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Standardization of fermentation process for mass production of fungal chitosan using agro-industrial waste

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Abstract

Agricultural wastes are available in large volumes at low cost and have been considered promising sustainable resources for the synthesis of many biopolymers. The agro-industrial waste products such as paddy straw, sugarcane bagasse, and potato peel waste can serve as economical nutritional sources for the cultivation of fungi. Our studies revealed that the growth media supplemented with agro-industrial waste @ 1% paddy straw +1% glucose (w/v) the yield of chitosan (0.315 g/100 ml) was increased. The fungal isolates (*Aspergillus flavus* strain AF211) showed maximum chitosan production in submerged fermentation (0.533 g/10g paddy straw) as compared to solid state fermentation (0.182 g/10g paddy straw). Therefore, mass production of chitosan from *Aspergillus flavus* strain AF211 was carried out by submerged fermentation in a bioreactor (BioFlo^R 120) and the yield of chitosan was found to be 5.37 g/l. Further, the chitosan extracted from fungi was estimated for an 88.5% degree of deacetylation.

Keywords: Fungal Chitosan, Fermentation, Chitosan characterization, degree of deacetylation

Introduction

Chitosan is a unique amino polysaccharide with diverse applications in the field of agriculture, pharmaceuticals, food, and nutraceuticals since it is nontoxic, biocompatible, and biodegradable. The presence of various functional groups (primary — OH, secondary — OH, and — NH₂) and its solubility in water at acidic pH are the major properties determining its functional properties. The major countries for commercial production of chitosan are Poland, India, Australia, Norway, Japan, and the USA (Komi and Hamblin, 2016) [15].

Commercially, chitosan is produced from the shells of crabs and shrimps, and squid bone plates. Chitosan mainly occurs in two forms in the fungal cell walls, as free chitosan molecules and covalently bounded to β-glucan strands. Today, the primary concern about chitosan derived from crustaceans is the molecular variability which leads to a broad unpredictable range of physicochemical properties. The production of chitosan from fungal biomass is more promising because this process can be employed for the production of pure chitosan with specific characteristics (Joseph *et al.*, 2021) [13].

Chitosan, besides chitin, occurs in fungal cell walls particularly of Ascomycetes, Basidiomycetes, and Zygomycetes (Wu *et al.*, 2019; Namboodiri and Pakshirajan, 2019) [25, 19]. The quality and quantity of chitosan extracted from fungal biomass mainly depends on the strain of fungi, type of fermentation process, media composition such as concentration of nutrients (carbon and nitrogen source) and trace metal content, time of harvesting the fungal biomass, temperature, pH of fermentation medium and extraction procedure for chitosan. Moreover, the production of fungi by fermentative process on cheap agro-industrial wastes is a continuous process throughout the year and a very economical source of chitosan.

The enzymatic DE acetylation of chitin is the major mechanism for the synthesis of chitosan in fungi. The two main biological alternatives to the chemical synthesis of chitosan are the use of chitin deacetylases and the fermentation of chitosan-containing fungi. Hence the present investigation was carried out standardization of fermentation process for the mass production of fungal chitosan using agro-industrial waste.

Materials and Methods

Effect of growth media supplemented with agro-industrial waste on chitosan production

Pretreatment of paddy straw: Paddy straw was dispensed in NaOH solution (2.0%) at a 1:10 ratio and autoclaved at 15 LBS for 1 hour. Then, it was washed with tap water and the pH was neutralized with 0.1 M acetic acid followed by drying at 50±2 °C.

Preparation of sugarcane bagasse: The sugarcane bagasse was washed, dried, and powdered in a grinder. Then it was mixed with 0.12 g of sodium hydroxide per gram dry weight followed by autoclaving at 121 °C for 20 minutes. After cooling, it was washed with distilled water and dried at 80 °C (Gutierrez-Correa *et al.*, 1999) [10].

Preparation of potato peel waste: The potato peels were thoroughly washed with distilled water and dried at 60 °C for 24 hours. The dried samples were ground and stored in an air-tight container for further use.

Analysis of paddy straw, sugarcane bagasse, and potato peel waste: The dried paddy straw, sugarcane bagasse, and potato peel waste were analyzed for various components *viz.*, cellulose, hemicellulose, lignin, organic carbon, total nitrogen, phosphorus, and potassium using standard methods.

