INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2004/06–6–1033–1036
http://www.ijab.org

# Production and Characterization of Fungal Chitosan Under Solid-State Fermentation Conditions

SALWA A. KHALAF
*Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt*
Corresponding author's e-mail: salwakhlf@hotmail.com

## ABSTRACT

A method for the laboratory-scale production, isolation, and characterization of chitosan by solid-state fermentation (SSF) from different fungal strains were developed. The maximum chitosan yield was 5.63g/kg of fermentation medium. It was attained by growing fungal strain *Rhizopus oryzae* on rice straw under SSF conditions for 12 days. Fungal chitosan, had a degree of deacetylation of 73-90% with a viscosity of 2.7-6.8 centipoises (cP). *R. oryzae* chitosan produced at concentration of 60 mg/L exhibited the maximum antibacterial activity, compared with crab shell chitosan.

**Key Words:** Chitosan; *Rhizopus oryzae*; Solid-state fermentation

## INTRODUCTION

The term solid-state fermentation (SSF) is a microbial process in which a solid material is used as the substrate or the inert support of microorganisms growing on it. In the past decade, SSF systems have generated increasing scientific interest, because they offer several economic and paractical advantages over submerged fermentation (SmF): higher and better product yield and quality, safety, improved product recovery, simpler fermentation technology, reduced waste-water output, lower capital investment, lower plant operation costs and the wastes could be useful as animal feed (Blandino *et al.*, 2002).

Chitosan (Polyglucosamine) is a natural and biodegradable biopolymer. Chitosan has recently been used in many areas, for example, in cosmetics, pharmaceuticals, food additives and agriculture. Its use as, a component of toothpaste, hand and body creams, shampoo, lowering of serum cholesterol, cell and enzyme immobilizer, as a drug carrier, material for production of contact lenses, or eye bandages, permeability control agent, as adhesive, chromatographic support, paper-strengthening agents, antimicrobial compounds, seed coats and flocculating and chelating agents in wastewater treatments (Synowiecki, 1986; Trzcinska & Pachlewski, 1986; Sandford, 1989; Crestini *et al.*, 1996; Yoko *et al.*, 1998; Shahidi *et al.*, 1999; Cetinus & Oztop, 2003; Gil *et al.*, 2004 ).

Chitosan is commercially produced by the deacetylation of chitin obtained from shellfish, shrimp waste and crab and lobster processing using strong alkalis at high temperatures for long periods of time (Knorr, 1991). There are problems with the seasonal supply of raw materials and the high processing costs associated with chemical conversion of the chitin to chitosan (Crestini *et al.*, 1996). Furthermore, the chitosans derived from such a process is heterogeneous with respect to their physio-chemical properties (Crestini *et al.*, 1996).

Recent advances in fermentation technology suggest that many of these problems can be overcome by culturing chitosan-producing fungi (White *et al.*, 1979; McGahren *et al.*, 1984; Rane & Hoover, 1993; Chiang *et al.*, 2003; Kucera, 2004).

The mycelia of various fungi including *Ascomycetes*, *Zygomycetes*, *Basidiomycetes* and *Deutermoycetes*, are alternative sources of chitin and chitosan (Ruiz-Herrera *et al.*, 1992; Hon, 1996; Pochanavanich & Suntornsuk, 2002).

However, some problems still remain, such as the high cost of culture medium. It appears that an economical process of chitosan production would be realized if chitosan could be produced by fungi using cheep lignocellulosic waste materials.

The objective of this study, was to evaluate the possibility to produce chitosan from different fungal species under solid state fermentation (SSF) conditions. Also, characterization of the properties of the produced chitosan were investigated.

## MATERIALS AND METHODS

**Cultures.** The microorganisms used in this study (*Aspergillus niger*, *Penicillium citrinum*, *Fusarium oxysporum* and *Rhizopus oryzae*) were previously, locally isolated from different lignocellulosic agriculture wastes. The cultures were maintained on potato dextrose agar (PDA) medium at 4°C.

