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SOLID-STATE FERMENTATIVE PRODUCTION AND BIOACTIVITY OF FUNGAL CHITOSAN

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ABSTRACT

Chitosan production was investigated using a laboratory-scale solid substrate fermentation (SSF) technique with four species of fungi: *Penicillium expansum, Aspergillus niger, Rhizopus oryzae* and *Fusarium moniliforme*.The peak growth for the organisms was after 16 days. *Aspergillus niger* had the highest growth with a maximal dry cell biomass of 15.8g/kg after 16 days cultivation on corn straw under solid substrate fermentation. This was closely followed by *Rhizopus oryzae* (14.6g/kg), *Penicillium expansum* (13.8g/kg) *and Fusarium moniliforme* (10.6g/kg) respectively. The fungus *Rhizopus oryzae* had the highest chitosan production with a maximum of 8.57g/kg in 16 days under solid substrate fermentation (SSF) with a medium containing corn straw. *Aspergillus niger* showed a modest chitosan yield of 6.8g/kg. *Penicillium expansum* and *Fusarium moniliforme* had low chitosan yields of 4.31g/kg and 3.1g/kg respectively. The degree of deacetylation of fungal chitosans ranged between 75.3-91.5% with a viscosity of 3.6-7.2 centipoises (Cp).Chitosan extracted from *Rhizopus oryzae* was found to have antibacterial activity on some bacterial isolates. At a concentration of 50mg/L, *Rhizopus oryzae* chitosan paralleled crab chitosan in susceptibility testing against some food-borne bacterial pathogens. *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Bacillus subtilis* showed inhibition rates of 83.2%, 67.9%, 63.8% and 62.4% respectively in response to 50mg/l *Rhizopus oryzae* chitosan in 24 h. The rate of inhibition (%) increased with increase in chitosan concentration.

Keywords: Fungal chitosan, solid-state fermentation, corn straw, bioactivity, bacteria

INTRODUCTION

The cationic biopolymer chitosan is a natural polymer derived from chitin, the principal fibre component of the exoskeleton of shell fish. This polysaccharide consists of repeating units of β- (1-4)2-amino-2-deoxy-D-glucose (or D-glucosamine). Chitosan also can be found in mucoralean fungi (*Zygomycetes*) **(Tan *et al.*, 1996).** In *Mucor rouxii,* it is the principal fibre polymer of the cell wall in addition to chitin **(Bartinicki-Garcia, 1968).** Chitosan has been isolated from *Absidia coerulea* **(Davoust and Hansson, 1991)** and *Mucor rouxii* **(White *et al.*, 1979).**The development of applications for chitosan has expanded rapidly in recent years. This non-toxic, biodegradable polymer has found applications in agriculture, pharmaceutical, cosmetics and food industries. In particular, it is used as a component of toothpaste, body creams, shampoo, lowering of serum cholesterol, cell and enzyme immobilization, as a drug carrier, seed coats, flocculating and chelating agents in wastewater treatments **(Muzzarrelli,1977).** Commercially prepared chitosan is obtained from industrial deacetylation of shrimp and crab shell chitin with the use of caustic alkali **(Tharanathan and Kittur, 2003).** Thus its isolation on an industrial scale is affected equally by the seasonable nature and by the limited availability of raw materials (chitin). The high processing costs associated with chemical conversion of chitin to chitosan coupled with negative environmental impact (pollution) due to the large amounts of waste generated in chemical conversion calls for alternative sources for this unique product **(Crestini *et al.*, 1996).** It is pertinent to note that the conversion of chitin to chitosan by means of strong alkali solution at high temperatures leads to inconsistent physico-chemical characteristics in the product formed **(*Arcidiacono* and Kaplan, 1992; Crestini *et al.*, 1996).** An alternative to overcome these challenges is the fungal production of chitosan, whose main advantage is that it is ecofriendly, can be grown on simple nutrients and the process does not require demineralization thus minimizing production cost. Therefore, physico-chemical characteristics and yields of chitosan isolated directly from a fungus may be optimized by controlling fermentation and processing parameters. Fungal biomass can be produced by solid state fermentation and submerged fermentation **(White *et al.*, 1997).** Some authors have reported the production and characterization of chitosan mostly in the fungal species of *Absidia* and *Mucor* **(white *et al*., 1979, Miyoshi *et al*., 1992; Rane and Hoover, 1993).**

The aim of this study was to investigate inexpensive lignocellulosic material (corn straw) as a suitable substrate for fungal chitosan production from different species using solid state fermentation conditions as well as the characteristics and bioactivity of the chitosan produced.

