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Extraction and Characterization of Chitosan from *Aspergillus flavus* strain AF2118

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ABSTRACT: Chitosan is a versatile natural hydrophilic polysaccharide which is composed of glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) units linked by β 1, 4-glycosidic bonds. It is mainly derived from chitin which is a component of crustacean exoskeleton such as shrimp, lobster, crab, insects and cell wall of fungi. Conventional method of chitosan production has harmful environmental effects since it produces millions of tons of basic and acidic residues, which are discharged into the ecosystem without any further treatment. The enzymatic deacetylation of chitin is the major mechanism for synthesis of chitosan in fungi. The chitosan produced from crustacean shell waste had inconsistent physicochemical properties because of variable and seasonal supply of raw materials and difficulties in processing methods. To overcome these problems, fungi have been treated as an alternative source for chitosan production. In our study 18 morphologically different fungal isolates, 17 bacteria and 3 actinomycetes were isolated. These isolates were further screened for chitin deacetylase activity. The isolate FC3 was the most efficient fungal isolate showing highest yield of chitosan (0.096g/100ml). The chitosan extracted from isolate FC 3 presented a degree of deacetylation of 88.5%. On the basis of ITS sequencing, the fungal isolate FC 3 showed 92 % similarity with *Aspergillus flavus* strain AF2118.

Keywords: Chitosan, fungi, degree of deacetylation, chitin deacetylase.

INTRODUCTION

Chitosan is a linear cationic biopolymer consisting of β (1-4) bonds between 2-amino-2-deoxy-D glucopyranose and 2-acetamido-2- deoxy-D-glucopyranose. It is derived from the deacetylation of chitin, which is the second most abundant polysaccharide found in nature after cellulose. Chitin and chitosan possess multiple functional applications in various field *viz.* agriculture, pharmaceuticals, environmental protection, food, cosmetics, paper making and textile industry. In winemaking, chitosan is used as a fining agent and antimicrobial agent to prevent spoilage of wine. It is used as a self- healing polyurethane coating in paint industry. In medicine, it is used in bandages to lower bleeding and as antimicrobial agent to prevent infections. In agriculture, chitosan is mainly as a natural plant growth promoter in seeds and as biopesticide compound that improves the ability of plants to fight against phytopathogens (Jeon *et al.*, 2000; Chakraborty *et al.*, 2020). In water processing engineering, chitosan has a potential role in filtration process which causes aggregation of fine sediment particles.

Chitosan, besides chitin, occurs in fungal cell walls particularly of Ascomycetes, Basidiomycetes and Zygomycetes (Wu *et al.*, 2019; Namboodiri and Pakshirajan 2019). Several yeasts and filamentous fungi including *Absidia coerulea*, *Candida albicans*, *Absidia glauca*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae Aspergillus niger*, *Gangronella butleri*, *Phycomyces blakesleeanus*, *Mucor rouxii*, *Coprinus cinereus*, *Neurospora crassa*, *Mortierella isabelina*, *Lentinus edodes*, *Trichoderma reesei*, *Agaricus bisporus* and *Rhizopus oryzae* have been reported as sources of chitosan (Pochanavanich and Suntornsuk, 2002). The enzymatic deacetylation of chitin is the major mechanism for synthesis of chitosan in fungi. Hence, the two main biological alternatives to the chemical synthesis of chitosan are the use of chitin deacetylases and fermentation of chitosan containing fungi. One of the main advantage of microbial chitosan production is to obtain chitosan free of allergenic animal proteins such as tropomysin. The degree of deacetylation and molecular weight of microbial chitosan can be controlled by varying the cultural conditions of fermentation. Fungal chitosan production is easy in handling, harvesting and production of high quality of chitosan under optimized conditions. There are many factors such as nutritional sources and cultivation conditions that strongly influence the chitosan production by microorganisms. Moreover, chitosan extraction from fungi does not need demineralization step and the starting material is available throughout the year at cheap cost (Berger *et al.*, 2018). The present study reports the isolation of fungi, extraction and characterization of chitosan from fungal biomass.

