acetic acid (v/v) was added to the pellet in 40:1 ratio (v/w) and kept in 60℃ for 6 hours. After 6 hours, it was centrifuged at 4000xg rpm for 15 minutes at 4°C. The supernatant was collected as chitosan. The pH of supernatant was adjusted to 9 by adding 4M NaOH solution. The chitosan was kept for 3 to 4 days in room temperature to dry [22, 5].

2.5. Preparation of chitosan solution in different pH and spectrophotometric characterization

Chitosan stock solution was made by dissolving 100 mg of chitosan in 100 mL of 1% (v/v) aqueous acetic acid; the chitosan dissolved readily on shaking. Six test tubes were taken for the experiment and the 2ml chitosan solution was poured into each test tube. Two test tubes were taken for pH 5, two for pH 8 and two for pH 11. One test tube from each pH was kept at 70°C in a water bath for 30 mins and rest of three test tubes of pH 5, pH 8 and pH 11 were kept at room temperature (27°C). Then 0.1ml of 0.5M $NaNO_2$ was added by micropipette in each test tube. The solution mixture was shaken briefly and was kept at 80°C for 30 minutes in a water bath to complete the reaction of depolymerization. After depolymerization, the pH was adjusted to 8 by addition of 0.2ml of 0.1M NaOH and the solution was then shaken. Then 0.5ml of 0.04M thiobarbuturic acid solution was finally added. The tubes were placed in a water bath at 80°C for 10 min for incubation. Then the solution was cooled. The pH of two test tubes (one test tube for room temperature and one for 70°C)

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were adjusted with 4M NaOH to pH5, two test tubes (one test tube for room temperature and one for 70°C) adjusted to pH 8 and rest two (one test tube for room temperature and one for 70°C) adjusted to pH 11. Then the solution of each test tube was then cooled and the absorbance measured at 555 nm by spectrophotometer against blank [11].

2.6. Antimicrobial assay of the *A. solani* chitosan

Antimicrobial assay of different concentration of chitosan was done on the common gram negative bacterium *E. coli* DH5α by disc diffusion method. Luria Bertani (LB) broth/agar was used to culture the bacteria. 100µl of fresh overnight culture of *E. coli* was uniformly spread on LB Agar plates. Sterilized paper discs of 5mm diameter with increasing concentrations of chitin in each disc such as 5mg/ml, 10mg/ml, 20mg/ml, 50mg/ml and 100mg/ml was used for the assay and incubated for overnight [23].

3. RESULTS AND DISCUSSION

3.1. Extraction of chitosan from *A. solani* mycelium

Chitosan was extracted from 3 weeks old mycelial cultures of *A. solani*. At the end of the extraction procedure, the supernatant was collected and dried as white coloured crystalline chitosan for characterization (Figure 1). With Potato dextrose broth as culture medium the amount of chitosan extracted expressed as mg per gram of dry mycelium biomass is 84mg/g, which is 8.4% of total weight. In spite of the importance of standardized extraction procedure of chitosan from filamentous fungi, the characterization of new chitosan in different pH/temperature and assay of its antimicrobial property, research in this field is scanty. Insight into the antimicrobial properties of the new *A. solani* chitosan towards a common gram negative bacterium not only has direct applications, but is of objective interest. Chitosan can be produced in higher amount by fungal mass culture applying large scale fermentation processes. Since sea food waste supplies are restricted to a particular season, the use of crustaceans as source of chitosan is not sustainable [1]. Finding of alternative sources such as filamentous fungi is of major interest. The research has gained momentum regarding the extraction of chitosan from filamentous fungi. The eco-friendly preparation of chitosan from filamentous fungi is still limited. The method of extraction of chitin from *A.solani* described here is a simple, eco-friendly and cost effective method as opposed to the chemical processes used in the chitosan extraction from crustaceans.

