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Optimization of cultural conditions for chitosan production from *Aspergillus flavus* strain AF2118

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Abstract
The cell wall of fungus mainly consists of chitin, chitosan, β-glucan and mannan. In recent years, fungi have been regarded as an alternative source of chitosan for many industrial applications due to significant advantages. The main objective of this study was to optimize cultivation parameters of *Aspergillus flavus* strain AF2118 for maximum yield of chitosan. The highest chitosan production was found to be 213 g/100ml after 96 hours of incubation. From the results, it appears that an initial pH of 5.0 and temperature of 35˚C lead to high-yield fungal chitosan (0.265±0.013 g/100ml). Among all the carbon sources, glucose plays an important role in the productivity of chitosan with the highest cell biomass (1.233±0.085 g/100ml) as well as fungal chitosan production (0.309±0.027 g/100ml). Moreover, our study revealed that yeast extracts as an organic nitrogen source showed a significant effect on the yield of fungal chitosan (0.332±0.035g/100ml). The above results proved the relevance of optimization of cultural conditions for maximum production of chitosan from fungal biomass.

Keywords: Chitosan, optimum conditions, *Aspergillus flavus*

1. Introduction
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 exoskeletons such as shrimp, lobster, crabs, insects and the cell wall of fungi (Bhuiyan *et al*., 2013) [4]. The global chitosan market exceeded 1.18 x 10^3 tons by 2018 and it is suggested for a shortage of suppliers to accomplish this demand (Gómez-Ríos *et al*., 2017) [13].

In most of the countries, commercial exploitation of chitosan is predominantly done by conversion of chitin to chitosan by treating shellfish waste with alkali. In India, Kerala have the largest production of the chitin and chitosan from crabs, lobsters and insects. The conventional method for the production of chitosan includes several steps such as demineralization with HCl, deproteinization, deacetylation of crustacean chitin with a hot concentrated sodium hydroxide solution for a long time, extraction of chitosan in an acidic medium and again precipitation in basic medium (Abdou *et al*., 2008) [1]. Furthermore, this process seems to have restricted potential for industrial application due to seasonal variation, confined manufacturing locations, high costs and difficulties in processing especially with generation of tremendous amount of unusable alkaline solution. Moreover, the marine chitosan is inconsistent in its physiochemical characteristics as a result of harshness in isolation, variability in the supply of raw materials and conversion processes, the difference in the levels of deacetylation and the presence of animal contamination. These 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. Hence, fungi can be the best alternative source of chitosan since it provides persistent physicochemical properties.

Chitin and chitosan are the main fibrillary components that act as a scaffold for assembling of various components of the fungal cell wall to provide skeletal support that is essential for the survival of cells and also to interact with the surroundings of fungi (Roncero, 2002) [21]. A part of the synthesized chitin is deacetylated to chitosan by chitin deacetylase (EC 3.5.1.41). Chitin deacetylase primarily acts on residues from a growing chain of chitin and possess low activity on preformed chitin molecule. Among the various components found in the fungal cell wall, chitosan is mainly associated with increased integrity of the cell wall thereby providing protection against high temperatures and cell inhibitors (Baker *et al.*, 2007) [3]. Chitosan is a major component in the cell walls of certain fungi such as *Aspergillus* sp., *Gongronella* sp., *Absidia* sp. and *Rhizopus* sp.

It is also observed in the stalks, spores and mycelia of Ascomycetes, Phycomycetes and Basidiomycetes (Logesh *et al.*, 2012) [15].

The chitosan yield from fungal biomass depends on various factors such as the fungal strain, method of cultivation (batch culture or continuous culture), and cultivation parameters such as temperature, pH, mixing rate and cultivation period. Moreover, an increase in the amount of chitosan can be obtained by an increase in the yield of biomass. The purpose of the present study was to optimize cultural conditions such as temperature, incubation period, pH, and carbon and nitrogen sources for maximum chitosan production from *Aspergillus flavus* strain AF2118.

2. Materials and Methods

2.1 Optimization of cultural conditions for chitosan production by selected fungal isolate

Different parameters (temperature, incubation period, pH, carbon and nitrogen sources) were optimized for chitosan production from *Aspergillus flavus* strain AF211. The basal media (glucose 2%, peptone 1%, $(NH_4)_2SO_4$ 0.5%, yeast extract 0.1%, K_2HPO_4 0.1%, $CaCl_2$ H_2O 0.01%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%) was inoculated with one ml of spore suspension (1×10^6 spore / ml) of the selected isolate and kept in orbital incubator shaker at 150 rpm for different incubation period. Four replications were maintained for different parameters. After incubation, the mycelia were harvested by filtering through Whatman filter paper no. 1 followed by washing with distilled water and dried at 60˚C to a constant weight to examine the chitosan content as described by Crestini *et al.*, (1996) [6].