MATERIALS AND METHODS

A. Isolation and screening of fungi for chitosan production

Soil and water samples were collected from different sites such as pond water, canal water, vermicompost, garden soil and wheat rhizosphere for the isolation of fungi for chitosan production using chitin agar medium. The isolates were screened for chitin deacetylase activity by spot inoculation method on chitin agar medium with 0.5g/L of p-nitroacetanilide as indicator.

B. Determination of chitosan levels in fungal isolates

The selected fungal isolates were used for the extraction of chitosan and for the determination of chitosan levels in the fungal biomass by using standard method (Crestini *et al.*, 1996).

C. Method for extraction of chitosan from fungal mycelia

The extraction of chitosan from fungi was determined by using modified method as described by Crestini *et al.*, (1996). The schematic representation for extraction of chitosan from fungal biomass was depicted in Fig. 1.

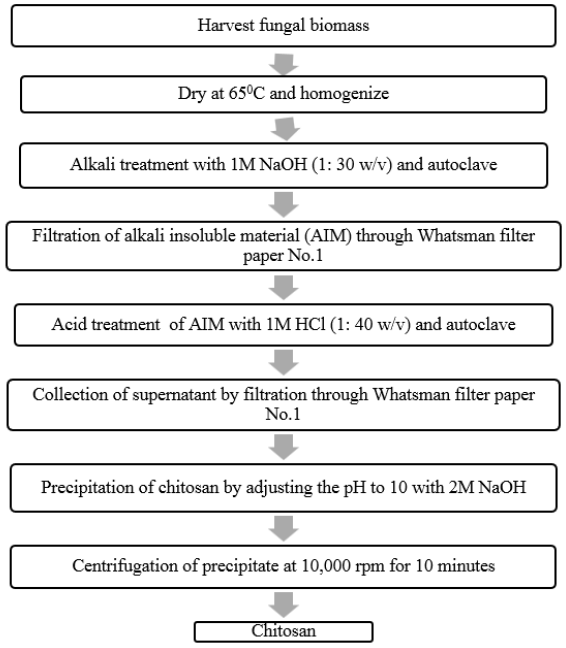

Fig. 1. Schematic representation for chitosan extraction from fungal biomass.

Preparation of alkali insoluble material (AIM). Dry fungal mycelia were finely ground and suspended with 1.0 M NaOH solution (1: 30 w/v) in 100 ml Erlenmeyer flasks followed by autoclaving at 121 °C, 15lbs pressure for 15 minutes. AIM were collected by filtering the autoclaved slurry through Whatman filter paper No.1 followed by washing with distilled water.

Acid extraction of chitosan from AIM. The residues were further extracted using 1.0 M HCl (1: 40 w/v) by autoclaving at 121°C, 15 lbs pressure for 15 minutes. The extracted slurry containing the chitosan was centrifuged at 10,000 rpm for 10 minutes and the acid insoluble fraction was discarded. The pH of the supernatant fluids was adjusted between 10 and 12 using 2.0 M NaOH to precipitate chitosan, followed by centrifuging the solution at 10,000 rpm for 15 minutes.

Purification of extracted chitosan. The precipitated chitosan was washed with distilled water, 95% ethanol (1: 20 w/v) and acetone (1: 20 w/v), respectively followed by drying at 60°C to a constant weight.

D. Quantitative estimation of chitosan

The chitosan extracted was washed with distilled water and resuspended in 1ml of distilled water. The weight of empty petriplates were taken and chitosan suspension was poured into it. Then the petriplates were kept at 60°C for drying and weighed again.

E. Qualitative estimation of chitosan

The chitosan extracted from the selected fungal isolates (6) were dried separately at 55°C for 2 hrs and 2-3 drops of potassium iodide solution were added on the dried precipitate. The mixture was further acidified with 2- 3 drops of 1.0% H_2SO_4 and the colour change was observed (Kaur *et al.*, 2012).

F. Characterization of fungal chitosan

Characterization of chitosan was done by determination of degree of deacetylation using method as described Muzzarelli and Rocchetti, 1985.

G. Morphological characterization of isolate

The colony morphology and appearance of the selected fungal isolate was studied on agar plates. The fungal isolate was cultivated on Potato Dextrose Agar (PDA) media. The plates were then incubated at 27±2°C for seven days for studying macroscopic characteristics such as colony growth, colony colour and colony texture.