Cultivation and production of biomass: Agro-industrial waste such as paddy straw, sugarcane bagasse, and potato peel were used as carbon sources for chitosan production. These were added to the basal mineral medium at various concentrations like 2.0% substrate alone, 1.0% substrate alone, 1.0% substrate +1.0% glucose, and 0.5% substrate +0.5% glucose (w/v).

Treatments

1. Paddy straw @ 2.0%
2. Paddy straw @ 1.0%
3. Paddy straw 1.0% + Glucose 1.0%
4. Paddy straw 0.5% + Glucose 0.5%
5. Sugarcane bagasse @ 2.0%
6. Sugarcane bagasse @ 1.0%
7. Sugarcane bagasse 1.0% + Glucose 1.0%
8. Sugarcane bagasse 0.5% + Glucose 0.5%
9. Potato peel waste @ 2.0%
10. Potato peel waste @ 1.0%
11. Potato peel waste 1.0% + Glucose 1.0%
12. Potato peel waste 0.5% + Glucose 0.5%

The *Aspergillus flavus* strain AF211 was transferred to sterilized Petri dishes containing PDA and incubated at 35°C for 5 days. After the period of incubation, one ml of spore suspension was inoculated into 100 ml of growth medium supplemented with various concentrations of paddy straw, sugarcane bagasse, and potato peel separately. Then the media was adjusted to optimized pH (5.0) and incubated at 35 °C for 96 hours in an orbital incubator shaker at 150 rpm. At the end of fermentation, the mycelium was separated by filtration with the aid of Whatman filter paper No. 1 and washed with distilled water. The biomass was dried at 60 °C biomass until a constant weight has reached and evaluated for the chitosan content in fungal biomass.

Standardization of the fermentation process for mass production of chitosan

The selected fungal isolate was used for standardizing the fermentation process (solid-state fermentation and submerged fermentation) under optimized conditions.

Comparison of solid-state fermentation and submerged fermentation

For submerged fermentation, fungi were transferred to PDA plates and incubated at 35 °C for 5 days. The basal media was supplemented with 1.0% pre-treated paddy straw + 1.0% glucose and pH was adjusted to 5.0 using 1.0N HCl followed by autoclaving at 121 °C for 20 minutes. After the period of incubation, one ml of spore suspension (3×10^6 spores/ml) was transferred to the 1.0 L of basal medium supplemented with 10.0 g pre-treated paddy straw. Each submerged flask was incubated in a shaking incubator (150 rpm) at 35 °C for 96 hours. At the end of fermentation, the fungal mycelia were separated by filtering through Whatman No.1 filter paper and dried in a hot air oven at 65 °C until constant weight biomass for extraction of chitosan.

For solid fermentation, 10g of pre-treated paddy straw was taken in a 500 ml Erlenmeyer flask followed by moistening with 60 ml basal media and autoclaved at 121 °C for 20 minutes. The spores suspension of the fungi was made from 4 to 5 days old cultures by adding 10 ml of sterilized distilled water to the Petri plates and scrapping the spores using sterile forceps thoroughly. Each flask was inoculated with one ml of spore suspension and incubated at 35 °C for 96 hours. At the end of fermentation, the mycelial mat was separated and dried in a hot air oven at 65 °C until constant weight biomass was obtained. Each experiment was carried out in three replicates. The chitosan from the mycelia was extracted by using an alkali-acid treatment as described by Crestini *et al.*, (1996) [7].

Mass production of chitosan in bioreactor

The *Aspergillus flavus* strain AF211 was used for the mass production of chitosan by submerged fermentation under optimized conditions.

The inoculum was prepared by taking spores suspension of the fungi from 4 to 5 days old culture. Ten milliliters of sterilized distilled water was added to the Petri plates and scrapped the spores using sterile forceps thoroughly. Growth media supplemented with 1.0% paddy straw +1.0% glucose (w/v) was sterilized in an autoclave at 121 °C 15 LBS pressure for 15 to 20 minutes. Then the bioreactor (Eppendorf BioFlo^R 120) was inoculated with 10 ml of spore suspension (3×10^6 spores/ml) and incubated at 35°C for 96 hours with constant agitation at 150 rpm. At the end of fermentation, the mycelia were separated and dried in a hot air oven at 65°C until constant weight biomass was obtained. The chitosan was extracted from fungi by alkali-acid treatment as described by Crestini *et al.*, (1996) [7].