2.1.1 Effect of incubation period on chitosan production

In order to determine the effect of the incubation period on the yield of mycelial chitosan, shake flask containing 100 ml of production media was incubated for different incubation periods *viz.* 24 h, 48 h, 72 h, 96 h, 120 h and 144 hours.

2.1.2 Effect of pH on chitosan production

The effect of pH on chitosan production by fungi was studied at different pH levels such as 3.0, 4.0, 5.0 and 6.0. The corresponding pH was obtained by using 1N HCl and 1N NaOH.

2.1.3 Effect of Temperature on chitosan production

The effect of temperature on biomass and yield of chitosan was studied at different temperatures of 25, 35, 40 and 45˚C.

2.1.4 Effect of carbon sources on chitosan production

The effect of carbon sources (glucose, fructose, sucrose and starch) @ 2% (w/v) concentration on chitosan production from fungal mycelia were studied at optimized pH, temperature and incubation period.

2.1.5 Effect of nitrogen sources on chitosan production

To evaluate the effect of different nitrogen sources on fungal chitosan production, organic and inorganic nitrogen sources were analyzed separately at optimized culture conditions. The different nitrogen sources selected for the study were yeast extract, beef extract, tryptone, peptone, potassium nitrate, sodium nitrate, ammonium sulphate and ammonium nitrate. The nitrogen sources like peptone (1.0% w/v), yeast extract (0.1% w/v) and ammonium sulphate (0.5% w/v) of the basal medium were replaced with different nitrogenous sources (both organic and inorganic) with concentrations of 1.6% w/v.

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 [19].

3. Results and Discussion

3.1 Optimization of cultural conditions for chitosan production from fungal isolate

The effect of different parameters such as temperature, pH, carbon and nitrogen sources and incubation period for chitosan production and dry cell biomass from *Aspergillus flavus* strain AF211 was observed under stationary conditions.

3.2 Effect of incubation period on fungal chitosan production

Cell biomass and chitosan production from *Aspergillus flavus* strain AF211 was studied at different incubation periods (Table 1). After 144 hours of incubation, the maximum fungal mycelium (1.433 g/100 ml) was observed. The yield of dry cell biomass at 72 and 96 hours were 0.523 g/100 ml and 0.566 g/100 ml, respectively. The least dry cell weight of mycelia was obtained after 24 hours (0.334 g/100 ml) and 48 hours (0.446 g/100 ml). The yield of chitosan extracted from fungal mycelia ranged from 0.043 to 0.213 g/100 ml. The extractability of chitosan from the mycelia increased with an increment of biomass until 96 h of incubation. The amount of chitosan extracted during the period of 24, 48, 72 and 96 hours of incubation were 0.043, 0.086, 0.091 and 0.213 g/100 ml, respectively. However, the amount of chitosan further decreased after 120 hours (0.160 g/100 ml) and 144 hours (0.143 g/100 ml) of incubation. Maximum chitosan production by *Aspergillus flavus* strain AF211 was found to be 213 g/100ml after 96 h of incubation.

Table 1: Effect of incubation period on fungal chitosan production

The incubation period (h)	Dry cell biomass (g/100ml)	Chitosan (g/100ml)
24	0.334±0.070	0.043±0.021
48	0.446±0.078	0.086±0.011
72	0.523±0.032	0.091±0.012
96	0.566±0.053	0.213±0.053
120	1.275±0.010	0.160±0.045
144	1.433±0.018	0.143±0.037
CD at 5%	0.346	0.109

It was inferred from Table 1 that the fungal biomass of the isolate *Aspergillus flavus* strain AF211 showed a tendency to increase from 24 to 144 hours. The chitosan extracted increased for the first 96 hours (0.213±0.053g/100 ml) with dry cell biomass (0.566±0.053 g/100 ml) after which there was a decline in the chitosan content with period. This reduction in the quantity of extractable chitosan from fungi may be due to physiological changes occurring in the fungal cell wall (McGahren *et al.*, 1984) [16]. When the fungi enter the late exponential phase, the ratio of free chitosan molecules present in the fungal mycelia was comparatively high due to its active growth. When fungi enter the stationary growth phase, the chitosan is more anchored to the cell wall by binding to chitin and other polysaccharides such as proteins,

lipids and carbohydrates (Tan *et al.*, 1996) [24]. Hence, the extraction of chitosan from fungal biomass becomes more difficult. Although, there was an increment in the fungal biomass, less chitosan was obtained at 122 hours while the maximum yield of fungal chitosan was extracted at the late exponential growth phase.