H. Molecular characterization of selected isolate by DNA sequencing

The molecular characterization of the selected isolate was done in collaboration with Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India, by DNA Barcoding universal primers of ITS.

I. Statistical analysis

The results were statistically analyzed using analysis of variance techniques (ANOVA) as applied to Completely Randomized Design (CRD) described by Panse and Sukhatme, 1985.

RESULTS AND DISCUSSION

A. Isolation of fungi for chitosan production

A total of 17 morphologically different isolates of bacteria, 18 fungi and 3 actinomycetes were obtained on chitin agar medium.

All the isolates were able to utilize chitin which was confirmed by the growth in chitin agar media. It was also presumed that these isolates were able to produce chitin deacetylase which hydrolysis chitin to chitosan, thereby generating glucosamine units and acetic acid. Mane *et al.*, (2022) isolated chitosan from different fungi namely, *Benjaminiella poitrasii* (Zygomycetes, dimorphic), *Hanseniaspora guilliermondii*, *Issatchenkia orientalis*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae* (Ascomycetes, yeasts), and *Agaricus bisporus* and *Pleurotus sajor-*

caju (Basidiomycetes) from various samples. Ssekatawa *et al.*, (2021) isolated chitosan from fungal cell walls of edible mushroom and also from Nile perch scales. Zininga *et al.*, (2019) isolated an oleaginous fungus *Mucor circinelloides* ZSKP from kitchen vegetable waste mixed with dried shrimp-shell waste which concurrently yielded 21.4% lipids and 11.2% chitosan per gram of dry biomass. Tasar *et al.*, (2016) isolated a fungus from the soil samples collected around Erzurum, Turkey and produced chitosan in molasses agar medium and identified as *Rhizopus oryzae* PAS17. Kaur *et al.*, (2012) isolated twenty bacteria for chitosan production from soils of different beaches of Chennai, India. Eighty strains of bacteria were isolated from Langoan hot spring water in North Sulawesi on minimal medium containing Bacto yeast extract, $(NH_4)_2SO_4$, KH_2PO_4 and chitin (Toharisman and Suhartono 2017).

B. Screening of isolates for chitin deacetylase activity

All the selected isolates were screened for chitin deacetylase activity (CDA) on chitin agar media supplemented with 0.5g/L of p-nitroacetanilide as indicator. Out of 38 isolates, only 6 fungal (FC1 to FC6), 2 bacterial (BC1, BC2) and one actinomycete (AC1) showed positive results by production of yellow colour in the chitin agar media (Table 1). The yellow colour was developed by different isolates on chitin agar media varied from 2-12 days of incubation.

Table 1. Screening of selected isolates for chitin deacetylase activity on chitin agar media.

Isolates	No. of positive isolates with CDA activity
BC1	+
BC12	+
AC1	+
FC1	+++
FC3	+++
FC7	++
FC8	++
FC9	++
FC16	++

+ -development of yellow colour after 12 days of incubation
++- development of yellow colour between 6 to 8 days of incubation
+++- development of yellow colour between 2 to 4 days of incubation

The production of yellow color was due to the conversion of colourless p-nitroacetanilide to yellow coloured p-nitroaniline by chitin deacetylase (Liu *et al.*, 2016). Chitin deacetylase catalyzes deacetylation of chitin resulting in production of chitosan and acetate ions. Similar results were reported by Pawaskar *et al.*, (2019), who screened microorganisms for chitin deacetylase activity from the marine crustacean dumped soil and water samples. Zhang *et al.*, (2023) isolated and screened chitin deacetylase enzyme producing *Bacillus cereus* from coastal mud. Similar results were also retrieved in studies conducted by Zou *et al.*, (2016) isolated and screened 142 chitin deacetylase producing microorganisms, out of which 91 isolates were found to be CDA positive using p-nitroacetanilide strip and p-nitroacetanilide agar method. Karthik *et al.*, (2017) investigated the production of chitin deacetylase activity from *Aspergillus flavus* under solid state fermentation (SSF) using wheat bran as substrate. Kaur *et al.*, (2012) screened microorganisms for chitin deacetylase activity by using the diagnostic strip test for conversion of p-nitroacetanilide which was indicated by development of yellow colour in the strip.

