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Kinetics of *Mucor rouxii* Fermentation in Relation to Chitosan Production

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Abstract: Some biochemical parameters during growth of *Mucor rouxii* and chitosan production in molasses-salt medium were determined. Growth of the organism started with an initial lag of 3 h; maximum growth and chitosan production were obtained at 32 h of fermentation. Fall in sugar concentration from 40.05 to 3.4 g L⁻¹ was noted during the course of fermentation. Degree of acetylation of chitosan was more or less the same whereas increase in molecular weight was noted with the time of fermentation. Maximum 0.61 g chitosan of molecular weight 2.48×10^4 with ~13.0% acetylation could be harvested from mycelia of *M. rouxii* grown in 1 L of molasses salt medium.

Key words: *Mucor rouxii*, chitosan, molasses, fermentation, biochemical changes

Introduction

Polymer of β -1,4-linked-D-glucosamine, commonly known as chitosan, is the deacetylated product of chitin - a major constituent of exoskeleton of arthropoda. Chitosan is widely used in food (Bégin and Calsteren, 1999; Chatterjee *et al.*, 2004; Roller and Covill, 1999; Tsai *et al.*, 2000) and pharmaceutical industries (Fernández-urrusuno *et al.*, 1999; Shu *et al.*, 2000; Kabbaj and Philips, 2001; Hu *et al.*, 2004). It also finds application in environmental biotechnology (Chatterjee *et al.* 2005b; Chatterjee *et al.*, 2002; Chiou and Li, 2003; Dambies *et al.*, 2001; Dantas *et al.*, 2001; Jin and Bai, 2002).

Chitosan is generally produced by deacetylation of chitin isolated from the exoskeleton of arthropoda with alkali (Chatterjee *et al.*, 2003). However, the process is not eco-friendly as it requires large volume of concentrated alkali. Moreover, chitosan prepared by this method lacks consistent physico-chemical properties, which are essential for specific application. With the advent of fermentation technology, chitosan having uniform physico-chemical properties can be prepared in an eco-friendly way from fungi belonging mainly to the zygomycetes group (Tan *et al.*, 1996). Organisms of this group contain chitosan as a major constituent of the cell wall; however, depending on the growth conditions, the amount of chitosan may vary. We have already reported the fermentative production and characterization of chitosan from *Mucor rouxii* grown in a cheap medium containing molasses, a byproduct of the cane sugar industry supplemented with inorganic salts (Chatterjee *et al.*, 2005a). However, details of the fermentative process with molasses are required for large-scale production of *M. rouxii* mycelia as well as chitosan on the same. In this direction, we describe in this paper the changes, in respect of utilization of sugar, different forms of nitrogen, phosphorous in relation to mycelial growth and chitosan production for better understanding of the fermentation process. This study was conducted in the Biological Chemistry Department of the institute during the past one year.

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Materials and Methods

Materials

All chemicals and biochemicals used in this experiment were purchased from E. Merck, Germany. Molasses was procured from local market.

Microorganism

Mucor rouxii (MTCC 386) used in this study was obtained from the Institute of Microbial Technology, Chandigarh, India and maintained on potato-dextrose agar slants.

Fermentation Medium

The medium contains 0.2% NaNO₃, 0.1% K₂HPO₄, 0.001% FeSO₄·7H₂O; 0.001% MgSO₄·7H₂O; 0.2% yeast extract and cane molasses. Molasses was added to the medium to attain the sucrose concentration at 4.0%. pH of the medium was adjusted to 5.1. The medium was distributed in 50 mL aliquots in 250 mL Erlenmeyer flask and autoclaved at 121°C for 15 min.

Inoculum and Fermentation

Each Erlenmeyer flask containing medium was inoculated with 1 mL spore suspension (~6.4×10⁶ spores) prepared by growing the organism in potato dextrose agar plates at 30°C for 3 days. Flasks were incubated under shaking condition (120 rpm) at 30°C up to 60 h. At the end of desired incubation period, mycelia were separated by filtration, washed with water and weights taken after drying by lyophilization. Both mycelia and culture filtrate (fermented broth) were kept at -20°C till used for analysis.