In the present study, the late exponential growth phase seems to be 96 hours for extraction of the maximum quantity of chitosan. Similar studies conducted by Nadarajah *et al.*, (2001) [17] showed that the strains of *Aspergillus niger*, *Rhizopus* sp. KNO1 and *Rhizopus* sp. KNO2 recorded the highest yield of chitosan at their late exponential growth phase of 72 hours of incubation. Similar findings were reported by Gawad *et al.*, (2017) [10] who found that the growth rate of *Aspergillus niger* gradually increased by increasing the incubation period however the maximum quantity of chitosan was obtained at the late exponential growth phase after the 7th day of incubation.

3.5. Effect of pH on fungal chitosan production

The effect of pH on chitosan production from *Aspergillus flavus* strain AF211 was examined at pH 3.0, 4.0, 5.0 and 6.0 (Fig.1). Maximum dry cell biomass (1.135 g/100 ml) was produced by fungal isolate at pH 5.0 after 96 hours of incubation. The least quantity of dry cell biomass was recorded at pH 3.0 with 0.328 g/100 ml. The amount of chitosan extracted from the fungi at various levels of initial pH ranged from 0.057 to 0.232 g/100 ml. Among the different initial pH ranges (3.0 to 6.0), it was observed that a significantly higher yield of chitosan (0.232 g/100 ml) was extracted at pH 5.0. Hence, further studies were carried out at pH 5.0. The lowest yield of chitosan from fungal mycelia was recorded at pH 3.0 (0.057 g/100 ml) after 96 h of incubation period. The cell biomass and chitosan production increased with increasing pH up to 5.0.

