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Original Research Article

Extraction, Optimization and Characterization of Chitosan from *Penicillium chrysogenum*

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A B S T R A C T

Extraction of chitosan from fungi is gaining significant importance in the world of industrial Mycology. Fungi are thus the promising alternative sources of chitosan. Chitosan extracted from fungi are found to be more consistent and have desired physiochemical properties when compared to the product from crustacean sources. In the present study, *P. chrsogenum* cultured in Czapex Dox broth and its wet mycelia after benzyl penicillin fermentation were selected for chitosan extraction. For the purpose of comparing the yields, commercial chitin obtained from shrimp shells was also used for chitosan extraction. The yield percentage of chitosan extracted from broth culture was found to be higher than that produced by wet mycelia and commercial chitin. Different acids were used for chitosan extraction and it was observed that acetic acid treatment was studied to give highest yield with all the three sources, followed by hydrochloric acid and sulphuric acid. It was also studied that when incubation period after acetic acid treatment during chitosan extraction was altered without changing the concentration, the yield was found to decrease drastically. When different ages of broth culture were used for chitosan extraction, 70 h culture gave a maximum yield. The extracted chitosan was then characterized based on properties such as nature, colour and solubility in water and different acids. The extracted chitosan from broth culture, wet mycelia and commercial chitin were analysed using FT-IR spectrophotometry and the results were found to be similar to the standard absorbance peak of chitosan. Colorimetric estimation of glucosamine units in chitosan from three sources were performed and it revealed higher glucosamine content (54%) in chitosan from broth culture than that extracted from wet mycelia (38%) and commercial chitin.

Keywords

Penicillium chrysogenum, Wet mycelia, Chitosan, FT-IR Commercial chitin.

Introduction

Chitosan is a derivative of chitin, the second most abundant natural polymer on earth after cellulose. It is the major component of skeletal or exoskeletal structure of lower animals, particularly crustacea, molluscs and insects. It is also present in a vast majority of fungi as the principle fibrillar polymer of the cell wall. Fungal cell walls and septa of

Ascomycetes, Zygomycetes, Basidiomycetes and Deuteromycetes contain mainly chitin, which is responsible for maintaining the shape, strength and integrity of cell structure (Ruiz-Herrera *et al.,* 1992; Hon 1996).

Chitin can be processed into many derivatives, the most readily available being chitosan. Rouget first discovered chistosan in □ □ □ □. He derived it from chitin. Chitosan is produced by alkaline deacetylation of chitin. Chitosan, a natural polymer is a fibre like substance formed primarily by repeated unit of ß (1,4) 2-amino, 2-deoxy, d-glucose or D-glucosamine (Varum *et al.,* 1994). Though it can be derived from chitin, it is also a natural component of fungi belonging to *Zygomycetes.* Chitosan occurs naturally in the Mucorales, in particular *Mucor, Absidia* and *Rhizopus sp.* There is apparently only one report on the presence of chitosan in a *Basidiomycete, Lentinus edodes* (Shiitake mushroom). The molecular formula for chitosan is $(C_6H_{11}O_4N)_n$.

Chitosan produced commercially from shrimp and crab shell. Recent advances in fermentation technology suggest that the cultivation of selected fungi provide an alternative source of chitosan is prepared from chitin contained in microbial biomass and in particular fungal biomass. Suitable biomass include *Aspergillus niger, A.terreus, A.oryzae, Lactarius vellereus, Mucor rouxii, Pencilliium chrysogenum, P.notatum, Saccharomyces cerevisiae* and in particular *Candida guillermondi, A.niger and A.terreus* (Fan *et al.,* 2005).

Pencillium oxalium and *P.chrysogenum* are good source of chitosan. Separation of chitosan from *P.chrysogenum* was performed by Tan Tianei *et al.,* 2002. *P.chrysogenum* can be subjected to alkali and acid treatment to isolate chitin and deacetylated to chitosan. The chitosan is then finally extracted. Though chitosan is extracted from broth culture of *P.chrysogenum*, wet mycelial waste after benzyl penicillin fermentation can also be used for chitosan extraction. The present study has been made with following objectives;

Chitosan extraction from broth culture of *P.chrysogenum.*

Chitosan extraction from wet mycelia of *P.chrysogenum.*

Chitosan extraction from commercial chitin.

