Appl Microbiol Biotechnol (2001) 56:220–224
DOI 10.1007/s002530000591

ORIGINAL PAPER

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The influence of supplemental components in nutrient medium on chitosan formation by the fungus *Absidia orchidis*

Received: 20 June 2000 / Received revision: 21 November 2000 / Accepted: 24 November 2000 / Published online: 6 June 2001

Abstract Chitosan, a derivative of chitin, is a natural component of some fungus cell walls. It is formed by the complex action of chitin synthase and chitin deacetylase. The in vitro activity of these two enzymes is known to be influenced by several factors. We investigated the influence of ferrous ions, manganese ions, cobalt ions, trypsin, and chitin, as individual supplements to the nutrient medium, on the in vivo activity of chitin synthase and chitin deacetylase to form chitosan in the fungus *Absidia orchidis*. Manganese and ferrous ions gave the most significant results. These ions increase chitosan yields through an increase in biomass production rather than an increase of chitosan content in cell walls. Manganese and ferrous ions lowered the activity of chitin deacetylase; however, their influence on the activity of chitin synthase was more complex. The effects of trypsin and chitin on biomass and cell wall chitosan content were negligible, while cobalt ions completely inhibited the growth of fungi.

Introduction

Chitin, poly-β-(1–4)-*N*-acetyl-D-glucosamine, is the most widespread biopolymer after cellulose. Chitin has rather limited uses, but chitosan (a copolymer of D-glucosamine and *N*-acetyl-D-glucosamine with β-1→4 linking), a derivative of chitin, has applications in medicine, agriculture, environmental protection, and as a food additive. Chitosan is produced on an industrial scale by chemical deacetylation of chitin with concentrated hydroxides (40–50%) at high temperature (100–150 °C).

Chitosan is also a natural component of cell walls of fungi belonging to Zygomycetes (e.g. *Absidia, Mucor, Rhizopus, Gongronella*) and can be produced by extraction from fungus cell walls (White et al. 1979). This method is not yet used on an industrial scale, although it can provide an alternative to chemical deacetylation, independent of chitin production at the fishery. Additionally, fungal chitosan is thought to have higher bioactivity than chitosan from other sources of chitin, which is especially important in medical applications.

The yield of chitosan from a fungal mass, or from a unit of culture medium, depends on several factors including: the strain of fungi used, cultivation method (shaking culture, bath culture, continuous culture, solid-state culture), and cultivation parameters (pH, temperature, mixing rate, length of cultivation). An increase in the chitosan yield can be obtained either by increased biomass yield or by an increase in the cell wall content of chitosan.

The formation of chitosan in fungi cell walls is a result of the complex action of two enzymes: chitin synthase and chitin deacetylase. In the first step, chitin synthase "builds" a chain of chitin using the chitin precursor, uridino-di phospho *N*-acetylglucosamine (UDP-GlcNAc). Next, chitin deacetylase hydrolyzes acetic groups from *N*-acetylglucosamine (GlcNAc), transforms it into glucosamine (GlcN) (Davis and Bartnicki-Garcia 1984; Bartnicki-Garcia 1989) and finally chitosan is formed.

$$\mathrm{UDF-GlcNAc} \xrightarrow{\text{chitinsynthase}} \mathrm{Chitin}$$
$$\mathrm{Chitin} \xrightarrow{\text{chitindeacetylase}} \mathrm{Chitosan}$$

The activity of chitin synthase influences the length of the chitin chain (that is, the molecular weight of the biopolymer), and the activity of chitin deacetylase influences the content of GlcNAc groups in the chain of chitosan (that is, the degree of acetylation). The properties of nascent chitosan (degree of acetylation and molecular weight) depend on the activity of these two enzymes.