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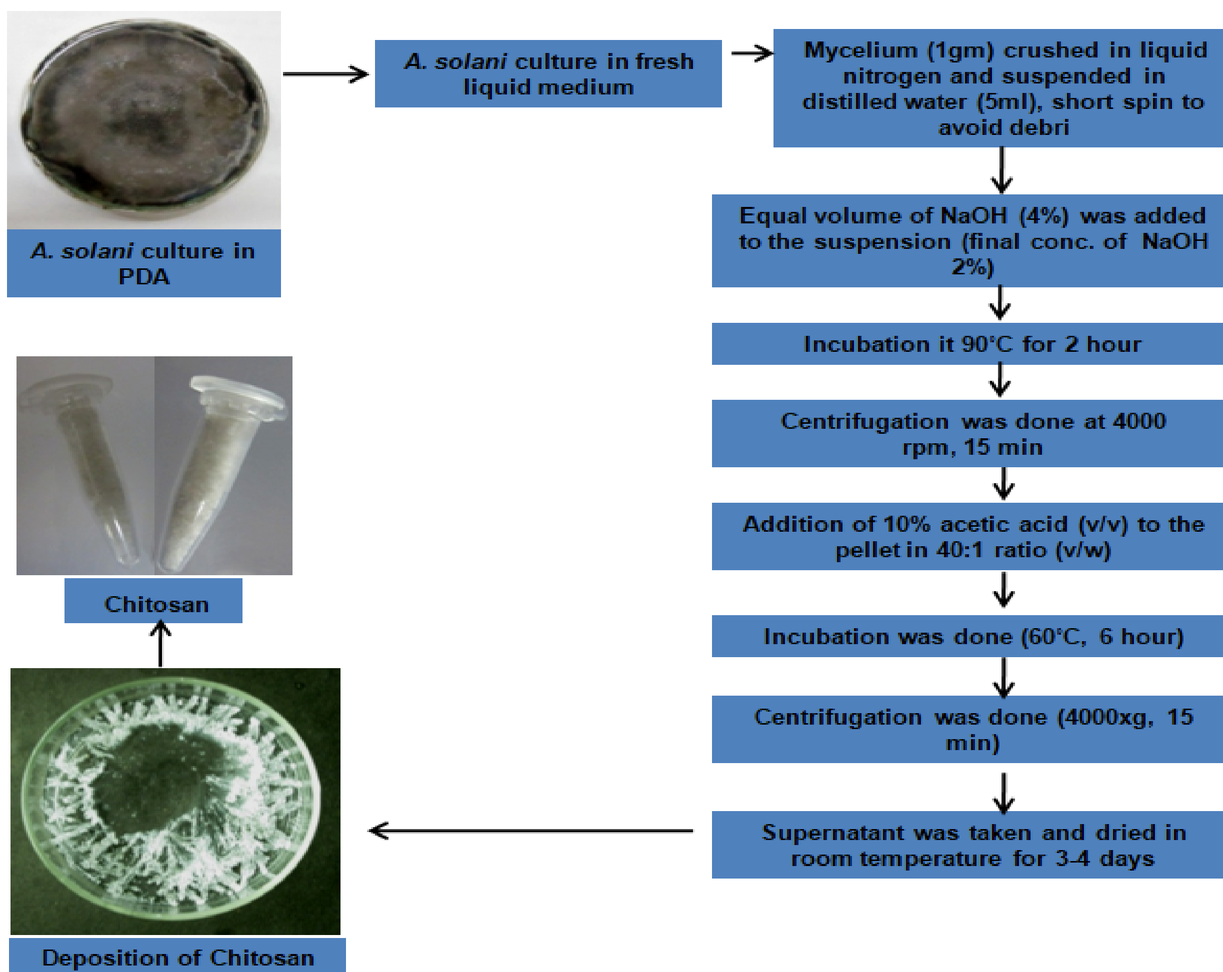