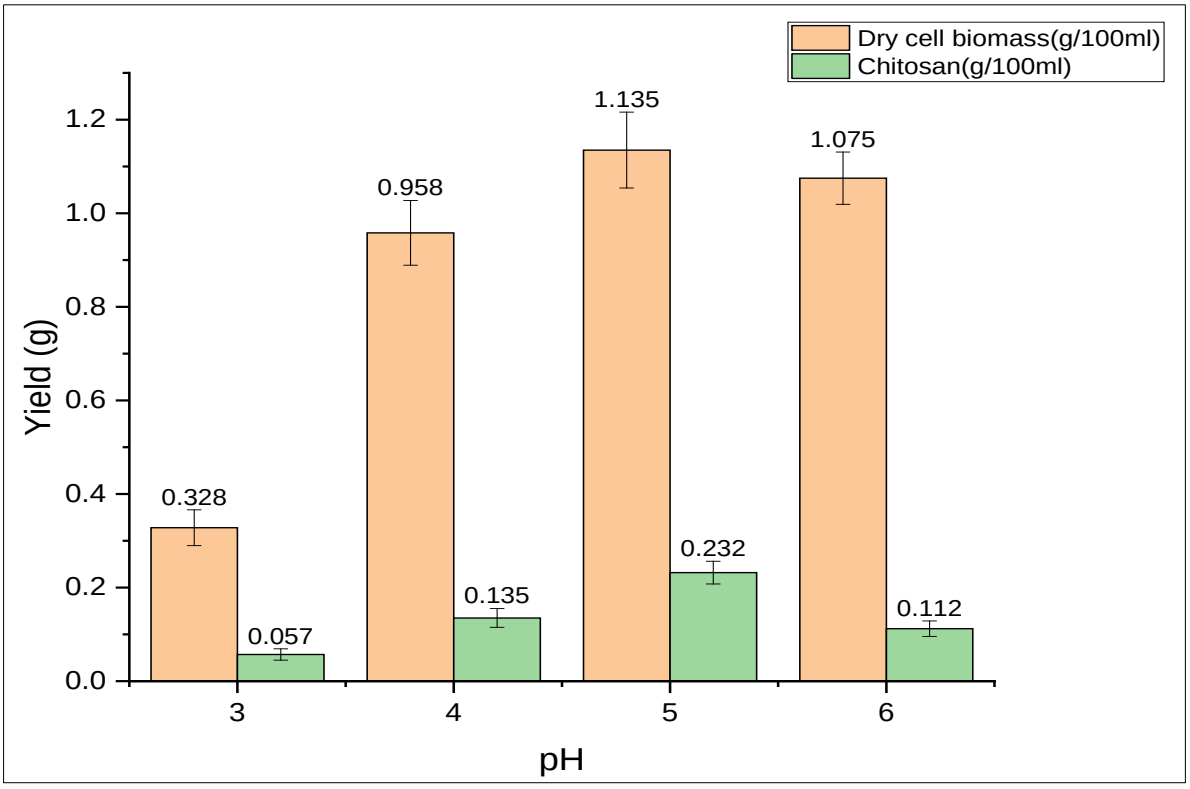

Fig 1: Effect of pH on fungal dry cell biomass and chitosan production

The pH of the growth medium always have an impact on the physiology of a microorganism by affecting enzyme activity, nutrient desirability, oxidative-reductive reactions and the morphology of the cell membrane. From the results, it appears that an initial pH of 5.0 leads to the maximum production of fungal chitosan (0.232±0.024 g/100 ml) (Figure 1). This might be due to the fact that most of the fungi grow best at slightly acidic pH values such as pH 4.0 and pH 5.0. A pH value lower than 3.0 may be inhibiting the growth of some fungi. The data in the present work was in agreement with the studies reported by Tasar *et al.*, (2016) [25] for *Rhizopus oryzae* PAS17, Vaingankar and Juvekar (2014) [26] for *Absidia butleri* NCIM 977, ElMekawy *et al.*, (2013) [8] for *Absidia coerulea* and Santos *et al.*, (2013) [22] for *Cunninghamella elegans* UCP/WFCC 0542 for fungal chitosan production. According to the studies conducted by George *et al.*, (2011) [11] and Synowiecki and Al-Khateeb (2003) [23], pH 5.0 supported the maximum production of fungal chitosan for *Mucor* (6.8 mg/50 ml) and *Cuninghamella* (30 mg/50 ml). This may be due to the fact that the activity of chitin deacetylase is favoured by pH ranging from 4.5 to 5.5 (Amorim *et al.*, 2001) [2].

3.6. Effect of temperature on fungal chitosan production

The effect of temperature on the yield of cell biomass and fungal chitosan were examined by incubating the culture medium at different temperatures like 25, 30, 35 and 45˚C. It was found that different temperature conditions had significantly influenced fungal growth and the chitosan content in the biomass. The amount of dry cell biomass recorded at different levels of temperature ranged from 0.642 to 1.102 g/100 ml. The yield of dry cell biomass increased

from 25 ˚C to 35˚C and further decreased at 45 ˚C. The maximum yield of dry cell weight was obtained at 35˚C (1.102 g/100 ml). At 30 ˚C and 25 ˚C, the quantity of dry mycelia obtained was 1.095 and 0.912 g/100 ml, respectively. However, the least amount of dry cell weight was observed at a temperature of 45˚C (0.642 g/100 ml). It is inferred from Table 2 that the chitosan production at different temperatures ranged from 0.067 to 0.265 g/100 ml. A maximum fungal chitosan production of 0.265 g/100 ml was observed at 35 ˚C. The lowest amount of chitosan was obtained at 45 ˚C (0.065 g/100 ml) followed by 25 ˚C (0.067 g/100 ml) and 30˚C (0.097 g/100 ml), respectively.

Table 2: Effect of temperature on fungal dry cell biomass and chitosan production

Temperature (°C)	Dry cell biomass (g/100ml)	Chitosan (g/100ml)
25	0.912±0.037	0.067±0.012
30	1.095±0.089	0.097±0.012
35	1.102±0.088	0.265±0.013
45	0.642±0.080	0.065±0.011
CD at 5%	0.276	0.098

Temperature is a critical parameter which plays a major role in various biological processes affecting the growth and synthesis of biopolymers. Maximum fungal chitosan production of 0.265±0.013 g/100 ml with the highest cell biomass yield (1.102 ±0.088 g/100ml) was measured at 35 ˚C (Table 2). This may be due to the higher activity of chitin deacetylase in the conversion of chitin to chitosan at this temperature. It was analyzed that beyond 35 ˚C, there was a considerable decline in the yield of chitosan. Similar results were reported by Ravikumar and Perinbam (2016) [20], that the maximum activity of chitin deacetylase was measured at 35 ˚C. However, the studies conducted by Gharieb *et al.,* (2015) [12] observed a maximum production of fungal chitosan at 30 ˚C for the *Cuninghamella* strain (17 mg/ 50 ml), *Mucor* strain (5.7 mg/50 ml) and *Rhizopus* strain (6.5 mg/ 50 ml).