C. Determination of chitosan levels in fungal isolates

A total of six fungal isolates (FC1, FC3, FC7, FC8, FC9 and FC16) were further assessed for the production of mycelial chitosan by culturing them in basal sterile media. The levels of chitosan in the mycelia of fungal isolates were determined qualitatively and quantitatively.

D. Qualitative estimation of chitosan

The chitosan obtained from selected fungal isolates were dried at 55°C for 2 hours and evaluated for the presence of chitosan. The addition of iodine solution to the dried precipitate of chitosan caused a colour change from cream yellow to dark brown. Further acidification with one percent H_2SO_4 lead to change of dark brown colour to dark purple colour (Plate 1). Hence the presence of chitosan was confirmed.

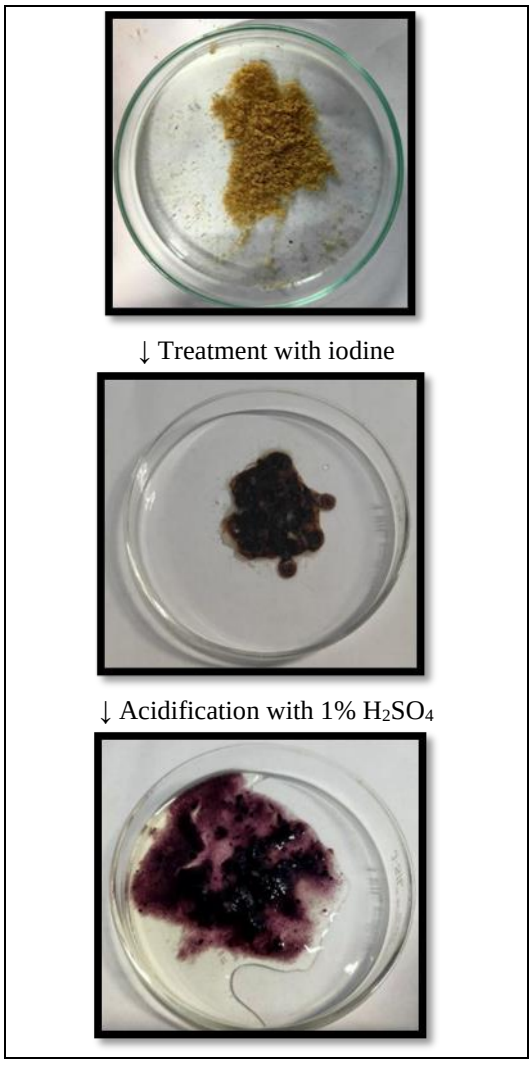

Plate 1: Qualitative estimation of chitosan from fungal mycelia.

Related results were also retrieved in studies conducted by Landge *et al.,* (2015) with microorganisms isolated from soil samples for chitosan production where the confirmation of extracted chitosan was proved by the production of dark purple precipitate when treated with 1.0 % H_2SO_4.

F. Quantitative estimation of chitosan

Further, the six fungal isolates were assessed for dry cell biomass, alkali insoluble material and chitosan levels in the mycelia (Table 2). The dry cell biomass among the fungal isolates ranged from 0.660 to 1.553 g/100ml. The highest yield of dry mycelial biomass was produced by the isolate FC3 (1.553 g/100ml) followed by FC7 (1.076 g/100ml) and FC16 (0.910 g/100ml), respectively. The lowest dry cell biomass was recorded for the isolate FC 8 (0.660 g/100ml). Whereas, the alkali insoluble fraction of fungal isolates varied from 0.323 to 1.266 g/100ml. The isolate FC3 was registered with maximum amount of alkali insoluble fraction i.e. 1.266 g/100ml followed by FC7 and FC16 with 0.696 g/100ml. The isolate FC8 showed the minimum amount (0.323 g/100ml) of alkali insoluble material (AIM).

Similarly, the dry weight of chitosan extracted from fungal isolates ranged from 0.096 to 0.027 g/100ml. Maximum chitosan was produced by isolate FC3 i.e. 0.096 g/100ml on a dry weight basis, followed by isolate FC16 and FC7 with 0.053 g/100ml. The least chitosan yield was obtained from the isolate FC8 with 0.027 g/100ml.