MATERIAL AND METHODS

Microbial cultures

The fungal cultures employed in this investigation namely: *Rhizopus oryzae, Aspergillus niger*, *Penicillium expansum* and *Fusarium moniliforme* were isolates from agricultural wastes. They were identified based on their microscopic and macroscopic morphological characteristics **(Barnett and Hunter, 1972)**. The cultures were maintained on potato dextrose agar (PDA) medium stored at 4 ^{o}C in a refrigerator. Bacterial cultures*: Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Salmonella typhi* were obtained from the Medical Microbiology Laboratory, University of Benin Teaching Hospital (UBTH), Benin City, Nigeria. They were characterized and identified based on their cultural, morphological and biochemical properties **(Cheesbrough, 2000; Collins *et al.*, 2004).**

Preparation of spore suspension

Spore suspension was employed as an inoculum for corn straw which was the solid substrate. A 5-day-old culture of the fungal organisms: *Rhizopus oryzae, Aspergillus niger, Penicillium expansum* and *Fusarium moniliforme* grown on potato dextrose agar (PDA) slant were used in preparing spore suspension for inoculating the fermentation medium. First sterile NaCl solutions were poured into prepared PDA slants, and after sufficient shaking, were returned to the original saline tube. Then spores suspension in the saline tube was homogenized by means of tube shaker. The spore concentration was adjusted to about 2.5 x 10^{7} spores/mL by 10-15 times dilutions.

Fermentation medium and conditions

The basic fermentation medium employed as the solid substrate was corn straw which was milled into 1-2 cm particles. For effective heat transfer and in order to

have a suitable porosity in solid substrate medium of fermentation, 40 g of dry substrate were weighted in 500 mL Erlenmeyer flasks. The straw being low in moisture for the growth of fungi was humidified to 60% water content with a synthetic medium (0.2% yeast extract, 1.0% peptone and 2.0% glucose). The flasks were then hand shaken to homogenize the solid mediums and autoclaved at 121 ^{0}C for 20 min. Following was inoculation with one mL of spore suspension (about 2.5 x 10^7 spores/mL) into sterilized flasks and shaken to distribute the spores. The flasks were cotton-plugged and remained static during incubation for 4, 8, 12, 16 and 20 days at 30 ^{0}C without air flow.

Extraction of chitosan

Chitosan extraction was carried out by a modified method of **Rane and Hoover (1993).** After cultivation for every 4days, the entire solid-state biomass were ground, suspended with 1M NaOH solution (1:30 w/v) and sterilized at 121 ^{0}C for 20 min (alkali treatment). Following, the alkali insoluble materials (AIM) was centrifuged at 6000rpm for 15min, washed with distilled water several times and re-centrifuged to a neutral pH. AIMs were dried at 40 ^{0}C in an oven. The dried alkali insoluble fraction were treated with acetic acid (2%v/v), as a chitosan solvent under reflux condition for 6-8 h at 95 ^{0}C (1:30 w/v). The extracted slurry was centrifuged at 6000rpm for 15- 20 min and the supernatant containing the chitosan was isolated. In order to precipitate fungal chitosan, the pH of the supernatant was adjusted with 2 M NaOH (pH 10) and then flocculated chitosan was centrifuged at 6000 rpm for 15 min. Thus the isolated chitosan was washed five to six times with distilled water to neutrality. At the same time ethanol (96%) and acetone were employed to rinse chitosan prior to drying in an oven at 60 ^{0}C to constant weight **(Nieder-hofer and Muller, 2004).**

Chitosan characterization

Determination of degree of deacetylation

The method of Donald and Hayes (1988) was used. The extent of chitosan deacetylation was obtained by titration with 0.01 M NaOH. First, the acetyl groups in chitosan was hydrolysed with a strong alkali and the salt converted to acetate, which was then evaporated as an azeotrope with water and titrated. Thus the acetyl percentage was determined from the equation:
% Acetyl = V x 0.04305/W
Where V= corrected volume of NaOH
W= weight of the sample. The degree of deacetylation was then calculated using the formula: % Deacetylation = 100 - % Acetyl.

Determination of viscosity

The viscosity was measured with an Ubbelohde viscometer (type/capillary no.53233/ 111C). Chitosan solution was prepared in 1% acetic acid at 1% concentration on a moisture-free basis. The chitosan solution was then filtered using Miracloth (rayon polyester; EMD Biosciences CA, USA) to remove insoluble materials. Thereafter the solution was allowed to stand for 2 h to remove air bubbles. Measurements were made in triplicate on each sample at 28$\pm$ 2 ^{0}C. Values were reported in centipoises units (cP).

