# SIMULTANEOUS PRODUCTION OF CHITOSAN AND ETHANOL WITH RHIZOPUS ORYZAE AND MUCOR INDICUS

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## Abstract

An alternative source of chitosan is cell wall of zygomycetes, which are saprophytic fungi and able to produce several metabolites such as ethanol and lactic acid. The current work deals with simultaneous production of ethanol and chitosan by zygomycetes *Rhizopus oryzae* and *Mucor indicus*. These fungi assimilated glucose and produced ethanol as the major metabolite, while their biomasses were future processed by alkali and acid treatment in order to obtain chitosan. The results indicate that cultivation time significantly influences both the amount of extractable chitosan in mycelia and the ethanol yield. As the fungi germinated, chitosan yield from dry biomass increased by increasing the cultivation time until a maximum and then decreased in the later phase of cultivation. Maximum chitosan and ethanol yields from *R. oryzae* were 4.9% of biomass and 0.37 g/g sugar respectively, which occurred at 48 h cultivation time. The corresponding numbers for *M. indicus* were 2.4% and 0.41 g/g which occurred at 24 and 18 h, respectively. The chitosan had degree of deacetylation higher than 80%. Glycerol was an important by-product of both fungi, while lactic acid was also produced by *R. oryzae* and not *M. indicus*.

## Introduction

Chitosan is deacetylated derivative of chitin, which is nowadays industrially produced from shellfish waste materials [1]. Chitosan is also available in fungal cell walls, where it is formed by progressive enzymatic deacetylation of chitin. Chitosan is a major component in walls of zygomycetes fungi, but probably a minor wall component in basidiomycetes, ascomycetes, chitridiomycetes, hypochitridiomycetes and oomycetes [2]. The biosynthesis of chitosan in fungi proceeds by coordinated action of chitin synthase and chitin deacetylase. The former enzyme synthesizes chitin by polymerization of *N*-acteylglucosaminyl residues from UDP-*N*-acteylglucosamine, whereas the latter hydrolyses the *N*-acetamido groups on the nascent chitin chains [3]. The chitosan can be purified from the biomass of these organisms by alkali-acid treatment. Chitin, chitosan and β-glucan as the structural components of the cell wall materials can be separated from interstitial components by hot water or dilute alkali treatment. Proteinaceous and other cell components are removed along with interstitial components as a result of their solubility at alkaline pH. Chitosan is isolated from remaining structural components by acid extraction. Chitosan is soluble in solutions that have a pH below 5.5, whereas chitin and β-glucan are insoluble in such acidic solutions. The efficiency of the purification of chitosan is dependent on the fermentation conditions and age of the culture [4]. Among different genera of zygomycetes fungi *Rhizopus oryzae* and *Mucor indicus* (formerly *M. rouxii*) were studied for bio-production of chitosan [5-8].

Zygomycetes are able to produce several different metabolites such as ethanol, lactic acid, citric acid and glycerol. Ethanol is now the most important product of biological processes in the world in terms of volume and market. It has several industrial, hygienic and food applications, but more than 70% of the produced ethanol goes to the fuel market. Ethanol is a renewable fuel, which is used either as substitute or additive to gasoline to adjust the octane number [9]. *Mucor indicus* and *Rhizopus oryzae* have recently been presented as ethanol producer organisms. These fungi are able to produce ethanol from glucose and other hexose with comparable yield and productivity with *Saccharomyces cerevisiae*. Furthermore, they can assimilate xylose and produce ethanol [10]. Therefore, it is probably possible to apply any of these zygomycetes to produce ethanol in industrial scale and process the biomass to obtain chitosan.

The aim of the current work was to study the effect of harvesting time on the yield of chitosan and ethanol form *Mucor indicus* and *Rhizopus oryzae*.

## Materials and methods
### The fungal strain and cultivation

*Rhizopus oryzae* CCUG 28958 and *Mucor indicus* CCUG 22424 obtained from Culture Collection, University of Göteborg (Göteborg, Sweden) were used in the experiments. These fungi were maintained on agar slants containing (g/l): D-glucose, 40; soy peptone, 10; and agar 20 at pH 5.5 and 30°C for 5 days.

Cultivations were performed in 200 ml working volumes in 500 ml cotton-plugged-Erlenmeyer flasks in a shaker incubator at 32°C and 150 rpm. A synthetic media contained (g/l): $(NH_4)_2SO_4$ 7.5, $KH_2PO_4$ 3.5, $MgSO_4.7H_2O$ 0.75, $CaCl_2.2H_2O$ 1.0, yeast extract 5, and glucose 20 was used. The spore suspension was prepared by adding 10 ml of sterile distilled water to slants and vigorously shaking by tube shaker. One ml of the suspension, which contained $5 \times 10^7$ spores/ml, was added to each flask. At the end of the desired fermentation period mycelia were harvested by filtration, washed three times with distillated water and dried at 60°C, weighted and stored. The liquid samples from fermentation media were taken and stored in a freeze.