3.7. Effect of carbon sources on fungal chitosan production

Different carbon sources such as glucose, fructose, sucrose and starch at 2% (w/v) concentration were used for chitosan production from *Aspergillus flavus* strain AF211 at 35˚C and pH 5.0 (Figure 2). All carbon sources showed different effects on the biomass yield and chitosan production. The fungal isolate grown on the media supplemented with glucose as a carbon source resulted in a significant increase in the cell biomass production (1.233 g/100 ml). When fructose was taken as a carbon source, the amount of dry cell biomass obtained was 1.227 g/100 ml which was found to be on par with the quantity of dry biomass (1.071 g/100 ml) obtained in the case of sucrose as a carbon source. However, the lowest yield of biomass (0.492 g/100 ml) was recorded when soluble starch was supplemented in the growth media.

The amount of chitosan derived from the fungal biomass with different carbon sources ranged from 0.027 to 0.309 g/100 ml. Among all the carbon sources, the maximum yield of chitosan production (0.309 g/100 ml) was measured when glucose was supplemented in the media after 96 h of incubation. The quantity of chitosan obtained when fructose and sucrose were used as carbon sources were 0.102 and 0.065 g/100 ml, respectively. The lowest yield of fungal chitosan (0.027 g/100 ml) was recorded when soluble starch was supplemented with the growth media as a carbon source.

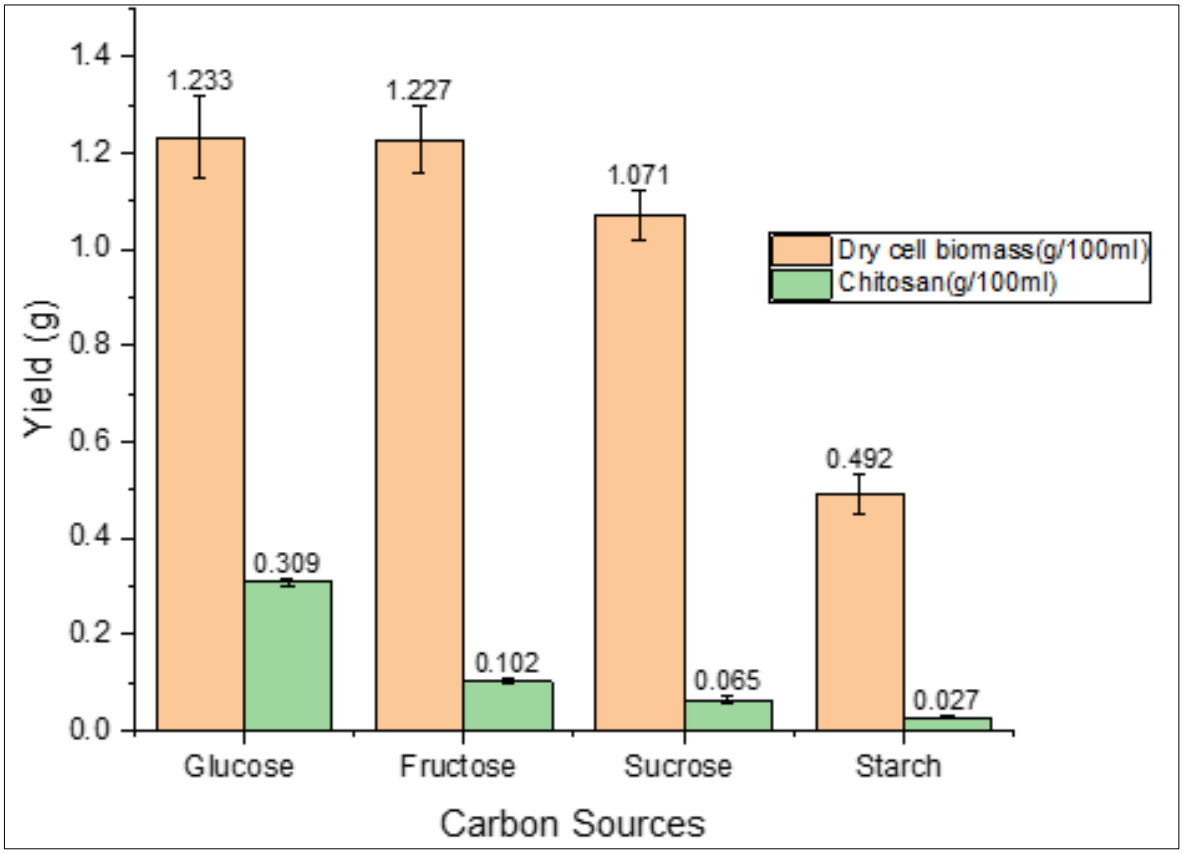

Fig 2: Effect of carbon source on fungal dry cell biomass and chitosan production