Analytical Techniques

Sugar content (expressed as sucrose) of the culture filtrate was determined by phenol sulphuric acid method as described by Dubois *et al.* (1956). Total and amino nitrogen were estimated by micro Kjeldahl (AOAC, 2000) and Sørensen's formol titration (Hawk *et al.*, 1954) method, respectively. Total phosphorus content of the broth was determined by the process described in AOAC (2000). pH of the broth was measured with a pH meter (Orion model 420A) while free acids were estimated by titration with 0.01 (N) NaOH. Chitosan was isolated from the mycelia (biomass) of *M. rouxii* by the method as described earlier (Chatterjee *et al.*, 2005a).

Characterization of Chitosan

Degree of acetylation was determined by the first derivative of UV absorbance spectrum (Muzzarelli *et al.*, 1997). Weight average molecular weight (M_w) of chitosan was measured from intrinsic viscosity method using Haake Rheometer (Rotovisco model RT20, con/plate sensor C60°/1° and Haake software version V₃).

Results and Discussion

The growth of *M. rouxii* as well as chitosan production was dependent on the nutrients used and the chemico-physical environment of the fermentation medium (Chatterjee *et al.*, 2005a). In this study, molasses was used since it is a cheap carbon source obtained as a byproduct of the cane sugar industries. As per objective of this study, the optimum conditions of growth and chitosan production by *M. rouxii* on molasses are presented with respect to sugar utilization, pH and titratable acidity of the broth at different stages of fermentation (Fig. 1). The mycelial growth started with an initial lag of 3 h and reached maximum at 32 h of incubation, beyond which fall in biomass production was noted

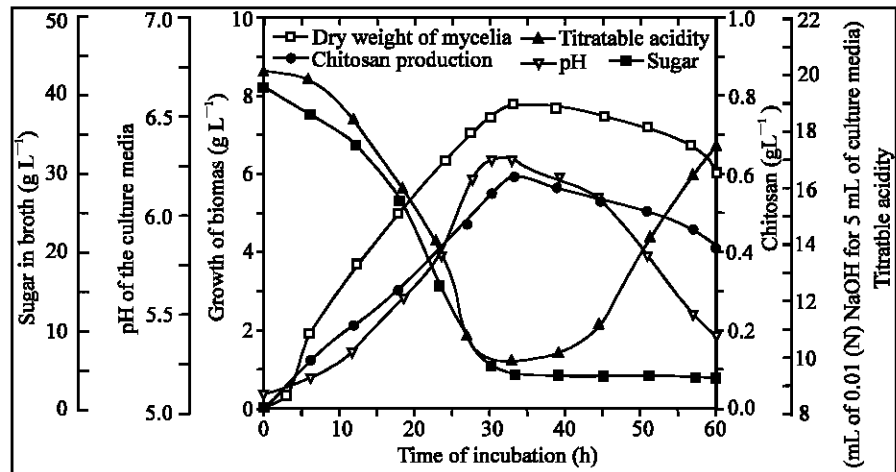


Fig. 1: Changes in sugar and acidity of molasses salt medium in relation to biomass and chitosan production by *M. rouxii*

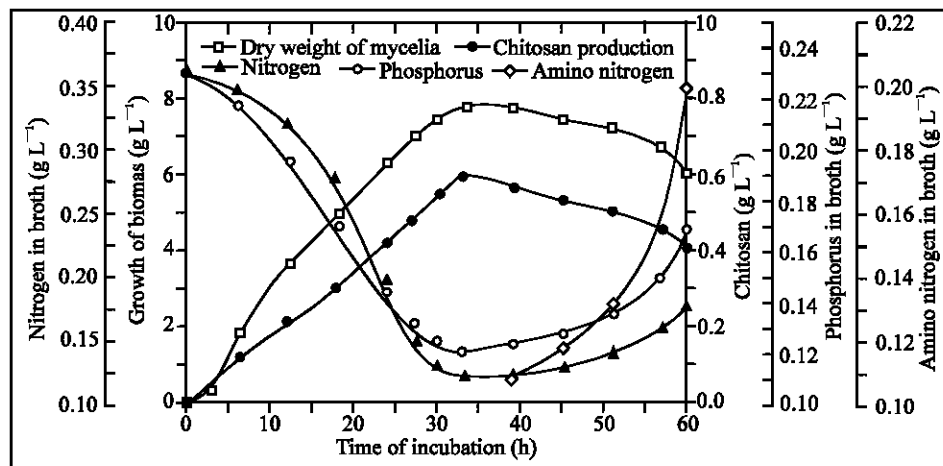


Fig. 2: Nitrogen and phosphorus nutrition during fermentation and chitosan production by *M. rouxii* in molasses salt medium

