# NITROGEN BALANCE OF FUNGAL CHITOSAN SYNTHESIS IN SOLID SUBSTRATE FERMENTATION

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## Abstract

The fungus, *Gongronella butleri* USDB 0201 was grown on sweet potato pieces supplemented with minerals and urea. Total nitrogen content of the substrate was 0.35 g N/100 g. The mycelia were harvested at the end of fermentation and dried. The amount of dry mycelia was 4.2 ± 0.1 g/100 g of solid substrate. The nitrogen content in the mycelia was 5.39 ± 0.01 g N/100 g of mycelia. Therefore about 0.23 g of nitrogen was absorbed by fungus as a nutrient from 100 g of solid substrate to synthesize the fungal mycelia. The nitrogen compounds in the fungal mycelia are mainly DNA, RNA, protein, chitin and chitosan. Chitosan was extracted from the fungal mycelia using 11 M NaOH at $45^0$C for 13 hr followed by Termamyl enzyme treatment. The amount of chitosan extracted from the mycelia was 8.09 g CTS/100 g of mycelia. The nitrogen content in the chitosan is 8 g N/100 g of chitosan. Therefore about 12 % of nitrogen from the fungal mycelia was utilized for the synthesis of chitosan in the fungal cell wall.

Key words: nitrogen content, fungal mycelia, chitosan, solid substrate

## Introduction

Naturally, fungi grow on solid materials and in solution. They are heterotropic, depend on external supply of ready food, like animal and some bacteria. On the solid material, fungi excrete the extracellular enzymes and convert the insoluble food into soluble form, which are then absorbed. After that fungi can synthesize their own proteins and cell components using supplied carbohydrates and nitrogen source [1].

A large amount of carbon assimilated by fungi is used in biosynthesis of the wall materials. Chitin/chitosan is an important component of the cell wall of certain fungi, particularly of those belonging to the class Zygomycetes [2]. Chitosan is a nitrogen containing biopolymer, which is the deacetylated form of chitin.

Chitin biosynthesis starts with the conversion of glucose into N-acetylglucosamine-1-phosphate, which requires a series of reactions. The N-acetylglucosamine-1-phosphate then reacts with UTP (the nucleotide uridine triphosphate) to form UDP-N-acetylglucosamine (the nucleotide diphosphate sugar). Finally, this sugar transfers the N-acetylglucosamine moiety to the growing chitin polymer chain, functioning as a primer, and becomes one of its subunits. The enzyme chitin synthetase and $Mg^{2+}$ ions are required for this polymerization step. The completed chitin molecule is a long chain of sugar subunits that are joined by β-1,4 links [3]. According to this biosynthesis pathway, nitrogen compound play an important role for the synthesis of chitin/chitosan from glucose molecule. Ammoniun ion can be utilized directly as a source of nitrogen, while other inorganic forms of nitrogen are reduced to the redox level of ammonium [3]. Therefore nitrogen source is one of the important factors for the biosynthesis of chitosan in fungi.

Therefore in this study, fungal *Gongronella butleri* USDB 0201 was grown on the sweet potato pieces supplied with additional nitrogen source. Fungal mycelia were harvested at the end of fermentation and extracted the chitosan from the fungal cell wall with the help of Termamyl enzyme. Nitrogen content in the solid substrate, fungal mycelia and chitosan were measured and studied the conversion of nitrogen from the solid substrate to synthesis of chitosan in the fungal cell wall.

## Materials and methods

*Cultures and chemicals*

*Gongronella butleri* USDB 0201 was obtained from the Department of Biological Sciences, National University of Singapore. The strain was maintained on 3.9 percent potato dextrose agar (PDA) slants at 4°C. Termamyl, Type LS enzyme, was kindly provided by Siam Modified Starch Co. LTD (density 1.2 g per ml, activity 120 Kilo Novo alpha-amylase Unit per gram). Concentrated NaOH used was commercial grade and the other chemicals were analytical grade.

*Spore Suspension*

Spores from a 10-day culture of *G. butleri* grown on PDA plates at 30°C were harvested in sterilized distilled water. The spores were counted using a haemocytometer.

*Growth of fungus, Gongronella butleri USDB 0201on solid substrate fermentation medium*

Peeled sweet potatoes (*Manihot esculenta*) were cut into 1 – 1.5 x 4 – 6 cm pieces and washed with water. Mineral solution was prepared with 1 l distilled water containing 5g $(NH_4)_2SO_4$; 1g $K_2HPO_4$; 1g NaCl; 0.5g $MgSO_4.7H_2O$; 0.1g $CaCl_2.2H_2O$, supplemented with 7.2 g urea and adjusted to pH 4.5 with 0.5M $H_2SO_4$. Sweet potato pieces (850 g) were sterilized together with 850 ml of mineral solution supplemented with urea. Fungus

was grown on these sweet potato pieces under a constant supply of filtered and humidified air [4].

*Chitosan extraction*

Dried mycelia were treated with 10 ml of 11 M NaOH per g mycelia at 45$^{o}$C for 13 hr. Alkali insoluble material (AIM) was collected and washed with water until neutral pH. Dried AIM (1 g) was treated with 200 ml of 0.35 M acetic acid at 95$^{o}$C for 5 hr. This suspension was treated with Termamyl, Type LS at optimal conditions. The clear chitosan solution was centrifuged at 6000 rpm for 15 min twice to remove the glucan from the chitosan solution. Chitosan was precipitated from the supernatant and treated with 1M NaOH to remove enzyme. The chitosan precipitate was washed with distilled water and freeze-dried [5].

The nitrogen content of the solid substrate, fungal mycelia and chitosan was measured by the Micro-Kjeldahl method.