**Cultivation conditions.** Rice straw, used as the basic substrate for SSF, was milled into 1-2 cm particles. The straw was humidified to 60% water content with a synthetic medium (0.2% yeast extract, 1.0% peptone and 2.0% glucose). The substrates were autoclaved at 121°C for 20 min, and inoculated with a spore suspension ($2x10^7$ cells/100 grams) in sterile plastic bags with cotton plugs,

without air flow, at 30°C. samples were taken every 3 days for 15 days.

**Chitosan extraction.** Chitosan extraction was carried out by a modified method of Rane and Hoover (1993) and Crestini, *et al.* (1996) The whole solid-state biomass were ground, suspended with 1M NaOH solution and autoclaved at 121°C for 30 min. Alkali-insoluble fractions (AIF) were collected after centrifugation at 8000 rpm for 15 min, washed with distilled water and recentrifuged to a neutral pH. The residues were further extracted using 2% acetic acid at 95°C for 8h. The extracted slurry was centrifuged at 8000 rpm for 15 min. The supernatant was separated from the precipitated chitin, adjusted to pH 10, and centrifuged. The obtained precipitate, chitosans air dried at 60 °C to a constant weight.

**Chitosan Characterization**

**Deacetylation.** The extent of chitosan deacetylation was determined by titration with 0.01M NaOH (Donald & Hayes, 1988). The method involved hydrolysing the acetyl groups in chitosan with a strong alkali and converting the salt to acetate, which was evaporated as an azeotrope with water and titrated. The acetyl percentage was determined from the equation :

$$\% \text{ acetyl} = V \times 0.04305 / W$$

where V is the corrected volume of NaOH and W is the weight of the sample. The degree of deacetylation was calculated using the equation :

% deacetylation = 100 - % acetyl.

**Viscosity.** The viscosity of 1% chistosan in 2% acetic acid solution was determined using a Brookfield digital Rheometer (Model DV-III, Brook Engineering laboratories, Inc., Stoughton, MA) at 25°C.

**Antibacterial activity.** Different concentration of *R. oryzae* chitosan, were separately added into 100 ml flasks containing 30 ml, tryptone yeast dextrose broth (TYD). The sterilized flasks were cooled at 45°C. then inoculated with 0.5 ml from cells of each pathogenic test bacterial species ($2.6 \times 10^6$ cells/ml). The inoculated flasks were incubated at 30°C for 24h. Surviving cells were counted by spreading on nutrient agar (NA) plates. Crab chitosan at concentration 60 mg/L was taken as a reference for comparison. The inhibition ratios were calculated with the following formula:

$$\text{Inhibition ratio (\%)} = C - E / C \times 100$$

where C is the average number of the surviving cells of the control groups (zero chitosan concentration), E is the average number of the surviving cells of the experimental groups (chitosan concentrations).

**Statistical analysis.** Each experiment was performed in three replicates and analyses were carried out in duplicate. Data given here are the averages of the measurements. The standard deviation of the duplicate never exceeded ± 10% of the mean through out present work .

**Table I. Degree of deacetylation (%) and viscosity of chitosan produced by different fungal strains**

| Chitosan source | Degree of deacetylation (%) | Viscosity (cP) |
|---|---|---|
| Crab shell (Sigma) | 96.8 | 316.2 |
| *A. niger* | 84.2 | 5.9 |
| *P. citrinum* | 78.5 | 4.6 |
| *F.oxysporum* | 73.4 | 2.7 |
| *R.oryzae* | 90.2 | 6.8 |

## RESULTS AND DISCUSSION

Under SSF, the percentage of AIF can be considered as a fungal growth parameter, since it has been reported to be mainly constituted by mycelial growth (Di Lenia *et al.*, 1994). The concentration of AIF was found fairly constant at the early stages of growth, and it was decreased slowly after 9-12 days of cultivation (Fig.1). *Aspergillus niger* had the highest growth rate with a maximal dry cell biomass of 16.6 g/kg after 9 days of cultivation, while *Fusarium oxysporum* grew very slowly with a minimal biomass of 8.6g/kg after 12 days of cultivation. The highest biomass of *Penicillium citrinum* and *Rhizopus oryzae* were 13.7 g/kg and 12.4 g/kg, respectively, after 12 days of cultivation (Fig. 1).