Antibacterial activity assay

Twenty milliliter (20 mL) of Muller-Hinton broth (MHB) was dispensed into 50 mL flasks to which different concentration of chitosan were separately added. The flasks were sterilized (121 ^{0}C, 15 min), cooled to 45 ^{0}C and then inoculated with 0.5 mL from cells of each pathogenic test bacteria species (2.5 x 10^6 cells/mL). The inoculated flasks were incubated at 37 ^{0}C for 24 h. Surviving cells were counted by spreading on Muller-Hinton agar (MHA) plates. For comparison and reference standard crab chitosan at a concentration of 50 mg/L was used. The inhibition ratios were calculated using the equation:
Inhibition ratio (%) = C-E/C X 100
Where C = average number of surviving cells of the control groups (zero chitosan concentration)
E = average number of the surviving cells of the experimental groups (chitosan concentrations)

RESULTS AND DISCUSSION

The dry cell biomass of fungal organisms cultivated in corn straw under solid substrate fermentation is shown in Figure 1. Growth was rapid for both *Aspergillus niger* and *Rhizopus oryzae*. Of the four fungi used, *Aspergillus niger* had the highest growth rate with a maximal dry cell biomass of 15.8 g/kg after 16 days of cultivation. The dry cell biomass of *Rhizopus oryzae* increased from 4.1 g/kg in 4h to 14.6 g/kg in 16 days. The biomass produced by *Penicillium expansum* and *Fusarium moniliforme* after 16 days cultivation on corn straw are 13.8 g/kg and 10.6 g/kg respectively.

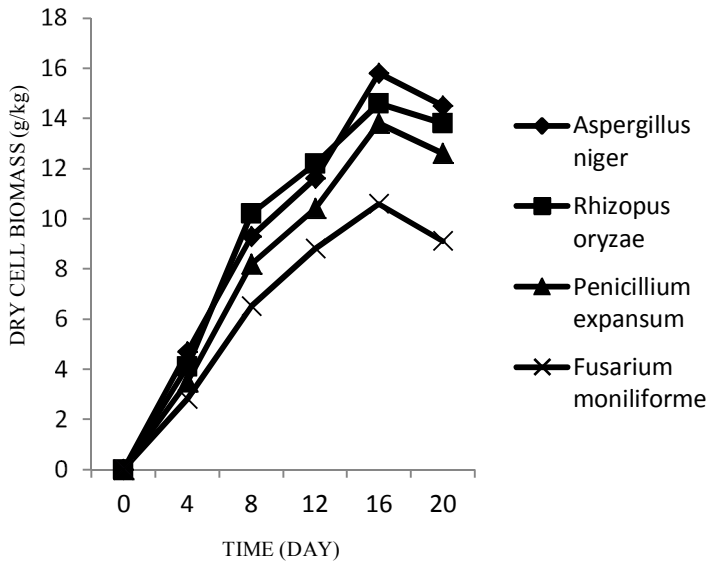

Figure 1 Dry cell biomass of fungal organisms cultivated in corn straw under solid substrate fermentation (SSF)

The chitosan yield of each fungal strain under solid substrate fermentation (SSF) using corn straw is depicted in Figure 2. With *Aspergillus niger* chitosan yield which started after 4 days with 1.8 g/kg increased steadily to a maximum of 6.80 g/kg in 16 days. The fungus *Rhizopus oryzae* had the highest chitosan production rate with a maximum of 8.57 g/kg in 16 days. The chitosan yields of *Penicillium expansum* and *Fusarium moniliforme* were little (4.31 and 3.1 g/kg respectively after 16 days), approximately half of what was produced by *Aspergillus niger* and *Rhizopus oryzae*. Longer periods of incubation of all the fungi beyond 16 days did not result in increase in chitosan but rather slow decrease (Figure 2). **Tan *et al.* (1996)** reported that the late exponential phase of growth produced the most extractable chitosan. However the result of the chitosan yield for *Rhizopus oryzae* in this study is at variance and a little lower than that produced by *Lentinus edodes* using wheat straw under SSF **(Crestini *et al*., 1996).** But in comparison with the reports of **Shimahara *et al.* (1989)** and **Yokoi *et al.* (1998)** the chitosan yield of *Rhizopus oryzae* in the present study is higher than the chitosan produced by other fungi.