**Results and Discussions**

Solid substrate fermentation (SSF) medium was prepared by peeled sweet potato pieces sterilized together with mineral and urea solution. The moisture content of the untreated sweet potato was 74 ± 1.73 %. During sterilization a part of the mineral and urea solution and its components will be absorbed by the sweet potato material. In volume about 20% of the added solution is absorbed. After sterilization, about 80 % of free solution is removed by decantation.

Therefore a nitrogen balance study has been carried out to put N supply in the proper perspective. However, with regard to additional nitrogen supply, the final nitrogen content available for SSF has to be measured because the amount of additional nitrogen that penetrates into the sweet potato pieces during the impregnation and sterilization steps is unknown. This does not take in account that components in the fluid may be preferentially be absorbed or refused by the sweet potato. The elementary N content in untreated sweet potato was found to be 2.63 g/kg. Mineral solution contains ammonium sulfate 5 g/L. This corresponds to 1.06 g elementary N per liter. In case 20 % of the ammonium sulfate is absorbed the total amount of nitrogen can be estimated to be 2.63 + 0.21 = 2.84 g per initial kg wet substrate.

In case of addition of urea, 7.2g was added per kg solid substrate to the mineral solution. This contain to 3.36 g N/l mineral solution. In the assumption that also 20 % of this extra nitrogen will penetrate in the solid substrate, its total nitrogen content will increase to 3.51 g N/initial kg wet substrate. The N from the mineral solution (0.21 g) and from the urea (0.67 g) will be available, whereas the sweet potato N (2.63 g) will be available only to a small extend. First, the majority of the solid material is in the interior of the sweet

potato tissue and will not participate in the fermentation. Secondly, most of the N will be bound in membrane, DNA and protein components of the sweet potato and be not available either. This could explain why an apparent low N contribution by mineral solution and by urea, has a pronounced effect on the mycelia and chitosan production. It could explain also why non-supplemented sweet potato could only support the production of a low amount of chitosan 0.69 g/100 g mycelia. It seems therefore justifiable to state that most of the endogenous nitrogen does not support the production of chitosan.

After sterilization, urea is partial decomposition into ammonia and carbon dioxide. The pH of the solid substrate increased to 2 units [6]. Therefore the final nitrogen compound impregnated to SSF medium is as ammonia nitrogen. Fungus, *Gongronella butleri* USDB 0201 was grown on the solid substrate fermentation medium for 7 days. Fungus can utilize directly ammonium ion as a source of nitrogen and synthesize amino acid in anabolic pathway [3]. He reported that about 60-70 % of the total nitrogen of the fungal cell is protein. The nitrogen that does not exist as protein occurs in nucleic acids, chitin, phospholipids, vitamins, and nonessential metabolites.

The α-ketoglutaric acid result from intermediate of TCA cycle is joined with ammonia in the fungal cell and forms an amino acid, L-glutamic acid. This reaction is coupled with the oxidation of NADPH + $H^+$, and is catalyzed by the enzyme glutamic acid dehydrogenase. Glutamine synthetase enzyme catalyzes the L-glutamic acid to join with second ammonia to yield glutamine. Chitin biosynthesis begins with the conversion of glucose into fructose-6-phosphate, which react with glutamine to form glucosamine-6-phosphate. After that this compound convert to N-acetyl glucosamine-1-phosphate with the help of acetyl Co-enzyme A. The N-acetyl glucosamine-1-phosphate react with UTP (the nucleotide uridine triphosphate) to form UDP-N-acetylglucosamine, which is the nucleoside diphosphate sugar. Finally this sugar transfers the N-acetylglucosamine moiety to the growing chitin chain [7]. Moreover the chitin synthetase and chitin deacetylase, operating in tandem, catalyze the conversion of UDP-N-acetylglucosamine to chitosan [8]. Therefore the conversion ratio of supplemented nitrogen to chitosan was investigated by extracting the chitosan from the fungal mycelia followed by determination of nitrogen content in the chitosan.

Fungal mycelia was harvested at the end of fermentation and dried. The amount of dry mycelia was 42.29 ± 1.01 g/kg SS. The nitrogen content in the mycelia was 5.39 ± 0.01 g N/100 g mycelia. Therefore about 2.28 g nitrogen was absorbed by fungus as a nutrient from the 1 kg of SS to synthesize the fungal mycelia. Apparently 2.28 g of 3.51 g total N is used. This means that the fungus consumed 1.40 g N of the endogenous nitrogen. Chitosan in the cell wall was extracted by using 11 M NaOH and followed by 0.35 M acetic acid and enzyme treatment. In the treatment with 11 M NaOH, chitin present in the mycelia cell wall is converted into chitosan and extracted during acid and enzyme treatment. The amount of chitosan extracted from the mycelia was 8.09 g CTS/100 g mycelia. The nitrogen content of chitosan from the mycelia is 8 g nitrogen/100 g

chitosan. Therefore about 12 % of nitrogen from the fungal mycelia was utilized for the synthesis of chitosan in the fungal cell wall.

## Conclusions

Fungal could synthesize only 0.69 g chitosan in 100 g of mycelia on the sweet potato medium without additional nitrogen source. However synthesis of chitosan in the fungal mycelia was increased to 8.09 g chitosan in 100 g of mycelia on the solid substrate fermentation medium supplemented with mineral and urea solution. Fungal absorb about 65% of nitrogen from the solid substrate and synthesize the nitrogenous compounds for their growth. About 12% of total absorbed nitrogen is used for the synthesis of chitosan in fungal cell wall. Therefore nitrogen source and amount of nitrogen supplied to the solid substrate fermentation medium were important factors for the synthesis of chitosan in fungal cell wall.

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