Figure 1: Extraction process of chitosan from *Alternaria solani.*

3.2. Spectrophotometric characterization of *A. solani* chitosan at different pH

UV-Vis spectroscopy was used for the optical characterization of the chitosan (Figure 2). The wavelength in the absorption spectrum (λmax) of extracted fungal chitosan in each solution was 500nm to 590nm. Higher peak of absorbance was found in the chitosan solution at room temperature at every pH level used i.e. pH 5, pH 8 and pH 11 (Figure 2.A, B, C) compared to the chitosan solution at 70°C of each pH applied i.e. pH 5, pH 8 and pH 11. Comparative graphical analysis show higher peak in the chitosan solution of pH 8, but the peak was lower in the chitosan solution of pH 5 and pH 11 (Figure 2.D). The absorbance was found to be lower in pH 5 and pH 11 at 500nm to 590nm wave length range whereas the chitosan solution of pH 8 showed higher absorbance at 500nm to 590nm wave length range. A sharp peak of chitosan was observed at 550nm (absorbance 0.747) in the chitosan solution of pH 8 at room temperature whereas at 550nm, the chitosan solution at pH 5 and 11 did not show any sharp peak at room temperature. The solubility and characterization of chitosan from crustacean waste in different solvents have been reported [24]. In the present study, chitosan solution of three different pH levels and two different temperatures were analyzed spectroscopically. Maximum absorbance was observed in the chitosan solution at room temperature of pH 8 rather than higher temperature (70°C). In the other chitosan solutions i.e. pH 5 and pH 11,

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the absorbance was lower compared to the solution of pH 8. This result may be explained by the fact that, the degree of charging of polymer chains decreases with an increase in pH value. As a result, the aggregation occurs owing to the repulsion and exerting osmotic pressure of counter ions trapped by the aggregates [25, 26]. Moreover, NH2 groups resulting from neutralization of pH may produce additional hydrogen bonds; this condition contributes also to stabilization of compact state [27]. Thus, with an increase in pH i.e. in the basic pH range, the tendency of chitosan towards aggregation becomes more pronounced. This effect is associated with the weakening of electrostatic repulsion related to decrease of charging of chains and formation of additional hydrogen bonds [28, 26]. So, in high pH range, the aggregates of hydrophobically modified chitosan become compact and retain stability in neutral or nearer to neutral pH solution. At the same time, in some cases the aggregation also occurs in acidic pH when polymer chains are fully charged and the attraction between associating group is so strong that it can overcome the electrostatic repulsion and loss in the entropy of counter ions resulting in the formation of aggregations [29, 30]. So, in neutral pH or neared to neutral pH, the chitosan become more stable which coincide with our present result where nearer to neutral pH i.e. pH 8 more stability was observed compared to the chitosan solution of pH 5 and 11.