Characterization of fungal chitosan

Characterization of chitosan was done by determination of the degree of DE acetylation using the method as described by Muzzarelli and Rocchetti, 1985 [18].

Statistical analysis

The results were statistically analyzed using analysis of variance techniques (ANOVA) as applied to a Completely

Randomized Design (CRD) described by Panse and Sukhatme, 1985 [21].

Results and Discussion

Effect of growth media supplemented with agro-industrial wastes on fungal chitosan production

Analysis of samples

Paddy straw, sugarcane bagasse, and potato peel were analyzed for different parameters. The cellulose, hemicellulose, lignin, organic carbon, total nitrogen, total phosphorus, and total potassium are presented in Table 1.

Table 1: Analysis of samples

Parameters	Paddy straw	Sugarcane bagasse	Potato peel waste
Cellulose (%)	30.01±0.84	32.10±0.58	1.91±0.06
Hemicellulose (%)	22.56±0.55	16.15±0.47	1.20±0.08
Lignin (%)	7.14±0.95	10.57±0.71	1.56±0.56
Organic carbon (%)	36.42±0.56	13.92±0.25	16.24±0.81
Total nitrogen (%)	0.81±0.07	0.79±0.04	1.21±0.038
Total phosphorus (%)	0.28±0.01	0.65±0.05	0.55±0.07
Total potassium (%)	3.65±0.08	2.25±0.04	1.31±0.02

The data presented in Figure 1, Tables 2 and 3 revealed that all substrates namely paddy straw, sugarcane bagasse, and potato peel waste showed different effects on the cell biomass yield and production of fungal chitosan. The dry cell biomass and chitosan production from *Aspergillus flavus* strain AF211 were observed at different concentrations of paddy straw with glucose ranging from 0.646 to 1.195 g/100ml and 0.052 to 0.315 g/100 ml. The highest yield of cell biomass was recorded in growth media supplemented with 1.0 % paddy straw +1.0 % glucose (w/v) (1.195 g/100ml). The lowest amount of dry cell biomass (0.646 g/100ml) was observed for treatments with 1.0 % paddy straw. Moreover, maximum chitosan production

(0.315 g/100ml) was observed when growth media supplemented with 1.0 % paddy straw +1% glucose (w/v) at 35 °C and pH 5.0 after 96 h of the incubation period. The quantity of chitosan extracted from the fungi for other treatments such as 0.5% paddy straw +0.5% glucose (0.073 g/100ml), 2.0 % paddy straw (0.052 g/100 ml), and 1.0% paddy straw (0.065 g/100ml) were found to be on par.

Table 2: Fungal chitosan production on growth media supplemented with paddy straw

Treatments	Dry cell biomass (g/100ml)	Chitosan (g/100ml)
Paddy straw @ 2.0%	0.747±0.041	0.052±0.001
Paddy straw @ 1.0%	0.646±0.070	0.065±0.007
Paddy straw 1% + Glucose 1%	1.195±0.053	0.315±0.007
Paddy straw 0.5% + Glucose 0.5%	0.687±0.040	0.073±0.009
CD at 5%	0.175	0.039

The fungal isolate grown in media supplemented with sugarcane bagasse 1.0% + glucose 1.0% (w/v) resulted in an increase in cell biomass production (0.452 g/100ml). The dry cell biomass yield recorded for other treatments such as 0.5% sugarcane bagasse + 0.5% glucose (0.370 g/100ml), 1.0% sugarcane bagasse (0.292 g/100ml), and 2.0% sugarcane bagasse (0.255 g/100 ml) were found to be on par.

The amount of chitosan extracted from fungi cultured in media supplemented with various concentrations of sugarcane bagasse ranged from 0.042 to 0.092 g/100ml (Fig.9). A higher yield of chitosan (0.092 g/100ml) was measured when 1% sugarcane bagasse + 1% glucose (w/v) was supplemented in the growth media. The quantity of chitosan extracted from other treatments such as 2% sugarcane bagasse (0.052 g/100ml), 1% sugarcane bagasse (0.045 g/100ml), and 0.5% sugarcane bagasse +0.5% glucose (0.042 g/100ml) were found to be on par.