The chitosan content of each fungal strain was estimated at different stage of cell growth as shown in Fig. 2. However, it has been previously reported that the late exponential phase produced the most extractable chitosan

**Table II. Effect of different chitosan concentrations on the growth of pathogenic bacterial strains**

| Chitosan conc. (mg/L) | No. of surviving cells and inhibition ratio (%) | | | |
|---|---|---|---|---|
| | ***B. cereus*** | ***P. aeruginosa*** | ***Salmonella sp*** | ***E. coli*** |
| 20 | $1.66 \times 10^6$ (36) | $1.24 \times 10^6$ (52) | $1.43 \times 10^6$ (45) | $9.62 \times 10^5$ (63) |
| 40 | $9.36 \times 10^5$ (64) | $7.02 \times 10^5$ (73) | $8.06 \times 10^5$ (69) | $4.16 \times 10^5$ (84) |
| 60 | $5.98 \times 10^5$ (77) | $3.64 \times 10^5$ (86) | $4.94 \times 10^5$ (81) | $2.08 \times 10^5$ (92) |
| 80 | $8.06 \times 10^5$ (69) | $6.50 \times 10^5$ (75) | $7.28 \times 10^5$ (72) | $3.64 \times 10^5$ (86) |
| 100 | $1.17 \times 10^6$ (55) | $8.86 \times 10^5$ (66) | $1.04 \times 10^6$ (60) | $5.98 \times 10^5$ (77) |
| Reference | $8.32 \times 10^5$ (68) | $5.46 \times 10^5$ (79) | $6.76 \times 10^5$ (74) | $3.90 \times 10^5$ (85) |

**N.B.**: 1- Initial count of each strain was $2.6 \times 10^6$ cells/mL (chitosan free).
2-Data between brackets were values of inhibition ratio (%).
3-Reference: Crab shell chitosan at conc. 60 mg/L.

**Fig. 1. Dry cell biomass of *A. niger*; *P.citrinum; F.oxysporum* and *R.oryzae* grown under SSF conditions at 30°C.**

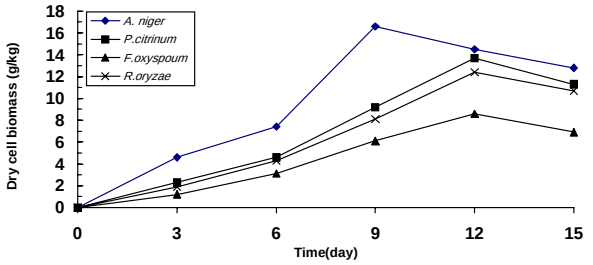



**Fig. 2. Yields of extracted chitosan/ fermentation medium of *A. niger* ; *P.citrinum; F.oxysporum* and *R.oryzae* grown under SSF conditions at 30°C.**

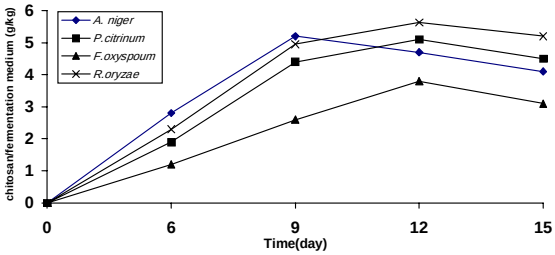
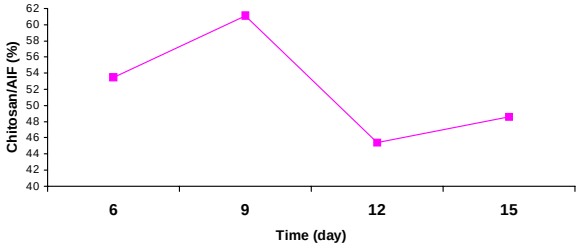



**Fig. 3. Yields of extracted chitosan/AIF from *R. oryzae* under SSF conditions at 30°C.**




(Su *et al.*, 1996). The chitosan content reached its maximum value of 5.63 g/kg by *R. oryzae* after 12 days of cultivation. For longer periods of incubation the chitosan content decreased slowly (Fig. 2). The other fungal strains were found to produce little chitosan content under SSF conditions. Chitosan yield by *R.oryzae* in present study was little lower than those of *Lentinus edodes* produced from wheat straw under SSF (Crestini *et al.*, 1996), but it was higher than the content of chitosan produced by other fungi, under submerged fermentation (Shimahara *et al.*, 1989; Yokoi *et al.*, 1998). The yield of chitosan from *L.edodes* grown on wheat straw under SSF was over 50 times higher than the chitosan yield by submerged cultivation (Crestini *et al.*, 1996). Therefore, the cultivation method is also an important factor for fungal chitosan production.