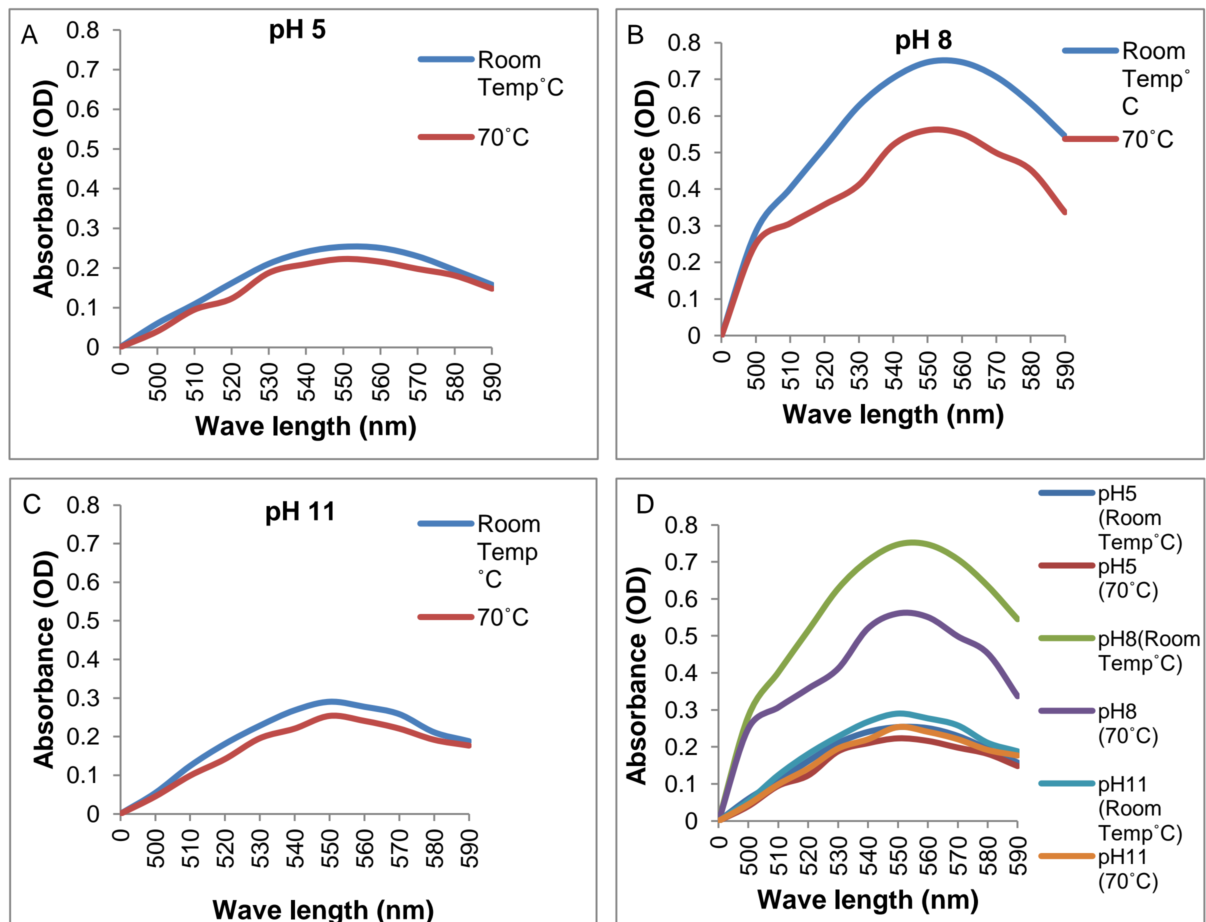

Figure 2: Spectrophotometric characterization of chitosan extracted from *Alternaria solani* at different temperature and different pH. (A) Absorbance at different temperatures and pH 5. (B) Absorbance at

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different temperature and pH 8. (C) Absorbance at different temperatures and pH 11. (D) Comparative representation of absorbance of chitosan solution at different temperatures in different pH.

3.3. Antimicrobial activity of *A. solani* chitosan

In vitro antimicrobial assay was done with different concentrations of chitosan on the common gram negative bacterium *E. coli* DH5α (Figure 3). The inhibition zones were visible in each applied concentration of chitosan (Figure 3.A). For comparison, the inhibition zones were plotted on a graph (Figure 3.B). The inhibition zones were found to increase with increasing concentration of chitosan. The inhibition zone was of diameter of 2.25 cm) in the highest applied chitosan solution of 100mg/ml. Results of the inhibition zones are given as average values (cm) in Table 1.