Comparing yield percentage of chitosan extraction from commercial chitin and *P.chrysogenum.*

Comparing yield percentage of chitosan extraction from different ages of broth culture of *P.chrysogenum.*

Characterization of extracted chitosan.

Determination of absorbance of chitosan using FT-IR spectrophotometry.

Colorimetric estimation of glucosamine units.

Materials and Methods

Collection of sample

Pencillium chrysogenum was obtained from Ponnaiyah Ramajayam College, Department of Microbiology, Thanjavur.

Sources used for Chitosan Extraction

Wet mycelia of *P.chrysogenum,* broth culture of *P.chrysogenum* and commercial chitin derived from shrimp were used as sources for chitosan extraction.

Collection of Wet Mycelia

Wet mycelia of *P.chrysogenum* obtained after benzyl Penicillin fermentation.

Cultivation of *P.Chrysogenum* in Broth

Broth culture of *P.chrysogenum* was prepared in laboratory by culturing it in Czapex Dox broth. About 400ml of Czapex Dox broth at pH 5.6 was prepared. 100 ml of broth was dispensed into each of the 4 conical flasks.

The broth was inoculated with slant and culture of *P.chrysogenum* maintained in Sabouraud's Dextrose agar slant and 4 flasks were inoculated in a rotary shaker operating at 220 rpm at 25^{o} C for 46, 70, 94 and 118 h respectively.

Experimental Design

Wet mycelia of *P.chrysogenum* was collected and was examined microscopically. Broth culture, wet mycelia and commercial chitin were subjected to alkali and acid treatment for chitosan extraction. Yield rate of chitosan was calculated and compared. Yield percentage for different ages of broth culture were also compared. Extracted chitosan was characterized and then analysed by FT-IR spectrophotometer. The amount of glucosamine content in the extracted chitosan was then finally estimated.

Calculation of Yield Percentage

The amount of chitosan from 100g of wet mycelia or commercial chitin was calculated as yield percentage as follows.

$$\text{Yield Percentage} = \frac{\text{Dry weight of chitosan extracted}}{\text{Dry weight of Broth culture / wet mycelia}} \times 100$$

Extraction Procedure (Mei – huei Chen *et al.*, 2002)

One hundred gram of broth culture was taken in clean conical flasks. The protein content in wet mycelia was removed by adding 400ml of 2N NaOH. The alkali treated mixture was then autoclaved at 121^{o}C for 30 minutes at 15 lbs pressure. Then 400 ml of 2% Acidic Acid added to the broth culture. The above procedure for extraction of chitosan from *P.chrysogenum* was modified in the deacetylation steps adding different acids such as conc. HCl (Rosa *et al.,* 2001) and H_2SO_4 instead of acetic acid. Yield percentage of chitosan was compared for 3 different acids added. Broth culture at different ages i.e., 46, 70, 94 and 118 h were subjected for chitosan extraction and the yield percentage were compared. The color of the final product was removed by adding activated charcoal powder (1%) before the crude chitosan extract was filtered after acid treatment.

Chitosan Analysis by FT-IR Spectrophotometry (Moore and Roberts, 1978)

Measuring the absorbance of the isolated chitosan fraction at the wave number 1655 cm^{-1} using FT-IR spectrophotometry, the chitosan analysis was done. FT-IR spectra determination of chitosan was carried out using the potassium bromide disk method.

Principle

FT-IR spectrophotometry determines the sample by measuring absorption of infrared radiation of wave numbers in a region of 4,000 to 400 cm^{-1} at various wave numbers when it passes through the sample. This method uses the property that the infrared absorption spectrum of a substance is characteristics of its chemical structure. FT-

IR spectra are shown in charts by plotting the wave numbers on the abcissa and transmittances on the ordinate..