Reports in the literature indicate the possibility of increasing the activity of pure chitin synthase and chitin deacetylase (see references in Table 1). Among all the tested cations, only the influence of Co^{2+} on chitosan formation in fungi has been investigated in vivo (Rane and Hoover 1993). It was shown that a Co^{2+} (as $CoSO_4 \cdot 7H_2O$) concentration of up to 0.5 g l^{-1} promotes

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Table 1 Compounds and ions influencing the activity of chitin synthase and chitin deacethylase

Enzyme	Compound/ion	Reference
Chitin synthase	Trypsin	Leal-Morales et al. (1994)[a] Cabib et al. (1996)[b] Deshpande et al. (1997)[c] Gooday et al. (1997)[d]
	Mn^{2+}	Cabib et al. (1996)[b] Gooday et al. (1997)[d]
	Co^{2+}	Cabib et al. (1996)[b]
	Mg^{2+}	Gooday and Schofield (1995)[d] Cabib et al. (1996)[b]
Chitin deacetylase	Co^{2+}	Tokuyasu et al. (1996)[e] Kołodziejska et al. (1999)[f]
	Mn^{2+}	Kołodziejska et al. (1999)[f]
	Zn^{2+}	Tokuyasu et al. (1996)[e] Kołodziejska et al. (1999)[f]
	Ca^{2+}	Kołodziejska et al. (1999)[f]

[a] *Neurospora crassa*
[b] *Saccharomyces cerevisiae*
[c] *Benjaminiella poitrassi*
[d] *Candida albicans*
[e] *Colletotrichum lindemuthianum*
[f] *Mucor rouxi*

chitosan formation; above this value a decrease in the amount of chitosan in cell walls was observed. It was also stated that Co^{2+} ions do not significantly influence the degree of acetylation of the biopolymer (Rane and Hoover 1993).

The work described here is part of a study on fungal chitosan production. The goal of the present experiments was to investigate the influence of selected additional medium components on chitosan formation in the fungus *Absidia orchidis*. This species was selected as the best "chitosan producer" based on earlier work (Jaworska and Szewczyk 1997).

In preliminary investigations, the addition of ferrous, manganese, and cobalt ions, as well as the addition of trypsin and chitin, to nutrient medium was investigated. On the base of these preliminary investigations, Fe^{+2} and Mn^{+2} ions were chosen for the final experiments.

Materials and methods

Fungi

The *Absidia orchidis* NCAIM F 00642 (Budapest, Hungary) strain was used. A 0.5-ml wash-out from an agar slant (for shaking cultures) or 2-day-old shaking cultures (for batch cultures) served as inoculum.

Cultivation medium

The fungus was cultured in YPG medium enriched with the following salts (amounts for 100 ml of H_2O): 0.5 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.1 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$. The pH of the medium was 6.3.

$FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 5H_2O$ and $CoCl_2$ were used for Fe^{+2}, Mn^{+2} and Co^{2+} ion experiments, respectively. Chitin was from shrimp, and trypsin from porcine pancreas (Sigma). All reagents were of analytical grade or higher.

Culture conditions

A shaking culture (100 ml of nutrient medium in a 300-ml Erlenmeyer flask, 250 rpm, 26 °C, cultivation time: 48 h) was used in all preliminary investigations. Bath cultures were carried out in a bioreactor (Bioflo III, New Brunswick, N.J.). The culture medium (4.5 l) was added to the bioreactor, sterilized (121 °C, 20 min), and inoculated with 500 ml of inoculum (total volume – 5.0 l). The fungi were incubated at 26 °C, pH 5.5 (stabilized with 1 N NaOH and 1 N HCl), aerated and mixed; the cultivation time was 48 h.

Biopolymer separation

Chitosan was separated by the following steps:

1. Biomass separation: The biomass was centrifuged from the culture broth (6000 rpm, 20 min) and washed once with deionized water.
2. Alkali-insoluble fraction (AIF): The fungal biomass was treated with 1 N NaOH at 121 °C, (10 min). The AIF was centrifuged (6000 rpm, 20 min), washed with deionized water, and dried for 24 h at 60 °C. Finally, the AIF was ground.
3. Biopolymer extraction: The AIF was treated with 1% CH_3COOH solution, centrifuged, and an acid-soluble fraction was collected and alkalized to pH 10.0 with 1 N NaOH. The biopolymer was precipitated, centrifuged (20,000 rpm, 20 min), washed with deionized water, dried at 50 °C, and stored at room temperature in an air-tight vessel.

Analytical methods

The degree of acetylation (AD) was calculated on the basis of infrared spectra (IR), according to the direct method of Shigemasa et al. (1996). Samples of chitosans were used as a KBr discs (2 mg/250 mg). IR spectra were measured on a Perkin-Elmer System 2000 spectrometer. The resolution was 4 cm^{-1}. An average of 32 scans for each spectrum was used.