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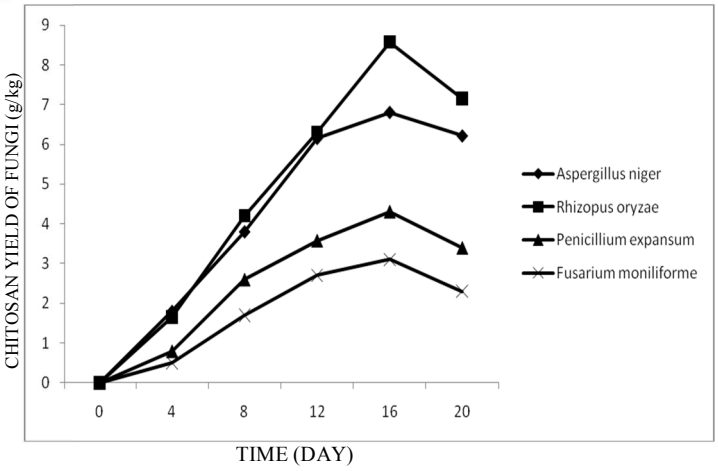

Figure 2 Chitosan Yield of Fungal strains under Solid Substrate Fermentation (SSF) using Corn Straw

The relationship between chitosan yield and alkali insoluble fraction (AIF) is depicted in Figure 3. **Di *et al.* (1994)** reported that the percentage of AIF can be considered as a fungal growth parameter since it is mainly constituted by mycelia growth using *Lentinus edodes* as a case study in solid substrate fermentation At the onset and early stages of fungal growth (Figure 1) the concentration of the AIF increased gradually and was fairly constant but experienced a fall after 16 days of growth. Amongst the four fungal strains used in this experiment, the relationship between AIF and chitosan yield was highest for *Rhizopus oryzae* (58.7%) after 16 days of cultivation (Figure 3).

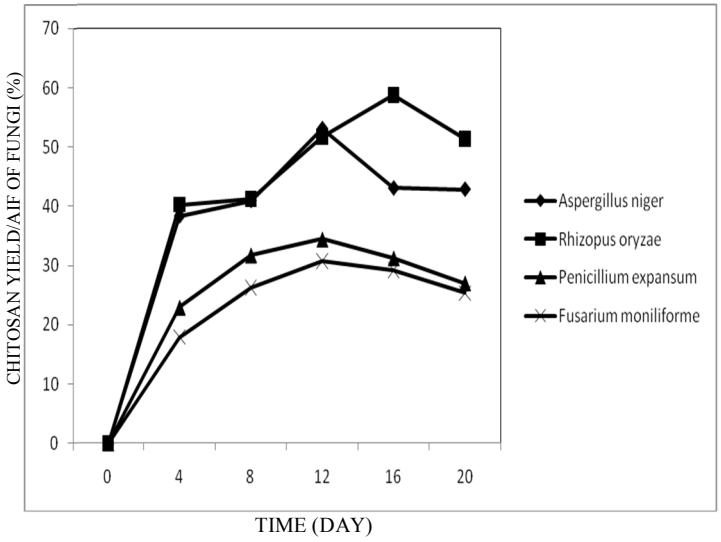

Figure 3 Ratio of Chitosan Yield to AIF from Fungal Strains grown in Corn Straw under SSF

For *Aspergillus niger, Penicillium expansum and Fusarium moniliforme*, the values were 53.0%, 34.4. % and 30.7% after 12 days respectively. **Yokoi *et al* (1998)** however reported that the maximum content of chitosan in dry cell weight for different isolates of *Absidia* sp. grown on shochu distillery wastewater in submerged fermentations conditions was 7.3%. On the contrary **Crestini *et al.* (1996)** found that the ratio of chitosan yields to AIF (%) was about 70% for *Lentinus edodes* growing under solid substrate fermentation conditions.

The functional characterisitics of fungal chitosans in comparison with a commercial chitosan derived from crab shell is shown in Table 1. The degree of deacetylation of fungal chitosans ranged between 75.3 -91.5%. Chitosan from *Rhizopus oryzae* had the highest degree of deacylation (91.5%) followed by *Aspergillus niger* (89.6%), *Penicillium expansum* (80.2%) and *Fusarium moniliforme* (75.3%). These values are however lower compared to that of commercially prepared crab chitosan with degree of deacetylation of 96.8%.The results in the present study were also slightly different from previous reports on degrees of deacetylation of fungal chitosans **(Arcidiacono and Kaplan, 1992; Miyoshi *et al*., 1992** and **Crestini *et al*., 1996).** The physico-chemical function of chitosan is affected by the degree of deacetylation. The higher the degree of deacetylation, the larger the positive charge density on the molecule which confers a greater chelating ability. This in turn enhance applications of the polymer as a coagulant in waste water treatment, food preservative and clarifier, as well as the controlled release of drugs in medicine.