Estimation of Glucosamine (Elson and Morgan, 1993)

The quantitative analysis of D-glucosamine units present in the isolated chitosan fraction was estimated.

In a test tube 0.5 mg of sample(chitosan), 1ml of acetyl acetone solution and 2ml of double distilled water was added. Simultaneously standard solution was prepared. The tubes were stirred and placed in boiling water bath for 10min. The tubes were cooled and 1ml of absolute ethanol was added. After stirring, the tubes were placed again in water set at 75ºC ± 20ºC for 5min. 1ml of Ehrlich's solution was added slowly and tubes were kept at 75ºC for 30Min. The tubes were then cooled and 5ml of absolute ethanol was added. Pink to red colour develops. It was then kept in dark for 30 min. The absorbance was measured at 520 nm using spectrophotometer. The colouration was stable for 24hrs in dark.

Results and Discussion

The results observed after chitosan extraction from *P.chrysogenum* and commercial chitin.

Microscopic Examination

The wet mycelia of *P. chrysogenum*, on microscopic examination prior to chitosan extraction showed paint brush like appearance.

Yield of Chitosan Extracted from Wet Mycelia, Broth Culture and Commercial Chitin

Chitosan was extracted from broth culture, wet mycelia and commercial chitin. Extraction procedure was similar to that described by Mei – chen *et al*., 2002. Chitosan was extracted and the yield percentage was compared by commercial chitin. Yield was found to be maximum with broth culture. This was because during the active growth, fungi attained high cell densities. The result produced agreed with White *et al*., 1979. Comparatively less yields were produced by wet mycelia and commercial chitin.

Comparison of Yield Percentage of Chitosan Extracted from Wet Mycelia, Broth Culture and Commercial Chitin

The yield percentage of chitosan extracted from wet mycelia, broth culture and commercial chitin were compared. The yield from broth culture was found to be maximum(42.25%) when compared to that produced by wet mycelia(31.66%) and commercial chitin(11.5%)(Table-I).

Comparison of Yield Percentage of Chitosan Extracted from Broth Culture by Different Acid Treatment

The yield of chitosan extracted from broth culture of *P.chrysogenum* varies with different acid treatment. The yield was found to be maximum(45.5%) with acetic acid treatment when compared to that produced by hydrochloric acid (19.0%) and sulphuric acid (16.25%).(Table-II).

Comparison of Yield Percentage of Chitosan Extracted from Wet Mycelia *P.chrysogenum* by Different Acid Treatment

The acetic acid treated wet mycelia of *P.chrysogenum* was observed to have yield of chitosan (34.72%) when compared to that produced by hydrochloric acid (25.25%) and sulphuric acid (15.97%) (Table-III).

Acetic acid treatment was studied to give highest yield than that produced by hydrochloric acid sulphuric acid. The chitosan extracted is soluble in acetic acid and gave higher yields. Less yield was produced by sulphuric acid because chitosan is insoluble in it.

Comparison of Yield Percentage of Chitosan Extracted from Commercial Chitin by Different Acid Treatment

Commercial chitin after acetic acid treatment represented a higher yield of chitosan (13'3%). Comparatively less yield of chitosan were produced by hydrochloric acid (3.66%) (Table-IV).

Comparison of Yield Percentage of Chitosan Extracted from Different Ages of Broth Culture of *P.chrysogenum*

Comparison of the yield percentage of chitosan extracted at different growth stages of *P.chrysogenum* was also done. The yield was maximum (22.7%) at 70 h. The chitosan yield was observed to decrease drastically from 94 h (Table-V). It represents that culture in late exponential phase produced highest chitosan yield as studied by Su *et al*., 1996. This suggests that yield of chitosan is dependent on the age of culture.