### Extraction of chitosan

Extraction of chitosan was performed according to the method presented by Synowiecki et al. [6] with slight modification. The process involved: deproteinization by 2% w/v NaOH solution (30:1 v/w, 90°C, 2 h), separation of alkali-insoluble material (AIM) by centrifugation, extraction of chitosan from AIM by acetic acid under reflux (10% acetic acid, 40:1 v/w, 60°C, 6 h), separation of acid insoluble material (AAIM) by centrifugation, and precipitation of chitosan from the extract at pH 9.0, adjusted with 4M NaOH solution. Precipitated chitosan was washed ten times with distilled water and then with ethanol and acetone and dried at 50°C.

### Analytical methods
#### Analysis of glucose and metabolites

The liquid samples were analyzed by HPLC, equipped with UV/VIS and RI detectors (Jasco International Co., Tokyo, Japan). Glucose, ethanol, glycerol, lactic, pyruvic, and succinic acids were analyzed by an Ion-exchange Aminex column (HPX-87H, Bio-Rad, Richmond, CA, USA) at 60ºC with 0.6 ml/min eluent of 5 mM sulfuric acid. Succinic and pyruvic acids detected and quantified on UV chromatograms, while the other components were determined from RI chromatograms.

#### Determination of degree of deacetylation

Degree of deacetylation (DD) of chitosan was measured by acid hydrolysis and HPLC analysis according to the method developed by Stevens et al.[11]. In this method a certain amount of chitosan (10-50 mg), 1.5 mL of 12 M $H_2SO_4$ and 1 mL of 1.4 mM oxalic acid solutions were

charged to a glass ampule. The ampule was then sealed by a hot flame and placed at 110 $^0$C for 40 min, where the acetyl groups were separated from the glucosamine units. After cooling to room temperature, diluted with deionized water and analyzed by HPLC. The acetic acid concentration was then measured by HPLC and degree of deacetylation was calculated by the following formula:

$$DD = \left(1 - \frac{A}{A + \frac{W - 204\,A}{161}}\right) \times 100$$

Where $W$ is dry weight of the sample and $A$ is moles of acetic acid which is produced during the hydrolysis reaction.

All experiments were performed in duplicated and average standard deviation of the duplicated experiments were less than 2.5% for glucose, 4.2% for ethanol, 3.2% for biomass, 3.3% for AIM and 5% for chitosan. The results presented in the paper are average of two replicates. Yields of different products during the cultivation were calculated based on consumed glucose and reported.

### Results
*Cultivation of R. oryzae*

Performance of filamentous fungus *R. oryzae* with respect to chitosan and ethanol production has been studied with glucose as carbon source and the most important results are summarized in Fig. 1 and Table 1, 3. During the first 36 h cultivation, glucose was completely consumed and ethanol was produced. The ethanol concentration was then practically constant up to the end of the experiment which lasted at 60 h (Fig 1a). The biomass concentration increased during the exponential growth phase on glucose and afterward. A similar trend was observed for the alkali-insoluble materials (AIM), whereas concentration of extractable chitosan passed through a maximum at 48 h. At this time the maximum chitosan yield was 4.9% (g/g biomass). Degree of deacetylation (DD) of the produced chitosan was higher than 80% (Fig. 1b and Table 1).

**Table.1:** Yields of AIM and chitosan and degree of deacetylation of chitosan during the cultivation time of *R. oryzae*

| Yields and DD | Time (h) | | | |
|---|---|---|---|---|
| | 18 | 36 | 48 | 60 |
| AIM Yield (g/g biomass) | 0.133 | 0.196 | 0.203 | 0.209 |
| Chitosan Yield (g/g AIM) | 0.233 | 0.236 | 0.242 | 0.107 |
| Chitosan yield (g/g biomass) | 0.031 | 0.046 | 0.049 | 0.022 |
| Degree of deacetylation (DD) | 82 ± 0.5 | 81 ± 1.0 | 83 ± 1.0 | 86 ± 2.0 |