Table 1 Functional Characteristics of Fungal Chitosans

Chitosan source	Degree of Deacetylation (DD)(%)	Viscosity(cP)
Rhizopus oryzae	91.5	7.2
Aspergillus niger	89.6	6.4
Penicillium expansum	80.2	4.8
Fusarium moniliforme	75.3	3.6
Crab shell(sigma-aldrich)	96.8	316.2

The viscosities of fungal chitosan shown in Table 1 reveals a range of 3.6 -7.2 Cp. This result is in agreement with the report of **Shimahara *et al.* (1989)** but lower compared to the viscosity of commercially produced crab chitosan (Table 1). Viscosity is an important factor which determines chitosan's commercial applications. Some studies have shown that viscosity of chitosan significantly affects its antimicrobial activities. **Cho *et al* (1998)** reported that the antimicrobial activity of chitosan against *Escherichia coli* and *Bacillus* sp increased with decreasing viscosity from 1000 to 10 Cp. Viscosity of chitosan is also closely related to its molecular weight. High molecular weight chitosan has higher viscosity than low molecular weight chitosan. This means that the molecular weight of fungal chitosan may be lower than that of crab chitosan. Thus fungal chitosan could have potential food, medicinal and agricultural applications as an antimicrobial and preservative agent.

Table 2 Susceptibility of food-borne Bacterial isolates to *Rhizopus oryzae* chitosan

Chitosan concentration mg/L	Number of Surviving cells (Log cfu/mL) and inhibition rate (%)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Salmonella typhi*
5	5.81 (38.4)	6.23 (29.1)	6.05 (34)	6.30 (27.2)
10	5.56 (41.0)	5.64 (35.8)	5.34 (41.8)	6.05 (30.0)
20	4.65 (50.7)	4.51 (48.7)	4.78 (47.9)	5.76 (33.4)
30	3.58 (62.0)	4.12 (53.1)	4.28 (53.4)	4.36 (49.6)
40	2.75 (70.8)	3.75 (57.3)	3.90 (57.5)	3.42 (60.4)
50	1.58 (83.2)	3.18 (63.8)	3.45 (62.4)	2.78 (67.9)
60	1.58 (83.2)	2.23 (74.6)	2.34 (75.0)	2.78 (67.9)
70	1.58 (83.2)	2.15 (76)	1.89 (79.4)	2.78 (67.9)
Reference[a]	2.00 (79.0)	3.15 (64.1)	3.40 (62.9)	3.02 (65.1)

NOTES: Initial bacterial count for each organism was 6.43 log cfu/mL.
Final bacteria count for *E. coli, Pseudomonas aeruginosa, Bacillus subtilis and Salmonella typhi* were 9.43, 8.79, 9.18 and 8.65 log cfu/mL respectively.
[a]: Crab shell chitosan at a concentration of 50 mg/L
Data in parathensis are values of inhibition ratio.

The susceptibility of some food borne bacterial pathogens to *Rhizopus oryzae* chitosan is shown in Table 2. Chitosan affected the growth of the test organisms in varying degrees when compared with the control (bacterial strains not treated with chitosan) and reference chitosan (50mg/l crab chitosan). Generally the cell numbers seem to decrease with increase in the concentration of chitosan. With 50mg/l of chitosan *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Salmonella typhi* showed log decrease from 6.43 log cfu/mL to 1.58, 3.18, 3.45 and 2.78 log cfu/mL respectively. The inhibition rate of fungal chitosan paralled that of the crab chitosan at 50mg/L (Table 2). Of the four test organisms *Escherichia coli* showed the highest inhibition rate of 83.2% at 50mg/L. *Salmonella typhi*, *Pseudomonas aerugisnosa* and *Bacillus subtilis* exhibited 67.9%, 63.8% and 62.4% inhibition rate respectively. The antimicrobial activity of chitosan has been reported by **Sudarshan *et al* (1992); Zheng and Zhu (2003)**. The results of this study shows that simple extraction from fungal biomass can provide fungal chitosan with similar or even higher efficiency as currently commercially available chitosan.

CONCLUSION

The production of higher amount and high quality chitosan by *Rhizopus oryzae* using a bench scale solid substrate fermentation technique can provide a new low cost and efficient means suitable for optimization to large scale commercial production of high quality chitosans.

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