In present study, because the AIF, was considered as a fungal growth parameter, the relationship between chitosan content and AIF was recorded, specially for *R. oryzae* (higher chitosan producer) strain. Fig. 3 showed that the higher percent of this relationship (61.11%) was observed after 9 days of cultivation. Yokoi *et al.* (1998) showed that the maximum content of chitosan in dry cell weight for different isolates of *Absidia* sp. grown on shochu media (shochu distillery wastewater) in submerged fermentations conditions was 7.3%. Also, Pochanavonich and Suntornsuk (2002) showed that, the maximal chitosan content of various fungal sp grown in synthetic medium was 14%, and recorded by *R. oryzae* TISTR 3189 strain. In contrast, Crestini, *et al.* (1996) found that the content chitosan/AIF (%) was about 70% for *L. edodes* by growing under SSF conditions.

The properties of fungal chitosan compared with those of a commercial ones derived from crab shells were shown in Table I.

The degree of deacetylation of fungal chitosan was73-90%, relatively lower than that of crab chitosan. Results were slightly different from the reported percentage degrees of deacetylation of chitosan from other fungi (Shimahara *et al.*, 1989; Arcidiacono & Kaplan, 1992; Miyoshi *et al.*, 1992; Crestini *et al.*, 1996). The degree of deacetylation is an important parameter affecting of physico-chemical properties of chitosan. In fact, the large positive charge density due to the high degree of deacetylation makes fungal chitosan unique for industrial applications, particularly as a coagulation agent in physical and chemical waste-treatment systems, as a chelating and clarifying agent in the food industry, and as an antimicrobial agent.

The viscosity of fungal chitosan was 2.7-6.8 centipoises (cP), considerably lower than the viscosity of crab chitosan (Table I). Results were similar to those reported by Shimahara *et al.* (1989) and Pochanavanich and Suntornsuck (2002). This means that the molecular weight may be lower than that of crab chitosan. Thus, fungal chitosan could have potential medical and agricultural applications (Pochanavanich & Suntornsuck, 2002).

Effect of *R. oryzae* chitosan on growth of different pathogenic bacterial strains is shown in Table II. Generally, it is clear that *R. oryzae* chitosan at concentration 60 mg/L was more effective on growth of all tested strains. Additionally, chitosan affected the growth of the test bacterial pathogens in varying degrees comparing with control (bacterial strains not treated with chitosan) and reference chitosan (60 mg/L crab chitosan). Wang (1992) showed that, chitosan exhibited antibacterial activity towards various bacteria where, as the concentration of chitosan increased, its effectiveness also increased.

In conclusion, the use of bench scale SSF technique gave higher amount and high quality of chitosan by *R. oryzae*. This technique provides a new economical, and efficient route, suitable for direct scaling up to large scale

industrial production for high-quality chitosans.

## REFERENCES


Arcidiacono, S. and D.L. Kaplan, 1992. Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. *Biotechnol. and Bioeng.*, 39: 281–6

Blandino, A., T. Iqbalsyah, S.S. Pandiella, D. Cantero and C. Webb, 2002. Polygalacturonase production by *Aspergillus awamori* on wheat in solid–state fermentation. *Appl. Microbiol. Biotechnol*., 58: 164–9

Cetinus, S.A. and H.N. Öztop, 2003. Immobilization of catalase into chemically crosslinked chitosan beads. *Enzyme and Microb. Technol.*, 32: 889–94

Chiang, C–L., C–T. Chang and H–Y. Sung, 2003. Production and properties of chitosanase from a mutant of *Bacillus subtilis* IMR–NKI. *Enzyme and Microb. Technol.*, 32: 260–7

Crestini, C., B. Kovac and G. Giovannozzi–Sermanni, 1996. Production and isolation of chitosan by submerged and solid–state fermentation from *Lentinus edodes*. *Biotechnol. and Bioeng.*, 50: 207–10