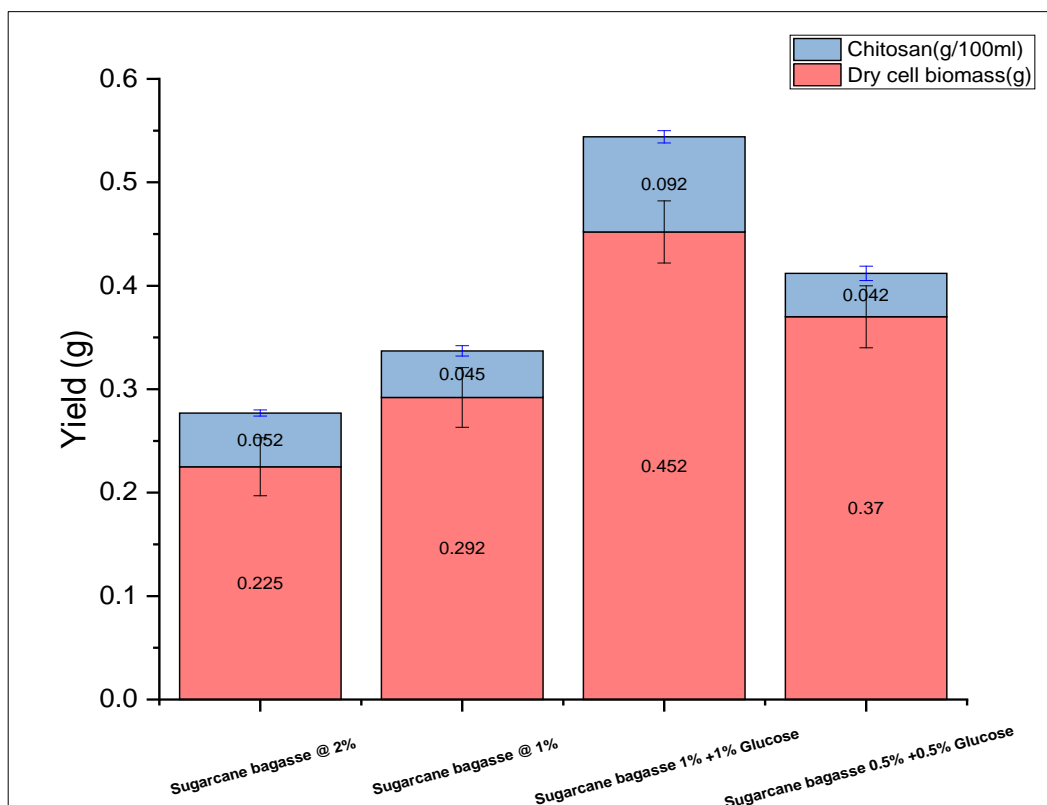


Fig 1: Fungal chitosan production on growth media supplemented with sugarcane bagasse

As shown in Table 3, the yield of dry cell biomass at different concentrations of potato peel waste and in combination with glucose ranged from 0.155 to 0.558 g/100ml. The highest yield of cell biomass was recorded in growth media supplemented with 1% potato peel waste +1% glucose (w/v) (0.558 g/100ml). The amount of dry cell biomass obtained for other treatments such as 2% potato peel waste, 1% potato peel waste, and 0.5% potato peel waste + 0.5% glucose (w/v) were 0.422, 0.288, and 0.155 g/100ml, respectively.

Among the different concentrations of potato peel waste, the growth media supplemented with 1.0% potato peel waste +1.0% glucose (w/v) recorded an increase in the yield of chitosan (0.078 g/100ml). The quantity of chitosan extracted from the fungi for other treatments such as 1% potato peel waste (0.042 g/100ml), 0.5% potato peel waste + 0.5% glucose (0.035 g/100ml), 2% potato peel waste (0.035 g/100ml) were found to be on par.