Table 2: Determination of dry cell biomass, alkali insoluble fraction and chitosan levels in fungal isolates.

Isolates	Dry cell biomass (g/100ml)	Alkali insoluble material (g/100ml)	Chitosan (g/100ml)
FC1	0.873± 0.082	0.530±0.046	0.033±0.009
FC3	1.553±0.185	1.266±0.084	0.096±0.008
FC7	1.076±0.102	0.696±0.156	0.053±0.004
FC8	0.660±0.079	0.323±0.024	0.027±0.007
FC9	0.780±0.070	0.503±0.029	0.036±0.008
FC16	0.910±0.109	0.696±0.099	0.053±0.006
CD at 5%	0.349	0.265	0.020

From the above experiment, it was found that the FC3 was the most efficient fungal isolates which produced highest amount of chitosan, AIM and dry cell biomass as compared to other isolates; therefore, it was used for further optimization of cultural conditions for chitosan production.

The fungal mycelium contains chitosan as natural compound in their cell wall and it depends up on several factors like fungal strain, mycelial age and media for cultivation. Furthermore, the enzyme chitin deacetylase carry out deacetylation of the growing chain of chitin which results in the production of fungal chitosan.

This study agrees with earlier findings of Rane and Hoover (1993) who observed the production of chitosan from *Absidia coerulea* (47-50 mg/100 mL), *Mucor rouxii* (29-32 mg/100 mL), *Gongronella butieri* (47-50 mg/100 mL), *Phvcomyces blakesleeanus* (6 mg/100 mL) and *Absidia blakesleeana* (7 mg/100 mL). Mane *et al.,* (2022) isolated *Benjaminiella poitrasii* and the yield of chitosan extracted was 60.89 mg/g of dry mycelial biomass. Kasongo *et al*., (2020) extracted chitosan from fungal biomass of *Termitomyces titanicus* with a yield of 38.40%. George *et al.,* (2011) isolated *Aspergillus flavus, Cladosporium cladosporioides* and *Phoma* sp. from medicinal plants and the yield of chitosan extracted from these fungal biomass were measured to be 57mg/g, 25.2mg/g and 31.1mg/g dry weight, respectively. Chang *et al.,* (2019) reported a yield of 5.81% of chitosan from the fruiting bodies of *Auricularia* sp. whereas Zamani *et al.,* (2007) examined 8% chitosan from the biomass of *Rhizomucor pusillus.* The amount of chitosan extracted from *Absidia glauca* var. *paradoaxa* IFO 4007 was estimated to 1.28 g/L of the growth media (Zininga *et al.,* 2019). Furthermore, Wu *et al.,* (2019) determined the chitosan content (5.74 ± 0.88%) in a special brown mushroom *Agaricus bisporus* (Lange) isolated from Chaidam basin. Ssekatawa *et al*., (2021) isolated chitosan from fungal cell walls of edible mushroom with a yield of 1.55% to 3.5%.

G. Characterization of chitosan derived from fungal biomass

The characterization of chitosan extracted from isolate FC3 was done by determination of degree of deacetylation (DA) on the basis of the absorbance of chitosan solution (in 0.1 M HCl) at 199 nm by UV spectrophotometry (Muzzarelli and Rocchetti 1985). A standard curve of concentration of N acetyl glucosamine versus absorbance was generated. In the present study, chitosan from isolate FC 3 presented a degree of deacetylation of 88.5%.

A degree of deacetylation higher than 50% implies that majority of the chitosan monomers are deacetylated and carries an NH_2 group at the C_2 position instead of acetamido group. Kasongo *et al*., (2020) measured the degree of deacetylation of chitosan from *Termitomyces titanicus* to be 69.50%. Aranaz *et al.,* (2009) stated that chitosan with DD of 70- 100% were observed to be efficient for biomedical and pharmaceutical applications. The degree of deacetylation of chitosan from white *Agaricus bisporus*, *Aspergillus oryzae* SU-B2 and *Aspergillus niger* were evaluated to be 50.00, 55.23 and 83.64%, respectively (Jebur *et al*., 2019; Ban *et al*., 2018; Gawad *et al*., 2016). A study conducted by Alshubaily and Al-Zahrani (2019), was in agreement with the above results and showed that the chitosan extracted from *Cunninghamella elegans* had a DD of