Table.1 Comparison of Yield Percentage of Chitosan Extracted from Wet Mycelia, Broth Culture and Commercial Chitin

S.No.	Source	Weight of residue /wet mycelia / commercial chitin (g)	Dry weight of residue/wet mycelia/commercial chitin (g)	Weight of chitosan (g)	Yield percentage (%)
1	Broth culture	100	20	8.45	42.25
2	Wet mycelia	100	24	7.6	31.66
3	Commercial chitin	100	100	11.5	11.5

Table.2 Comparison of Yield Percentage of Chitosan from Broth Culture by Different Acid Treatment

S.No.	Treatment	Weight of residue (g)	Dry weight of residue (g)	Weight of chitosan (g)	Yield percentage (%)
1	Acetic acid	100	20	9.1	42.25
2	Hydrochloric acid	100	20	3.6	31.66
3	Sulphuric acid	100	20	3.25	16.25

Table.3 Comparison of Yield Percentage of Chitosan from Wet Mycelia by Different Acid Treatment

S.No.	Treatment	Weight of wet mycelia (g)	Dry weight of wet mycelia (g)	Weight of chitosan (g)	Yield percentage (%)
1	Acetic acid	100	24	8.33	34.70
2	Hydrochloric acid	100	24	6.06	25.25
3	Sulphuric acid	100	24	3.83	15.96

Table.4 Comparison of Yield Percentage of Chitosan from Commercial Chitin by Different Acid Treatment

S.No.	Treatment	Dry weight of commercial chitin (g)	Weight of chitosan (g)	Yield percentage (%)
1	Acetic acid	100	13.3	34.70
2	Hydrochloric acid	100	3.66	3.66
3	Sulphuric acid	100	1.9	1.9

Table.5 Comparison of Yield Percentage of Chitosan from Different Ages of Broth Culture

S.No.	Ages of clture (h)	Weight of initial material (g)	Dry weight of residue (g)	Weight of chitosan (g)	Yield percentage (%)
1	46	100	20	0.11	0.55
2	70	100	20	4.54	22.7
3	94	100	20	1.68	8.4
4	118	100	20	0.69	3.45

Table.6 Comparison of Yield Percentage of Chitosan from Broth Culture at Different Incubation Time

S.No.	Weight of residue (g)	Dry weight of residue (g)	Incubation time (h)	Weight of chitosan (g)	Yield percentage (%)
1	100	20	12	8.45	42.25
2	100	24	8	7.6	31.66
3	100	100	4	11.5	11.5

Comparison of Yield Percentage of Chitosan Extracted from Broth Culture of *P.chrysogenum* at Different Incubation Time

Chitosan extracted from broth culture was employing different incubation period after acetic acid treatment. Yield was found to be high with 12 h incubation and did not increase on decreasing the incubation time (Table-VI,). This reveals that the yield was depending on the incubation time and the

result was similar to that studied by Pochanavanich and Suntornsuk, 2002.

Characterization of Chitosan

The extracted chitosan was characterized based on colour, nature, texture and its solubility. The colour of the extracted chitosan was noted to be pale yellow. After activated charcoal treatment the colour was pure white. The extracted chitosan was hygroscopic in nature. The extracted chitosan was observed to have a flabby texture.

Chitosan was noted to be insoluble or sparingly soluble in water. Chitosan was found to dissolve in acetic acid and hydrochloric acid. It was insoluble in sulphuric acid. The solubility of chitosan in various acid agreed with the observation of Domand, 1998.

Chithsan Analysis by FT-IR Spectrophotometry

Chitosan extracted was found to give a peak near 1655 cm^{-1} which is the characteristic peak of chitosan in FT-IR spectrum. Chitosan extracted from broth culture, wet mycelia and commercial chitin had absorbance peak at 1655.42, 1650.67 and 1655 cm^{-1} respectively. Commercial chitin was observed to have a peak at 3454.04 cm^{-1} (Fig- 3,4,5&6).The chitosan extracted from three sources were analysed using FT-IR spectrophotometry and the peak of absorbances were similar to the standard peak described by Roberts, 1992.

Estimation of Glucosamine

The amount of glucosamine present in chitosan sample extracted from broth culture, wet mycelia and commercial chitin on treatment with acetic acid are depicted. Highest amount glucosamine was found to be present in chitosan extracted from broth culture (54%). About 38% and 20% of glucosamine units were found in chitosan extracted from wet mycelia and commercial chitin.

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