Table 3: Fungal chitosan production on growth media supplemented with potato peel waste

Treatments	Dry cell biomass (g/100ml)	Chitosan (g/100ml)
Potato peel waste @ 2%	0.422±0.048	0.035±0.004
Potato peel waste @ 1%	0.288±0.050	0.042±0.010
Potato peel waste 1% +1% Glucose	0.558±0.036	0.078±0.007
Potato peel waste 0.5% +0.5% Glucose	0.155±0.026	0.035±0.003
CD at 5%	0.235	0.021

Among the various agricultural waste (paddy straw, sugarcane bagasse, and potato peel) analyzed, the growth media supplemented with 1% paddy straw +1% glucose (w/v) recorded a significant increase in the yield of chitosan (0.315 g/100ml) (Fig.2). Hence this concentration was further selected for standardization of fermentation process.

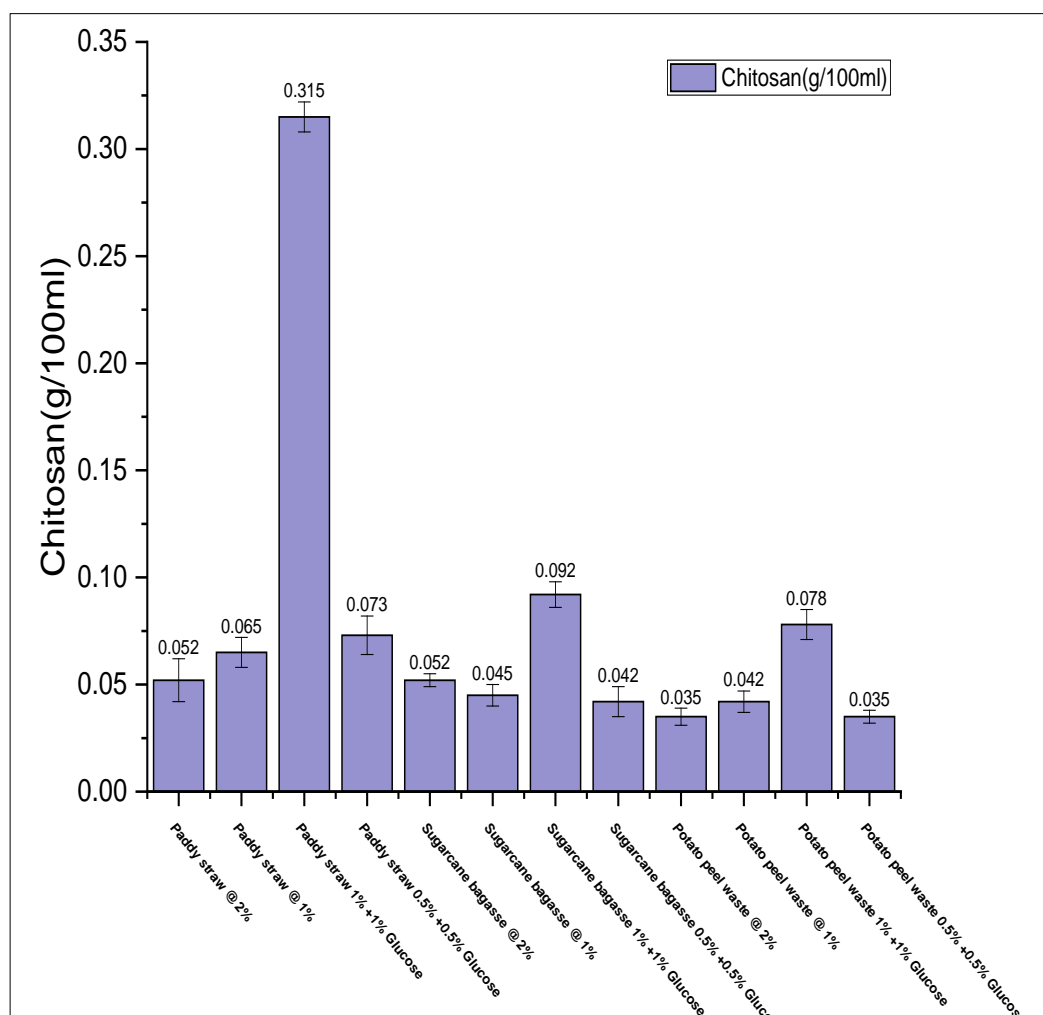


Fig 2: Comparison of the effect of agricultural waste at different concentrations on the production of fungal chitosan