It should be noticed that the highest yields of chitosan and ethanol occurred simultaneously at the end of exponential growth phase. The maximum yield of chitosan and ethanol were 0.007 and 0.367 g/g of glucose, respectively. Glycerol and lactic acid were the main by-products of the fungus, with the yields of 27 and 23 mg/g respectively. Acetic, pyruvic, and succinic acids were also produced in trace amounts (Table 3).
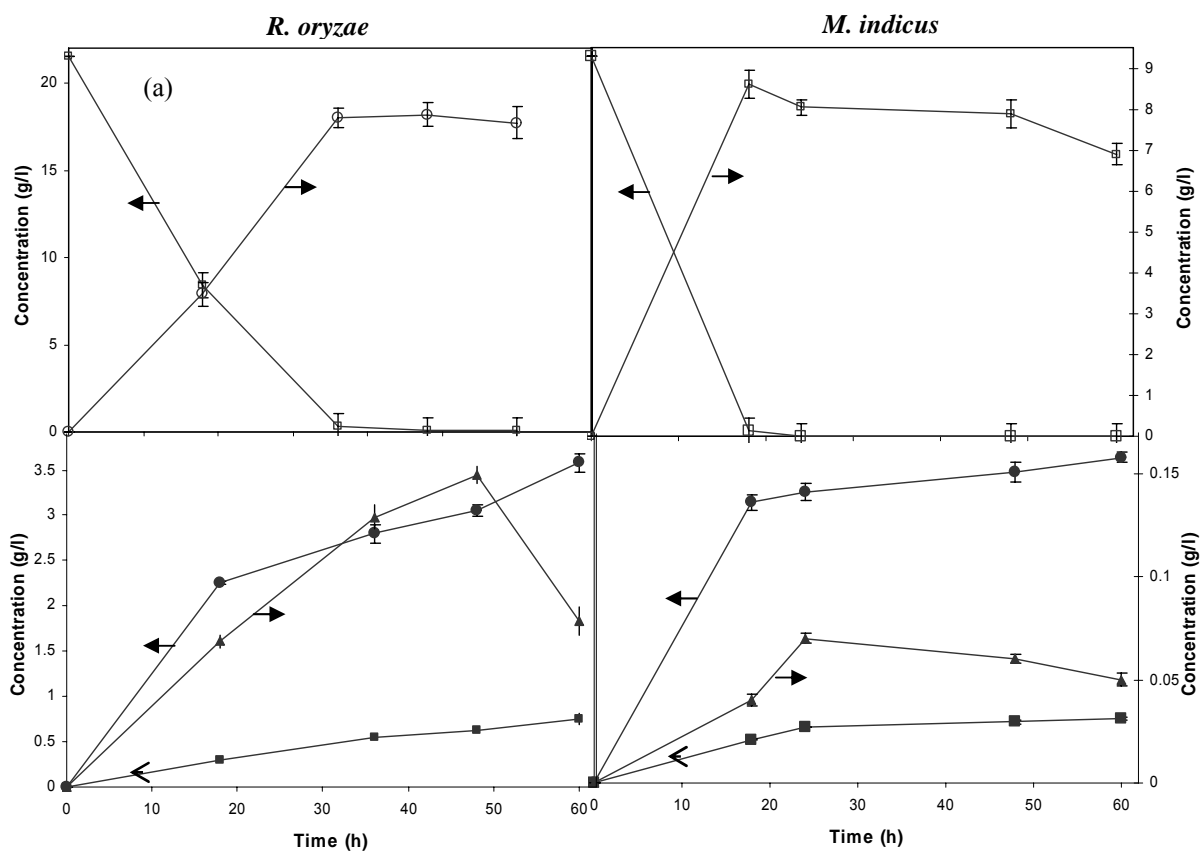
*Cultivation of M. indicus*

Similar to the previous experiments with *R. oryzae,* chitosan and ethanol production were studied by *M. indicus* and the most important results are summarized in Fig. 1 and Table 2, 3. This filamentous fungus was similar to *R. oryzae* in terms of formation of chitosan and ethanol. The results showed that sugar conversion by *M. indicus* is much faster than by *R. oryzae*. The maximum ethanol concentration observed while glucose was completely consumed, and then decreased slowly (Fig. 1a). Maximum rate of biomass production obtained within the first 20 h of cultivation.

Concentration of AIM increased by cultivation time thorough the experiments, whereas concentration of extractable chitosan passed a maximum at 24h. At this time chitosan yield was 2.4% (g/g biomass). Degrees of deacetylation of produced chitosan were more than 80% and decreased during the cultivation from 96% to 80% (Fig. 1b and Table 2).

**Table.2:** Yields of AIM and chitosan and degree of deacetylation of chitosan during the cultivation time of *M. indicus*

| Yields and DD | Time (h) | | | |
|---|---|---|---|---|
| | 18 | 24 | 48 | 60 |
| AIM Yield(g/g biomass) | 0.154 | 0.193 | 0.197 | 0.197 |
| Chitosan Yield (g/g AIM) | 0.093 | 0.125 | 0.098 | 0.078 |
| Chitosan yield (g/g biomass) | 0.014 | 0.024 | 0.019 | 0.015 |
| Degree of deacetylation (DD) | 96 ± 4.0 | 91 ± 1.0 | 86 ± 2.5 | 80 ± 3.0 |



**Fig.1.** (a) Profile of ethanol and glucose and (b) dry biomass, AIM and chitosan in cultivation of *R. oryzae* (left side) and *M. indicus* (right side) on glucose. The symbols present glucose (□), ethanol (○), dry biomass (●), alkali insoluble material (■) and chitosan (▲)

The maximum yields of ethanol and chitosan by *M. indicus* observed at different cultivation times. Maximum yield of ethanol was 0.41 g/g sugar and observed after 18 h, which was 6 h earlier than maximum yield of chitosan. Glycerol was the most important by-product in the experiments and its yield at maximum concentration was 33 mg/g sugar (Table 2, 3). Other by-products such as acetic, pyruvic, and succinic acids were also analyzed and their maximum yields were 5, 13 and 4 mg/g respectively (Table 3).