indicating beginning of the death phase. Like growth, chitosan production also reached maximum after 32 h of fermentation and then decreased slowly. The change in pH of the medium should be taken into consideration as it greatly influences microbial growth. pH of the medium increased from 5.1 to 6.3 after 30 h of incubation then it started decreasing. Initial increase in medium pH may be due to the utilization of nitrate by the organism and low buffering capacity of the medium. As expected titratable acidity of the broth corroborated to the findings of pH change. Steady fall in sugar concentration from 40.5 to 4 g L⁻¹ was noted during the course of fermentation. This is due to the bioconversion of sugar for biomass production. The results suggest that chitosan production varies directly with the biomass as expected because it is a natural constituent of *M. rouxii* mycelia.

The change in concentration of the total and amino nitrogen along with residual phosphorus of the fermented broth in relation to mycelial growth of *M. rouxii* and chitosan production at different phases of growth are presented in Fig. 2. Phosphorus concentration of the medium decreased from 0.23

to 0.11 g L^{-1} till maximum biomass production was obtained. This is apparently due to uptake of phosphorous from the medium by the cells to synthesize cellular organic phosphorus compounds. Slight increase in phosphorous concentration of the broth was noted after 32 h of incubation, probably due to release of phosphorous upon death of the cells. Amino nitrogen concentration in the broth was initially low (below detection limit) as the medium contained only a small amount of this nitrogen from the yeast extract and molasses, which was utilized during growth of the organism. The amino nitrogen content could be detected in the broth after 39 h of fermentation with an increasing trend, which may be attributed to the release of protein and peptides due to lysis of the cells. Total nitrogen of the medium declined from 0.36 to 0.14 g L^{-1} until maximum mycelial growth was obtained, which was probably due to incorporation of nitrogen in the cells. Similarly, lysis of the cells contributed to the rise in nitrogen of the culture filtrate after 32 h of fermentation. The results of phosphorus and nitrogen utilization from the culture medium corroborated excellently with the growth phase of *M. rouxii*.

Maximum 0.61 g of chitosan could be obtained from the mycelia grown in one litre of molasses-salt medium after 32 h of incubation. The ($\bar{M}_w$) of chitosan isolated from the mycelia harvested at different growth phases at 12 h, 20 h, 32 h and 50 h of incubation were 1.35×10^4 , 1.89×10^4 , 2.48×10^4 , 2.35×10^4 , respectively. The ($\bar{M}_w$) was hence found to increase with the time of incubation and reached a maximum at 32 h of incubation. Further incubation resulted in a decrease in the molecular weight. These findings corroborated to the earlier report of Arcidiacono and Kaplan (1992). Slight decrease in ($\bar{M}_w$) of chitosan noted at death phase of the organism may be due to modification in the cell wall or chitosanase activity, as this enzyme has been reported to be present in *M. rouxii* (Reyes *et al.*, 1985). Degree of acetylation of isolated chitosan was around 13% and no significant change was noted (data not shown). Thus, the chitosan obtained from the *M. rouxii* mycelia may be regarded as a good quality product since its solubility is expected to be high due to its low acetyl content. It may be noted that chitosan of various ($\bar{M}_w$) having specific end use may be produced at different periods of incubation. Towards commercial utilization of molasses for the production of chitosan, further study on scaling up is required.

Conclusions

Production of chitosan by *M. rouxii* depends on the time of fermentation and the highest amount of chitosan could be obtained when the mycelial growth was maximum. Molecular weight but not degree of acetylation of chitosan depends on the phase of growth of *M. rouxii*. 0.61 g chitosan having molecular weight 2.48×10^4 and degree of acetylation ~13% could be harvested from the mycelia of *M. rouxii* grown in one litre of molasses salt medium after 32 h of fermentation.

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