87.4%. Mane *et al.,* (2022) extracted chitosan from different fungi namely, *Benjaminiella poitrasii* (Zygomycetes, dimorphic), *Hanseniaspora guilliermondii*, *Issatchenkia orientalis*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae* (Ascomycetes, yeasts), and *Agaricus bisporus* and *Pleurotus sajor-caju* (Basidiomycetes) and observed the degree of deacetylation was in the range of 70-93%. Furthermore, Ssekatawa *et al*., (2021) measured the degree of deacetylation with 79.1% using FTIR spectra from Ugandan edible mushrooms.

H. Identification of promising fungal isolate for chitosan production

The fungal isolate FC 3 with maximum chitosan production was characterized based on morphological and molecular studies.

Colony morphology. The fungal isolate FC3 was sub cultured on potato dextrose agar medium for morphological studies. Initially the colonies were white and later changed to light green colour as culture matured. When the colonies were immature, the aerial mycelia showed a white fluffy texture. The matured colonies was covered with light green spores and reverse of the colony was cream coloured (Plate 2).

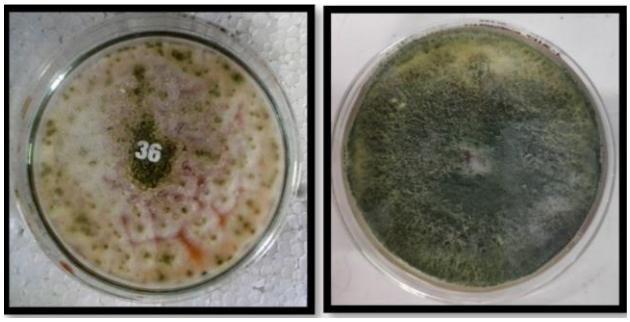

Plate 2: Colony morphology of isolate FC 3 on PDA media.

Microscopic examination. The microscopic examination of the fungi was determined by lacto phenol cotton blue staining. From the microscopic observation, the mycelia showed septate hyphae. The conidial head was large and appeared green colour. The conidiophores were unbranched, aseptate and terminate with swollen head. The vesicle appeared globose in shape and covered with radiating sterigmata on the entire surface. The chains of conidia carried by the sterigmata was arranged in acropetal succession (Plate 3).

Molecular characterization of selected isolate by DNA sequencing. The molecular characterization of the fungal isolate FC 3 was done in collaboration with Rajiv Gandhi Centre for Biotechnology, Trivandrum. The genomic DNA was extracted from the isolate FC 3. The agarose gel electrophoresis of the DNA bands on the gel are shown in Plate 4.

Sequencing. On the basis of ITS sequencing, the fungal isolate FC 3 showed 92 % similarity with *Aspergillus flavus*. The phylogenetic tree of isolate FC 3 was made by using neighbour joining of BLAST programme (Fig.1).

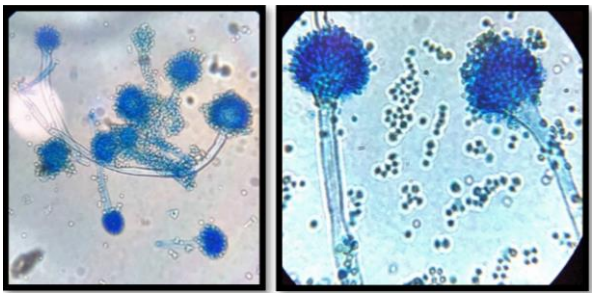

Plate 3: Microscopic examination of isolate FC 3 showing conidiophores.