The mean viscosimetric molecular weight (MVMW) of the biopolymers was determined by measuring the viscosity of a chitosan solution in acetate buffer (0.3 M CH_3COOH+0.2 M CH_3COONa) at 25 °C using the Mark-Houwink equation, with constants evaluated by Roberts and Wang (1996): a=0.76, K=0.075 ml g^{-1} (0.074 ml g^{-1} for chitosan with AD above 20%).

Results

Preliminary investigations

The aim of preliminary experiments was to test whether selected medium components increased the in vivo formation of chitosan by the fungus *Absidia orchidis*. The effects of supplemented compounds in nutrient medium on the activity of chitin synthase (trypsin, Mn^{2+}, Co^{2+}) and chitin deacetylase (Mn^{2+}, Co^{2+}), as well the effect of the addition of chitin and Fe^{2+} ions, were investigated. The results were expressed as the amount of chitosan produced per unit of culture medium (g l^{-1}). In Table 2, the mean value from three parallel shaking cultures are presented.

The most significant increase in chitosan formation was observed in cultures containing ferrous and manganese ions (from 0.40 g l^{-1} in standard culture to 1.28 and

Table 2 The effect of supplemental compounds in nutrient medium on biomass, alkali-insoluble fraction (*AIF*), and chitosan yields from a unit of culture medium

Concentration (g l^{-1})[a]	Yield of:		
	Biomass (g l^{-1})[b]	AIF (g l^{-1})	Chitosan (g l^{-1})
Control culture	22.5	1.7	0.40
+Mn^{2+}			
1.13	35.3	4.0	1.15
2.27	36.2	4.7	1.84
+Co^{2+}			
2.30	<0.5	–	–
4.50	<0.5	–	–
+Fe^{2+}			
1	21.8	3.7	1.28
2.01	12.3	4.0	0.78
+Trypsin			
0.02	19.0	1.4	0.36
0.05	21.4	1.5	0.36
+Chitin			
5	31.9	4.8	0.60

[a] All the experiments were performed by the addition of a single compound
[b] Biomass as dry biomass

1.84 g l^{-1} in cultures containing Fe^{2+} and Mn^{2+}, respectively). These ions also increased the yields of dry biomass (Mn^{2+}) and AIF (Mn^{2+}, Fe^{2+}). The influence of trypsin and chitin on chitosan yield was negligible. Contrary to literature data, the addition of Co^{2+} ions completely inhibited the growth of fungi. Fe^{2+} and Mn^{2+} ions were chosen for further experiments.

Bath culture

The aim of the next set of experiments was to study the effect of the concentration of Fe^{2+} and Mn^{2+} on chitosan formation and on the properties of the obtained chitosan (AD, MVMW). The concentrations of ions were chosen on the basis of preliminary investigations carried out in shake cultures. The data are presented in Table 3.

Both Mn^{2+} and Fe^{2+} ions increased the chitosan yield per unit of culture medium (1.78 and 1.79 g l^{-1} respectively). The optimal concentrations of these ions were different from those obtained in the preliminary investigations. This can be explain as an effect of the cultivation methods (Jaworska 1999).

The chitosans separated from the fungal biomass were characterized by two parameters: AD and MVMW (Table 4). The AD of chitosans separated from fungus biomass was higher in all of the cultures that had received supplementary nutrient compounds. The only lower AD value was in the culture containing Mn^{2+} (0.56 g l^{-1}). The MVMW of all separated chitosan samples was three to four times lower than chitosan obtained from the biomass derived from the control culture. The only exception was chitosan obtained from fungi cultivated in nutrient medium containing Mn^{2+} ions at a concentration of 1.13 g l^{-1}.

Table 3 The effect of the concentration of Mn^{2+} and Fe^{2+} in culture medium on the dry biomass, alkali-insoluble fraction (*AIF*), and chitosan yields from a unit of culture medium

Concentration (g l^{-1})[a]	Yield of:		
	Biomass (g l^{-1})[b]	AIF (g l^{-1})	Chitosan (g l^{-1})
Control culture	7.4	2.51	0.71
Mn^{2+}			
0.56	6.5	1.75	0.55
1.13	15.2	4.53	1.05
2.27	10.6	2.95	0.78
Fe^{2+}			
0.2	16.9	4.83	0.63
0.5	31.2	13.15	0.85
1	45.3	11.95	1.79

[a] All the experiments were performed by the addition of a single compound
[b] Biomass as dry biomass