The carbon source acts as a major constituent for building cellular material as well as a primary energy source during microbial fermentations. The data on Figure 2 reveals that all carbon sources had different effects on the biomass yield and production of fungal chitosan. Among all the carbon sources, glucose supported the highest cell biomass (1.233±0.085 g/100 ml) as well as fungal chitosan production (0.309±0.027 g/100 ml). This might be due to the easy assimilation of glucose in the metabolic pathway for biosynthesis (Dhillon *et al.*, 2013) [7]. Similar findings were reported by Gharieb *et al.*, (2015) [12] who measured maximum fungal chitosan production when glucose was employed as sole carbon source for *Cuninghamella* (20 mg/50 ml), *Mucor* (10 mg/50 ml) and *Rhizopus* (7 mg/50 ml). Elsoud *et al*., (2023) obtained high yield of chitosan from glucose as carbon source for *Aspergillus terreus*. Vaingankar and Juvekar (2014) examined that when glucose was supplemented in the medium as a carbon source, a maximum yield of fungal chitosan (683.16 mg/L) with a dry cell weight of 8.3 g/L was obtained. ElMekawy *et al.*, (2013) [8] observed that the highest amount of fungal chitosan (0.182 g/L) from *Absidia coerulea* was obtained with glucose at 2.0% concentration in the growth media. This is in contrast to the finding of Wang *et al.*, (2008), wherein these authors observed that sucrose as a carbon source yielded the highest chitosan production (3.24 g/L) from *Absidia*.

3.8. Effect of nitrogen (organic and inorganic) sources on fungal chitosan production

The nitrogen content in the growth medium is a crucial factor which was to be optimized for the synthesis of fungal chitosan. The effect of different nitrogen sources (organic and inorganic nitrogen) on cell biomass and fungal chitosan production were analyzed at 35˚C and pH 5.0 (Table 3 and Figure 3). The different organic nitrogen sources like yeast extract, beef extract, tryptone and peptone as well as different inorganic nitrogen sources such as potassium nitrate, sodium nitrate, ammonium sulphate and ammonium nitrate were assessed for the fungal chitosan production.

The range of dry cell biomass recorded from growth media supplemented with different nitrogen sources was 0.722 to 1.272 g/100 ml. The yield of dry cell biomass obtained when beef extract was added to the basal medium was 1.022 g/100 ml and found to be on par with dry cell weight (0.985 g/100 ml) with tryptone as source of nitrogen. The least yield of cell biomass (0.722 g/100 ml) was observed with peptone used as a source of organic nitrogen in growth media. Maximum dry cell biomass of *Aspergillus flavus* strain AF211 was found to be 1.272 g/100 ml with yeast extract used as a nitrogen source at 35 ˚C and pH 5.0 after 96 h of incubation. However, the chitosan production from fungal biomass for various sources of organic nitrogen ranged from 0.025 to 0.332 g/100ml. The highest fungal chitosan production was found to be 0.332 g/100 ml when yeast extract was supplemented to the basal medium as a source of organic nitrogen. The amount of chitosan obtained with beef extract as a source of organic nitrogen was 0.116 g/100ml and found to be on par with the yield of chitosan extracted for tryptone (0.072 g/100ml). The lowest yield of chitosan from fungal biomass was recorded with peptone as a source of organic nitrogen in growth media (0.025 g/100ml).