The data in Figure 2 reveals that among the various agricultural waste analyzed, the growth media supplemented with 1% paddy straw +1% glucose (w/v) recorded a significant increase in the yield of cell biomass (1.195 ±0.053 g/100ml) and chitosan (0.315±0.007 g/100ml) at 35°C and pH 5.0 after 96 h of incubation. This might be due to the fact that paddy straw has high water-holding capacity and high carbon content which makes it a potential growth substrate for fungi.

The substrates such as rice straw (Khalaf, 2004) [14], soybean and mungbean residues (Suntornsuk *et al.*, 2002) [23], and potato pieces (Wang *et al.*, 2008) [24] have been employed to culture fungi for chitosan production. Satari *et al.*, (2015) [22] reported the production of large amounts of chitosan (62–67% of the fungal alkali insoluble materials) from the fungus *Mucor indicus* using rice straw as substrate. Wu *et al.*, (2019) [25] extracted 19.7% chitosan from 15 days old biomass of *Agaricus sp.*, *Pleurotus sp.*, and *Ganoderma sp.* using paddy straw and hardwood sawdust in a ratio of 1:1.

Standardization of the fermentation process for mass production of chitosan

The fungal isolate was used for standardizing the fermentation process (solid state fermentation (SSF) and submerged fermentation (SmF)) under optimized physio-chemical and nutrient conditions. Fermentation was carried out at 35 °C and pH 5.0. The highest yield of dry mycelia (2.036 g/10g paddy straw) was obtained in submerged

fermentation when compared with solid-state fermentation (1.266 g/10g paddy straw). The alkali-insoluble material for submerged fermentation was found to be 1.473 g/10g paddy straw whereas for solid-state fermentation, it was 0.628 g/10g paddy straw. The quantity of chitosan extracted from fungi grown in submerged fermentation (0.533 g/10g paddy straw) was significantly higher when compared with the solid-state fermentation (0.182 g/10g paddy straw), (Fig. 3).