Table 4 The effect of of Mn^{2+} and Fe^{2+} concentration on acetylation degree and mean viscosimetric molecular weight (*MVMW*) of chitosans

Concentration (g l^{-1})[a]	Acetylation degree (%)	MVMW
Control culture	15.6	750,900
+Mn^{2+}		
0.56	18.8	98,100
1.13	31.7	1,156,000
2.27[b]	–	–
+Fe^{2+}		
0.20	30.8	88,800
0.50	27.1	123,300
1.00	26.8	298,800

[a] All the experiments were performed by the addition of a single compound
[b] The IR spectrum differs from that of chitosan, suggesting that the separated biopolymer was not chitosan

Discussion

There are only a few compounds that have been reported to increase the in vitro activity of chitin synthase and chitin deacetylase. Amongst them, manganese and cobalt ions, as well as trypsin, have been chosen for further experiments. On the basis of these data, in vivo experiments with the fungus *Absidia orchidis* were undertaken. The results of preliminary experiments do not agree with those reported in the literature.

Mn^{2+} ions and trypsin influence the chitin synthase enzyme. As a result, an increase in the AIF was expected. This effect was observed for manganese ions but there was no changes in comparison to control cultures when trypsin was added to nutrient medium.

According to literature data, Mn^{2+} and Co^{2+} ions influence the chitin deacetylase enzyme, but contrary to data published so far, cobalt ions completely inhibited the growth of fungi *Absidia orchidis* in our experiments.

The addition of chitin to nutrient medium was also investigated. The aim of these experiments was to determine whether chitin was incorporated into cell walls directly or by depolymerization (via the action of chitinolytic enzymes of *Absidia orchidis*) and incorporation of GlcNAc. Significant increases in the biomass and AIF were observed; the yield of chitosan was greater than in control culture but less than in cultures containing Mn^{2+} ions. These were the expected results.

The influence of ferrous ions was also investigated. Although Fe^{2+} ions inhibit chitin deacetylase (Kołodziejska et al. 1999), increases in biomass, AIF, and chitosan were observed, with values comparable to those obtained in cultures with Mn^{2+} ions.

The positive effects of Mn^{2+} and Fe^{2+} were the most significant among all the nutrient medium supplements that were tested. Thus, the effect of the concentration of these ions on the formation of chitosan in a submerged culture subsequently determined.

Ferrous ions increased the yield of biomass from a volume unit of culture medium (up to 45 g l^{-1}), the AIF (up to 13 g l^{-1}) and chitosan (up to 1.8 g l^{-1}). However, they also decreased the chitosan content of the biomass and cell walls (Fig. 1). Thus, the increased amount of chitosan is a result of the increase in biomass rather than an increase in chitosan content in fungal cell walls. For a Fe^{2+} concentration equal to1 g l^{-1}, the biomass yield from a volume unit of supplemented culture medium was six times higher and the chitosan yield 2.5 times higher than obtained in the unsupplemented medium. By comparison, the chitosan yield from a mass unit of dry biomass was two to three times smaller than that in standard medium.

The decrease of chitin deacetylase activity by ferrous ions was confirmed in our experiments. Chitosan from fungi cultivated with Fe^{2+} ions had a higher degree of acetylation (26–30%) than chitosan from unsupplemented medium (15%).

Ferrous ions also decreased the activity of chitin synthase. The MVMWs of chitosan from modified cultures were lower (88,000–300,000) than those of controls (750,000).

Manganese ions act in similar way as ferrous ions. Above a concentration of 1.13 g l^{-1}, they increase the yield of biomass (up to 15 g l^{-1}), AIF (up to 4.5 g l^{-1}) and chitosan (up to 1.05 g l^{-1}). Similar to Fe^{2+}, the increased production of chitosan was also caused by an increase of biomass yield (Fig. 2). In contrast to ferrous ions, however, manganese ions do not influence the chitosan yield from either a mass unit of dry biomass or the chitosan yield from a mass unit of cell walls.