**Table.3:** Yields of the metabolites and chitosan in cultivation of *R. oryzae* and *M. indicus* on glucose

| Strain | $Y_{Ethanol/S}$ (g/g) | $Y_{Glycerol/S}$ (mg/g) | $Y_{Lactic/S}$ (mg/g) | $Y_{Acetic/S}$ (mg/g) | $Y_{pyruvic/S}$ (mg/g) | $Y_{Succinic/S}$ (mg/g) | $Y_{Chitosan/S}$ (mg/g) |
|---|---|---|---|---|---|---|---|
| *R. oryzae* | 0.37 | 27 | 23 | 5 | 18 | 6 | 7 |
| *M. indicus* | 0.41 | 33 | - | 5 | 13 | 4 | 3 |

**Discussion**

In recent decades, zygomycetes have been considered as sources for production of chitosan as well as ethanol [7,12]. Ethanol market has increased aggressively in the recent years. Therefore, it might be possible to produce ethanol with zygomycetes and process the residual biomass for production of chitosan. The results of the current work show that *R. oryzae* and *M. indicus* produce ethanol as the major metabolite and the content of their chitosan in the biomass are maximum while glucose is completely consumed and ethanol is produced.

Chitosan yields from *R. oryzae* and *M. indicus* were previously reported between 4.4-7.4 g/l [7-8] and 4.3-7.7 g/l [5,7], respectively. In this study, *R. oryzae* showed a better performance than *M. indicus* in chitosan production. Maximum chitosan obtained from 100 g of dry biomass of these strains were 4.9 g and 2.4 g, respectively. The low chitosan yields of these fungi are probably due to the applied cultivation conditions such as T, pH and lack of aeration. As expected, the main product of both of the fungi was ethanol. The yield of ethanol by *R. oryzae* in this work was in line of the results of the previous works. Taherzadeh et al. [13] reported the maximum yield of ethanol as 0.37 g/g during cultivation of *R. oryzae*. Millati et al. [12] reported ethanol yield 0.37-0.43 g/g by different strains of *R. oryzae* which is in the range of results in this work. *M. indicus* showed a yield of ethanol as high as 0.41 g/g which is similar to the results presented by Karimi et al [14], but higher than the results by Millati et al. [12] which reported the ethanol yield of 0.39 g/g.

AIM, the fraction of the fungi cell wall, increased by cultivation time but the amount of extractable chitosan in AIM increased only during the growth phase on glucose and then declined (Fig. 1). The decline of the extractable chitosan is probably depend on physiological changes in the fungal cell wall [7]. Chitin is synthesized in fungi cells by action of chitin synthase and then is sent into the cell wall, where enzymatic deacetylation of chitin for producing chitosan and covalent cross-linking of chitin with other polymers such as glucan take place simultaneously [2]. In the mycelia of the filamentous fungi β-D-glucan is covalently associated with chitin and presence of the two biologically active polysaccharides in the complex may enhance its pharmacological effect [15]. Formation of chitin-glucan complex reduces free chitin which can be converted to chitosan. On the other hand, the ratio of free chitosan molecules in the cell wall is relatively high during the exponential phase, which is due to the active growth. Once the culture enters the stationary growth phase, more of the chitosan is anchored to the cell wall of the zygomycetes by binding to chitin and other polysaccharides and extraction becomes more difficult [7].

It is interesting that in this work the results show a close optimum time for both chitosan and ethanol production. The maximum yield of ethanol and chitosan from *R. oryzae* in aerobic condition occurred simultaneously at 48 h, while maximum chitosan yield from *M. indicus* occurred 6 h later than the time of obtaining maximum ethanol yield. In this way one can use these strains for production of ethanol and at the same time it is possible to use fungal biomass as a rich source of chitosan.

It can be concluded that chitosan as well as ethanol can be produced by *R. oryzae* and *M. indicus*. Cell wall of these fungi contain appreciate level of extractable chitosan. On the other hand, these fungi are able to produce relatively high level of ethanol. Considering the ability of the fungi in assimilation of pentoses and converting them to ethanol, tolerant of the fungi to a series of inhibitors, together with the appreciate level of extractable chitosan make the fungi as good alternatives for ethanol production from different sources.

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