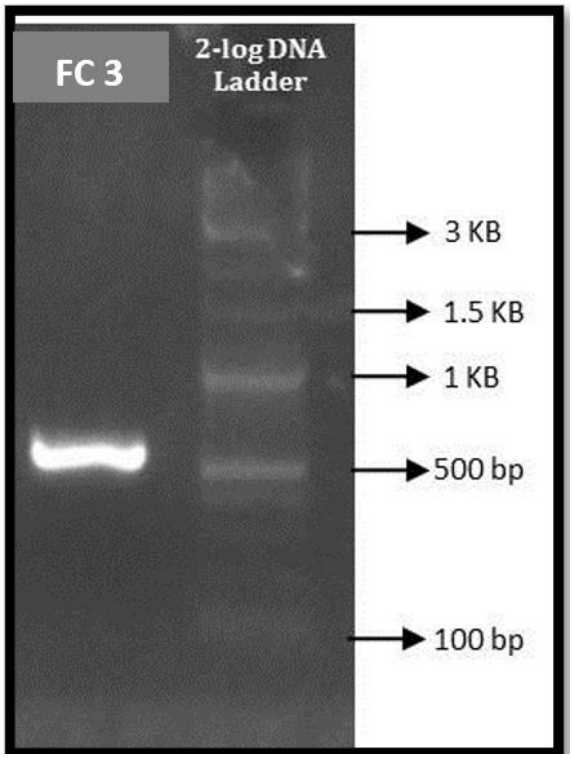

Plate 4: Agarose gel (0.8%) electrophoresis showing band of genomic DNA.

According to their morphological and molecular characteristics, the fungal isolates FC3 was identified as *Aspergillus flavus* strain AF2118.

The studies conducted by Muslim *et al.* (2018) demonstrated that chitosan extracted from *Aspergillus flavus* would serve as an alternative source from the shells of crustaceans. Yonis *et al.,* (2019) recorded a high chitin deacetylase activity (219.5 U/g solid substrate) in *A. flavus.* The yield of chitosan extracted from biomass of *A. flavus* was determined to be 57mg/g dry weight (George *et al.,* 2011).

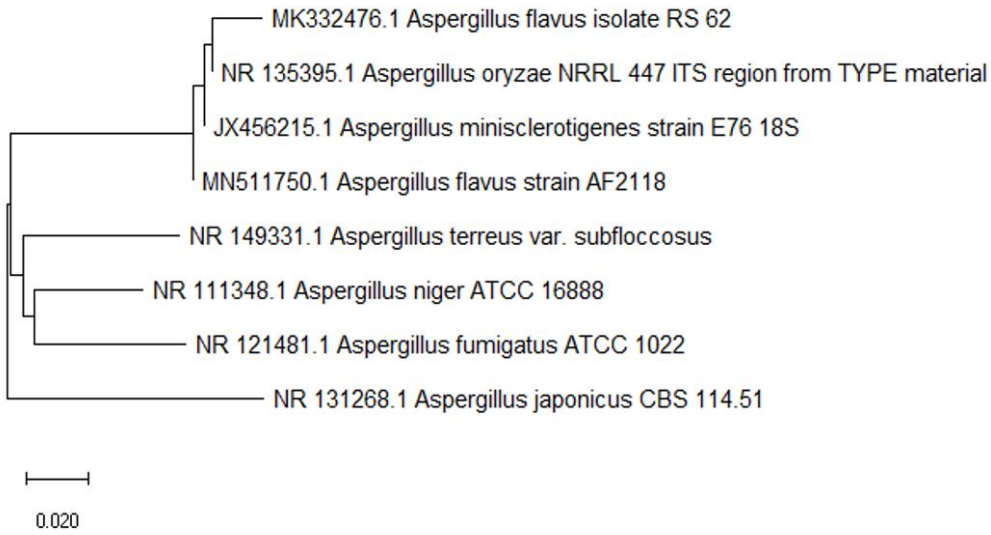

Fig. 1. Phylogenetic tree of fungal isolate FC 3 by using neighbour joining method of BLAST programme.

CONCLUSION

Based on the quantitative estimation of chitosan extracted from the biomass, among the different isolates *Aspergillus flavus* strain AF2118 (FC3) was selected as an alternative source of chitosan from the shells of crustaceans. Henceforth, an economical process of chitosan production can be organized by growing fungi on agro-industrial waste materials which provides a continuous and unlimited source of chitosan.

FUTURE SCOPE

Standardization of a fermentation process for mass production of fungal chitosan using agro-industrial waste as substrates is prerequisite to achieve high product yield which would not only improve the yield and quality of chitosan but also render the process more economical.

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Conflict of Interest. None

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