Table 3: Effect of organic nitrogen on the production of chitosan

Organic Nitrogen	Dry cell biomass (g/100 ml)	Chitosan (g/100 ml)
Yeast extract	1.272±0.103	0.332±0.035
Beef extract	1.022±0.068	0.116±0.070
Tryptone	0.985±0.074	0.072±0.076
Peptone	0.722±0.089	0.025±0.071
CD at 5%	0.225	0.068

The data presented in Fig. 3 revealed that all inorganic nitrogen sources showed different effects on the cell biomass yield and production of fungal chitosan. The fungal isolate grown with ammonium sulphate as a source of inorganic nitrogen resulted in a significant increase in the cell biomass production (1.105 g/100 ml). When media supplemented with ammonium nitrate, the amount of dry cell biomass obtained was 0.815 g/100ml which was found to be on par with the quantity of dry cell biomass (0.582 g/100ml) obtained in the case of sodium nitrate as a nitrogen source. The lowest yield of cell biomass (0.422 g/100 ml) was recorded when potassium nitrate was supplemented in the growth media at 35˚C and pH 5.0.

The amount of chitosan extracted from the fungal mycelia with different inorganic nitrogen sources ranged from 0.055 to 0.137 g/100ml. A significantly higher yield of fungal chitosan (0.137 g/100ml) was measured when ammonium sulphate was supplemented in the growth media. The quantity of chitosan extracted with potassium nitrate as a nitrogen source was 0.095 g/100 ml which was followed by ammonium nitrate with an amount of chitosan of 0.061 g/100 ml. The least amount of chitosan was recorded with sodium nitrate as a nitrogen source (0.550 g/100 ml) at 35 ˚C and pH 5.0 after 96 hours of incubation period.

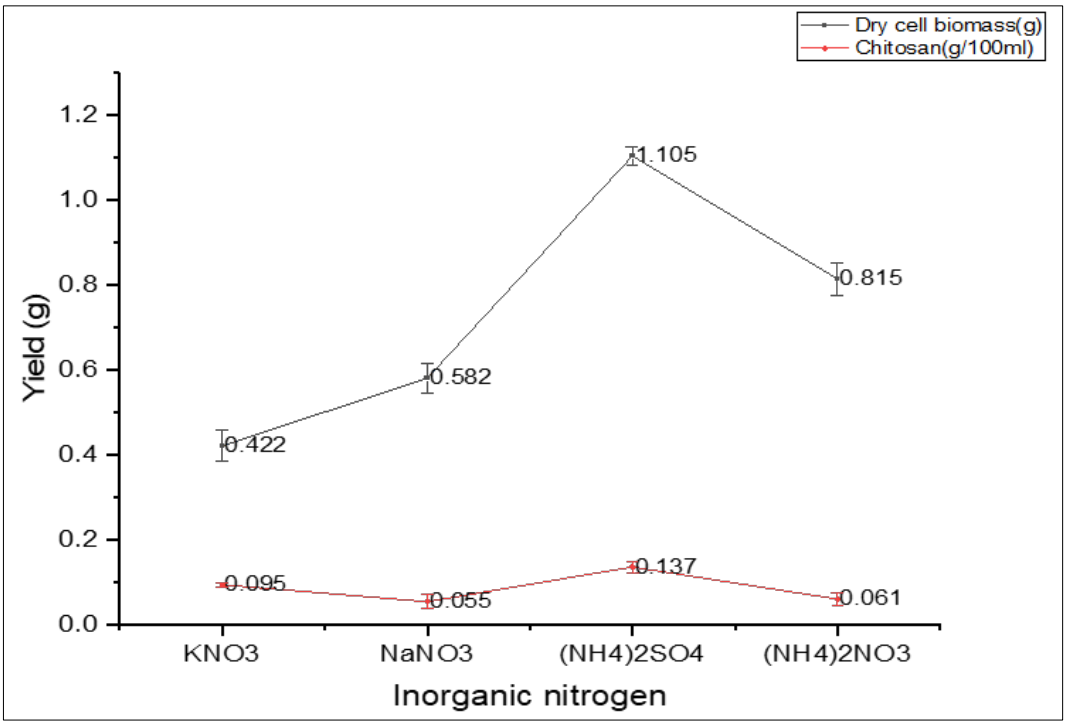

Fig 3: Effect of inorganic nitrogen on the production of fungal chitosan

From the above results, it has been revealed that among the organic and inorganic sources of nitrogen, yeast extract @ 1.6% established a prominent effect on cell biomass and chitosan production from FC3. Hence, yeast extract was selected as the source of nitrogen for further studies (Fig.4).