Di, Lena, G., A.D. Annibate and G. Giovannozzi–Sermanni, 1994. Influence of the age growth conditions on the mycelial chitin content of *Lentinus edodes*. *J. Basic Microbiol.*, 34: 11–6

Donald, H.D. and E.R. Hayes, 1988**.** Determination of degree of acetylation of chitin and chitosan. *Methods in Enzymol.*, 161: 442–6

Gil, G., S. del Monaco, P. Cerrutti and M. Galvagno, 2004. Selective antimicrobial activity of chitosan on beer spoilage bacteria and brewing yeasts. *Biotechnol. Lett*. 26: 569–74

Hon, D.N.S., 1996. Chitin and chitosan: Medical application. *In*: Dumitri S. (ed.), *Polysaccharides in Medicinal Application*, pp: 631–49. Marcel Dekker, New York

Knorr, D., 1991. Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.*, 26: 114–22.

Kucera, J., 2004. Fungal mycelium–the source of chitosan for chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*., 808: 69–73

McGahren, W.J., G.A. Perkinson, J.A. Growich, R.A. Leese and G.A. Ellestad,1984. Chitosan by fermentation *Process Biochem.*, 19: 88–90

Miyoshi, H., K. Shimura, K. Watanabe and O. Kasuki, 1992. Characterization of some fungal chitosans. *Biosci. Biotechnol. Biochem.*, 56: 1901–5

Pochanavanich, P. and W. Suntornsuk, 2002. Fungal chitosan production and its characterization. *Lett. in Appl. Microbiol.*, 35: 17–21

Rane, K.D. and D.G. Hoover, 1993. An evaluation of alkali and acid treatment for chitosan extraction from fungi. *Process Biochem.*, 28: 115–8

Ruiz–Herrera, J., R.R. Sentandreu and J.P. Martinez, 1992. Chitin biosynthesis in fungi. *In*: Dilip, K.A., P.E. Richard and K.G. Mukerji (eds.), *Handbook of Applied Mycology*, pp: 281–312. Fungal Biotechnology. Marcel Dekker, New York

Sandford, P.A., 1989. Chitosan: commercial uses and potential applications. *In*: Braek, G., T. Anthonsen and P. Sandford (eds.), *Chitin and Chitosan*. pp: 51–69. Elsevier Appl. Sci. London–New York

Shahidi, F., J.K. Arachchi and J.J. You, 1999. Food applications of chitin and chistoan. *Trends in Food Sci. Technol.*, 10: 37–51

Shimahara, K., Y. Takiguchi, T. Kobayashi, K. Uda and T. Sannan, 1989. Screening of mucoraceae strains suitable for chitosan production. *In*: Skjak–Brack, G., T. Anthonsen and P. Sanford (eds.), *Chitin and Chitosan*. pp: 171–8. London, Elsevier Applied Science

Su, C.T., K.T. Teck, M.W. Sek, and E. Khor, 1996. The chitosan yield of zygomycetes at their optimum harvesting time. *Carbohydrate Polymer*, 30: 239–42

Synowiecki, J., 1986. Use of krill chitin as an enzyme support. *In*: Muzzarelli, R.A., C. Jeuniaux and G. Gooday (eds.), *Chitin in Nature and Technology*. pp: 417–20. Plenum Press, New York

Trzcinska, M. and W. Pachlewski, 1986. Chitin content of vegetative mycelia of some mycorrhizal fungi. *Acta Microbiol.*, 22: 89–93

Wang, G.H., 1992. Inhibition and inactivation of five species of food–borne pathogens by chitosan. *J. Food Prot*., 55: 916–9

White, S.A., P.R. Farina and L. Fultong, 1979. Production and isolation of chitosan from *Mucor rouxii*. *Appl. and Environ. Microbiol.*, 38: 323–8

Yokoi, H., T. Aratake, S. Nishio, J. Hirose, S. Hayashi and Y. Takasaki, 1998. Chitosan production form *Shochu* distillery wastewater by funguses. *J. Ferment. Bioeng.*, 85: 246–9


**(Received 10 September 2004; Accepted 20 October 2004)**