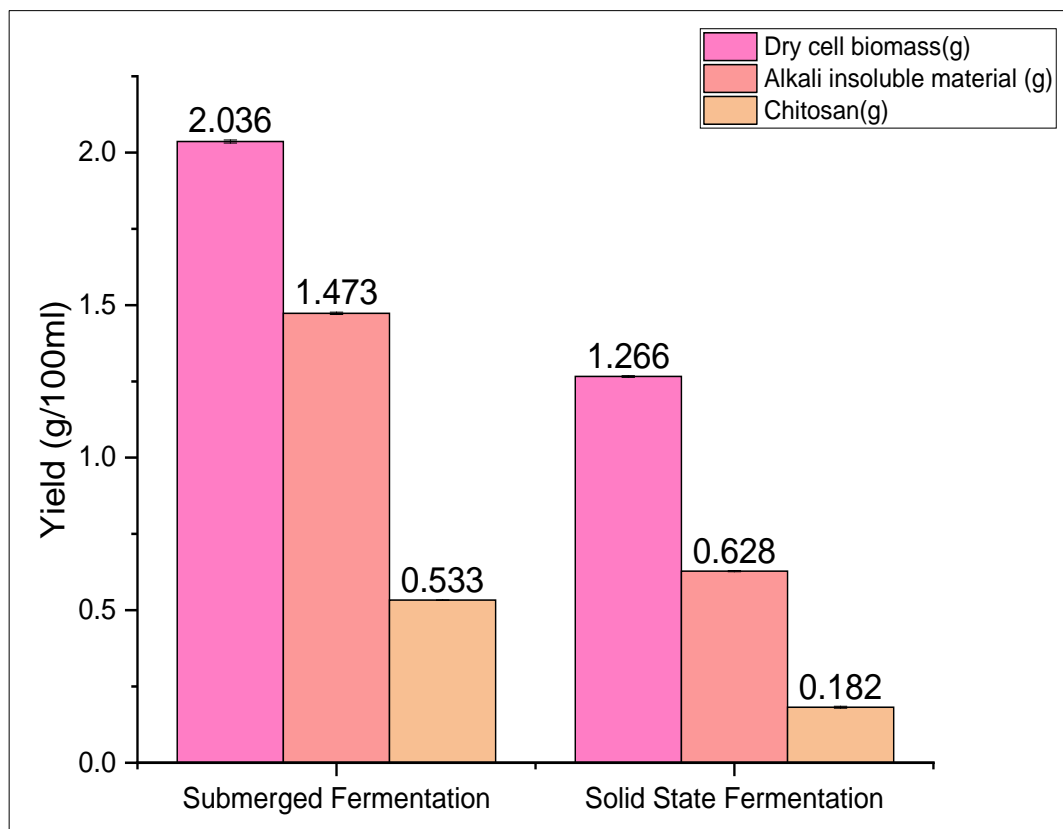


Fig 3: Comparison of the fermentation process for mass production of chitosan

The fermentation process was standardized at 35 °C and pH 5.0. From the data in Fig.3, it was observed that the quantity of chitosan (0.533 g/10g paddy straw) extracted from fungi grown in submerged fermentation has shown a significant increase when compared with the solid-state fermentation (0.182 g/10g paddy straw). This can be due to the fact that solid-state fermentation requires a longer fermentation period for microbial growth than submerged fermentation. Moreover, submerged fermentation provides easier control of fermentation parameters and harvesting of biomass than solid-state fermentation. Many researchers have assessed the production of chitosan from various fungi (*Absidia*, *Aspergillus*, *Penicillium*, *Gongronella*, *Cunninghamella*, *Mucor*, *Phycomyces*, and *Rhizopus*) grown in submerged fermentation and solid-state fermentation (Suntornsuk *et al.*, 2002; Khalaf, 2004) [23]. New *et al.*, (2011) examined a double yield in the fungal biomass and 9.2% of chitosan for *Gongronella butleri* USDB 0201 when grown in submerged fermentation. Similarly, *Mucor rouxii* when grown on different media under submerged conditions gave the yield of chitosan in the range 0.51-0.61g/L. Logesh *et al.*, (2012) [17] further reported a chitosan production of 1.3g per liter of media by *Aspergillus niger* under submerged conditions. The results were also in accordance with the findings of Amorim *et al.*, (2001) [2] who reported the fungi *Mucor racemosus* and *Cunninghamella elegans* by submerged

fermentation, produced a chitosan yield of 35.1 mg/g and 20.5 mg/g dry mycelia weight, respectively. Habibi *et al.*, (2021) [11] further investigated chitosan production through submerged fermentation of *Aspergillus terreus* using apple waste extract as the sole carbon source.

Mass production of chitosan in bioreactor

Aspergillus flavus strain AF211 was used for mass production of chitosan by submerged fermentation in a bioreactor (Eppendorf BioFlo^R 120) under optimized conditions of temperature (35 °C), pH (5) and incubation period (96 h), (Fig. 12 and 13; Plate 6). The maximum dry cell biomass of FC3 was found to be 20.08g/L at 35 °C and pH 5.0 after 96 hours of incubation. On alkali treatment, the amount of alkali-insoluble material obtained after filtration was 8.30g/L. Finally, the yield of chitosan extracted from the fungal isolate FC 3 on mass production in the bioreactor was evaluated to be 5.73 g/L (27%).

The production of chitosan in higher quantities can be done by mass culturing the fungi by utilizing large-scale fermentation techniques. The highest chitosan yield of 27% was obtained from the fungal isolate after 96 hours of submerged cultivation in the bioreactor (BioFlo^R 120). Similar results were obtained by Kumaresapillai *et al.*, (2011) [16] who concluded that the maximum yield of chitosan (26.1%) was obtained from *Aspergillus Niger*

MTCC 2208. It was also found that the results were superior to the values ranging from 1.2 to 24% reported by Gawad *et al.*, (2017) [8] and George *et al.*, (2011) [9], respectively from different fungal strains. This difference in the amount of chitosan extracted from fungal sources might be due to the nature of the fungal strain and the type of production media.