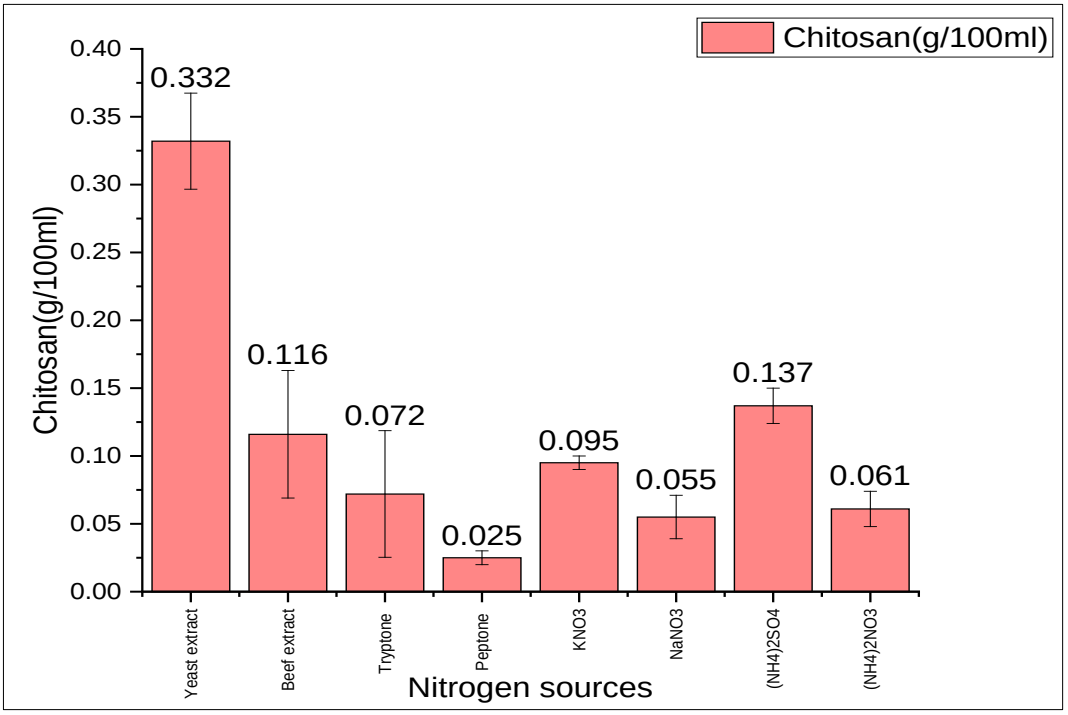

Fig 4: Comparison between organic and inorganic nitrogen sources on chitosan production

The nitrogen source is an important parameter which should be optimized for chitosan production from fungal biomass. Chitosan is a nitrogen-containing biopolymer that is formed as a result of deacetylation of chitin by the enzyme chitin deacetylase. Most of the fungi require an inorganic or organic nitrogen source as a nutrient for the synthesis of chitin and chitosan. Therefore, the nitrogen source has a critical role in the production of chitosan by fungi (New and Stevens, 2004) [18]. In the present study, different organic nitrogen sources like yeast extract, tryptone, beef extract and peptone as well as different inorganic nitrogen sources such as potassium nitrate, sodium nitrate, ammonium sulphate and ammonium nitrate were evaluated for the chitosan production by *Aspergillus flavus* strain AF211. Among the organic and inorganic nitrogen sources, the organic nitrogen source showed a significant effect on the yield of fungal chitosan

(Fig. 4). The highest yield of fungal chitosan (0.332±0.035 g/100ml) with maximum cell biomass yield (1.272±0.103 g/100ml) was recorded when yeast extract was added to the growth medium. The studies conducted by Zhou *et al.,* (2013) and Kashyap and Garg (2014) [14] reported that yeast extract was an ideal nitrogen source for the production of chitin deacetylase. Chhabra *et al.,* (2014) examined approximately 10-fold increases in the yield of chitosan (0.24 mg/ml) with yeast extract from indigenously isolated *Aspergillus Niger* (ITCC 7635.09). However, studies conducted by ElMekawy *et al.,* (2013) [8] reported ammonium sulfate as the best nitrogen source for maximizing the production of fungal chitosan.

4. Conclusion

The effect of different physical parameters such as incubation period, pH and temperature, were investigated for chitosan production from *Aspergillus flavus* strain AF211. An incubation period of 96 hours, pH 5.0 and temperature of 35˚C were found to be the optimum physiochemical conditions with a maximum chitosan production of 0.213, 0.232 and 0.265 g/100ml, respectively. Glucose and yeast extract severed as the best carbon and nitrogen source for highest production of chitosan from *Aspergillus flavus* strain AF211. Hence the present study provides the relevance of optimization of cultural conditions for maximum production of chitosan from fungal biomass.

5. Acknowledgements

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