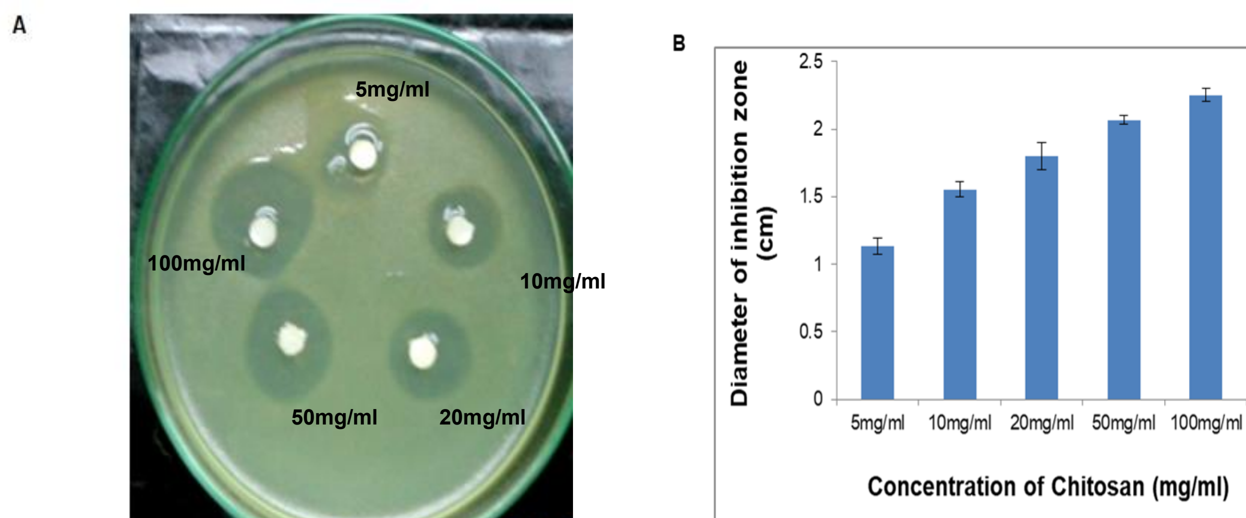

Figure 3: Anti-microbial effect of different concentrations of chitosan extracted from *A. solani* on gram negative bacterium *E. coli* DH5α. (A) Inhibition zones formed around on *E. coli* DH5α in disc diffusion assay using increasing concentration of chitosan. (B) Graphical representation of comparison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

Table 1: Inhibition zones around the bacterium *E. coli* DH5α with different concentrations of chitosan (mg/ml) extracted from *A. solani.*

Chitosan concentration (mg/ml)	Diameter of inhibition of zone (cm)
5mg/ml	1.13
10mg/ml	1.55
20mg/ml	1.8
50mg/ml	2.07
100mg/ml	2.25

In recent times, considerable attention has been given to the antimicrobial property of chitosan against different groups of microorganisms like bacteria, fungi and yeast [1]. The present study

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shows the significant inhibition zones around the gram-negative bacterium *E. coli* DH5α when tested with extracted chitosan of *A. solani*. Chitosan generally interacts with anionic groups on the bacterial cell surface due to its polycationic nature resulting in the formation of an impermeable layer around the cell that prevents the transport of essential solutes [31]. Earlier workers reported the antimicrobial activity of chitosan extracted from *Pleurotus sp.* [1] and *Aspergillus terreus* [5]. Present study coincides with these previous observations made on chitosan from other sources. Thus chitosan extracted from *A. solani* has significant antibacterial activity and may be utilized for wide applications in the biomedical field and agriculture.

4. CONCLUSION

In summary, we can conclude that a simple and eco-friendly method of chitosan extraction from a phytopathogen *Alternaria solani* was developed. The extracted chitosan was characterized using different pH levels with two different temperatures. This work also concluded that the best pH and temperature for the extracted chitosan amongst the pH and temperature tested, are pH 8 and room temperature (25°C) respectively. The knowledge generated from this work can be applied in isolating chitosan from the necrotrophic pathogen *A. solani* which can be employed as an elicitor in host defence study. The antibacterial properties can be utilized in agricultural, biomedical, pharmaceutical and food industries after further studies.

ACKNOWLEDGEMENT

This work was partially supported by DBT and UGC CAS, Govt. of India. The authors are thankful to the Department of Botany, University of Calcutta for providing necessary research facilities and instruments facilities. The authors declare that there is no conflict of interest.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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