Characterization of chitosan derived from fungal biomass

The characterization of chitosan extracted from isolate FC3 was done by determination of the degree of DE acetylation

(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) [18]. A standard curve of concentration of N acetyl glucosamine versus absorbance was generated (Fig. 4). The degree of DE acetylation of chitosan was a key parameter affecting its physiochemical properties because it is associated with the cationic functions of chitosan. In the present study, chitosan from *Aspergillus flavus* strain AF211 presented a degree of DE acetylation of 88.5%.

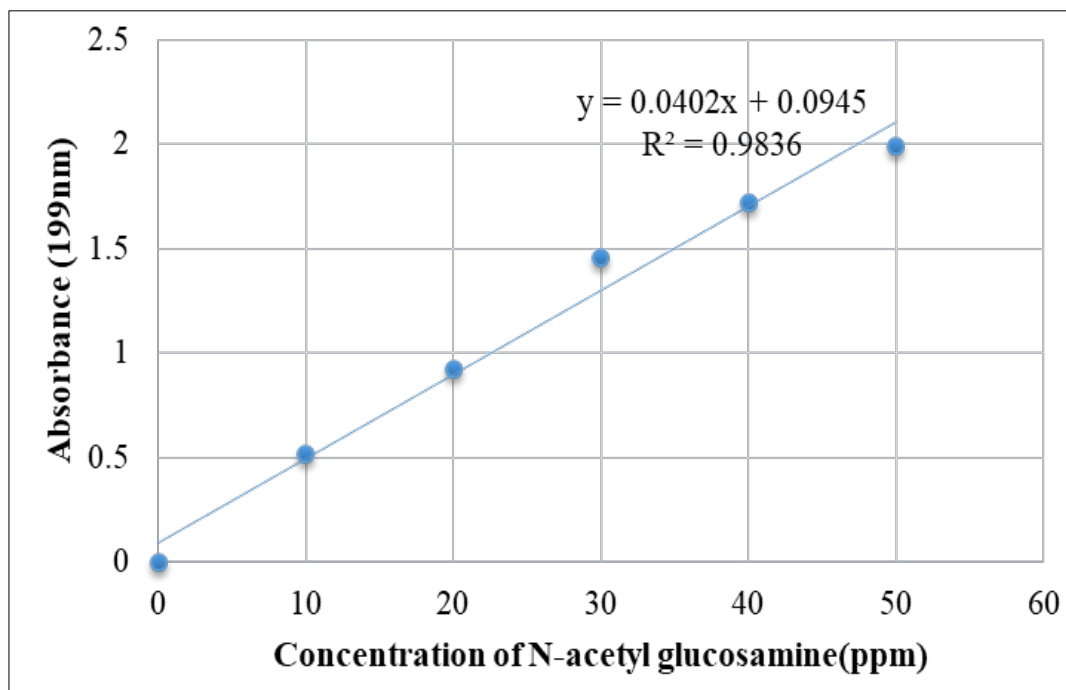


Fig 4: Linearity Curve of N - acetyl glucosamine

The degree of DE acetylation (DD) is one of the important chemical characteristics that influence the chemical reactivity, solubility, biodegradability, and antimicrobial properties of chitosan (Batista *et al.*, 2018) [6]. The chitosan extracted from *Aspergillus flavus* strain AF211 had a degree of DE acetylation of 88.5%. A degree of DE acetylation higher than 50% implies that the majority of the chitosan monomers are DE acetylated and carry an NH₂ group at the C₂ position instead of the acetamido group. Aranaz *et al.*, (2009) [3] stated that chitosan with DD of 70- 100% was observed to be efficient for biomedical and pharmaceutical applications. The degree of DE acetylation 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.*, 2017) [12, 5, 8]. 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%.

Conclusion

Agro-industrial wastes or their residues are rich in nutrient composition and bioactive compounds. In this study, it was demonstrated that the growth media supplemented with 1% paddy straw +1% glucose (w/v) recorded a significant increase in the yield of chitosan (0.315 g/100ml). Fungi have the potential to reuse waste as raw materials for their

growth through fermentation processes. Standardization of farm the enation process *i.e.* solid state fermentation/ submerged fermentation were also conducted under optimized conditions. The quantity of chitosan extracted from fungi grown in submerged fermentation was significantly higher. A culture media using cheap agricultural waste as substrates is a 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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