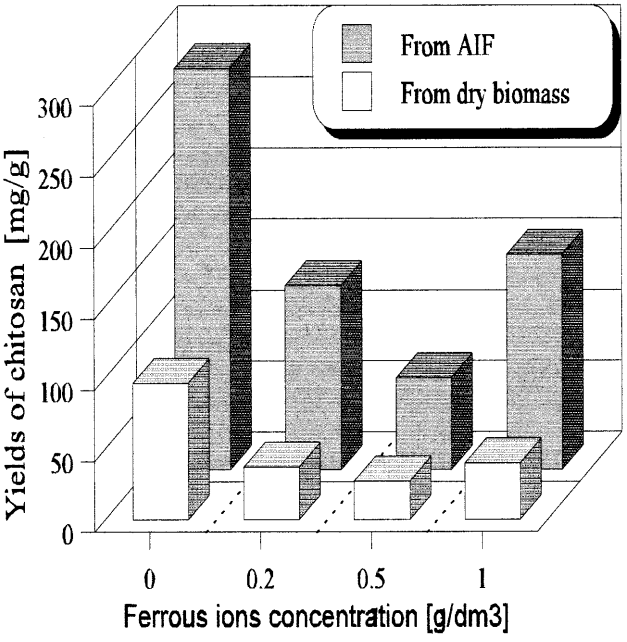

Fig. 1 The influence of ferrous ions on the chitosan yields from a mass unit of dry biomass and a mass unit of the alkali insoluble fraction (*AIF*)

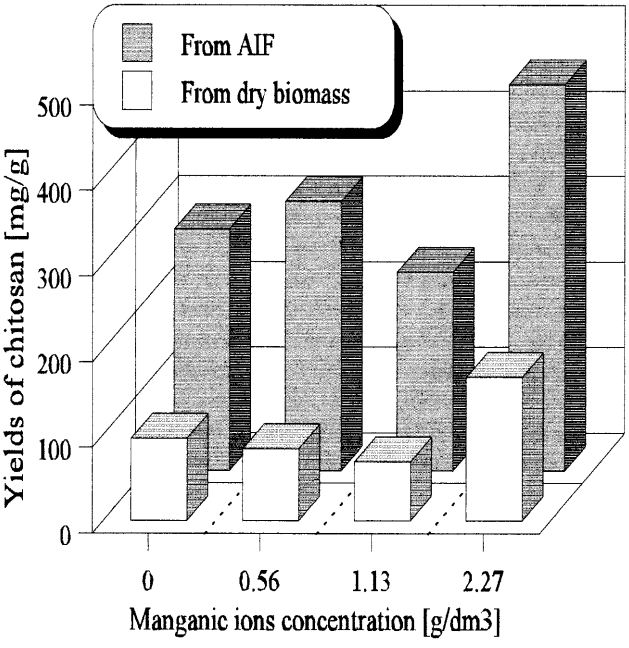

Fig. 2 The influence of manganic ions concentration on chitosan yield from a mass unit of dry biomass and a mass unit of the alkali-insoluble fraction (*AIF*)

While manganese ions have been reported to activate chitin deacetylase (Kołodziejska et al. 1999), this effect was not observed in our experiments. The degree of acetylation of chitosan produced from fungi cultivated in cultures containing Mn^{2+} ions was higher (above 30%) than that in unsupplemented medium (15%). This was

probably caused by an inhibition of chitin deacetylation by Mn^{2+} ions.

The relationship between the MVW of chitosan and the concentration of manganese ions in medium is complex. At lower Mn^{2+} concentrations (0.58 g l^{-1}), chitosan with a lower mean molecular weight (96,000) was separated, while at higher Mn^{2+} concentrations (1.13 g l^{-1}), chitosan with a higher mean molecular weight (1,156,000) was obtained (the MSMW for the chitosan in unsupplemented medium was 750,000).

The work presented here is part of a study on chitosan production by the fungus *Absidia orchidis*. The goal of the experiments described above was to increase the amount of chitosan formed in cell walls of the fungi by activating the enzymes (chitin synthase and chitin deacetylase) responsible for this process. The higher yields of chitosan observed in the cultures containing Mn^{2+} and Fe^{2+} ions were related to the increased biomass production rather than the increased chitosan content in cell walls. Ions, as activators of chitin deacetylase or chitin synthase in vitro, were probably recruited into fungal metabolism rather than only activating chitosan formation. Further work will be focused on increasing the yield of *Absidia orchidis* biomass from 1 l of culture medium and on the continuous cultivation of biomass for chitosan production.

Acknowledgements This work was supported by the Committee for Scientific Research, Poland, in the framework of the project number T09C 039 14. We declare that the experiments described here complied with the current laws of Poland.

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