Annual chitin production

In freshwater lakes, the crustacean planktonic community is mainly composed of branchiopods, copepods and mysids. Considering the mean chitin content of branchiopods and mysids to be equal to this of freshwater cladocerans and anostracans respectively, the mean chitin production of the planktonic community in lakes and ponds would be equal to 1.96 (branchiopods) + 4.12 (copepods) + 0.02 (mysids) = 6.10 grams of chitin produced by square meter and by year. Concerning the benthic community, we only have data about the malacostracan production. The chitin content of freshwater malacostracans has been taken equal to 8 % of the body dry mass on the basis of the chitin content of marine amphipods (7.3 % of the body dry mass) and decapods (8.8 % of the body dry mass). The annual production of malacostracans being equal to 11.8 $g.m^{-2}.yr^{-1}$, the chitin production by the benthic community in lakes and ponds would amount to at least 1.04 grams of chitin produced by square meter and by year. On the basis of an estimated extent of lakes and ponds equal to 1.2×10^6 km^2, the total chitin production for this type of ecosystems would reach 8.5 million tonnes by year (table 3).

Table 3: Total chitin production by natural freshwater and marine ecosystems

Ecosystem community	mean chitin production ($g.m^{-2}.yr^{-1}$)	Extent ($\times 10^6$ km^2) [1]	Total production (10^6 tons. yr^{-1})
CONTINENTAL WATERS			
◆ Freshwater lakes and ponds			
– planktonic community	6.10	1.2	7.3
– benthic community	1.04	1.2	1.2
◆ Rivers and streams			
– planktonic community	0.03	149.0	4.5
– benthic community	3.93	149.0	585.6
◆ Saline lakes and inland seas			
– planktonic community	?	0.7	?
– benthic community	?	0.7	?
Estimation of the total chitin production in continental waters [2]			603.5
OCEANS AND SEAS			
◆ shores and continental shelf			
– pelagic community	2.65	13.8	36.6
– benthic community	1.67	13.8	23.0
◆ continental slope	?	30.6	?
◆ deep sea floor	?	316.6	?
Estimation of the total chitin production in oceans and seas [3]			1,559.5

[1] After Korzun [146], Jeuniaux et al. [5] and Percier[147];
[2] considering the production in saline lakes and inland seas to be equal to that of freshwater lakes;
[3] considering the production in continental slope and deep sea regions to be equivalent to the production upon the continental shelf.

Assuming for rivers and streams the same assumptions as for lakes and ponds, the chitin production of the planktonic and benthic crustacean communities would amount to 0.03 and 3.93 grams of chitin by square meter and by year, respectively. The extent of rivers and streams being equal to 149×10^6 km^2, the total production of this type of ecosystem would be equal to 590.0 million tonnes by year.

We have no data about crustacean secondary production in saline lakes and inland seas. If we consider for this kind of ecosystem a similar areal chitin production as for lakes and ponds, the total chitin production by crustaceans in continental waters would amount to 603.5 million tonnes by year (table 3).

In marine environments, all the production data that we have collected concern the shores and the continental shelf. Considering a mean chitin content of mysids equal to this of euphausiids, the mean chitin production in the pelagic zone reaches 1.58 (copepods) + 1.03 (euphausiids) + 0.04 (mysids) = 2.65 grams of chitin by square meter and by year. Our estimations of euphausiid chitin production are 5 to 7 times higher than to those presented by Nicol and Hosie (0.14, 0.17 and 0.20 grams of chitin by square meter and by year for *Euphausia superba*, *Nyctiphanes australis* and *Euphausia pacifica*, respectively) [4], mainly because we have taken into account the recent estimation of annual production by *Euphausia superba* in the Southwest Atlantic [134].

Considering that isopods and leptostracans have a similar chitin content to amphipoda, the mean chitin production by benthic malacostracans would equal 0.73 (amphipods) + 0.13 (decapods) + 0.54 (isopods) + 0.27 (leptostracans) = 1.67 grams of chitin by square meters and by year. As far as benthic community is concerned, our results are in good agreement with those calculated by Jeuniaux *et al.* for benthic crustaceans on rocky shores (2.0 grams of chitin by square meters and by year) [5,6].

If we make the assumption that the mean chitin production by crustaceans is similar in all part of the oceans and seas, it would reach 1,560 million tonnes of chitin by year for the whole marine environment (table 3). This value is more or less two times lower than that given by Jeuniaux *et al.* [5,6]. The main reason for this difference is that these authors used an estimate of the chitin production by krill about seven times higher than ours.

Our estimation underestimates certainly the actual chitin production. The first reason is that we have few information about some regions which are thought to be very productive such as hydrothermal plumes [148] and upwelling areas [149]. The second reason is that the production of exuviae has generally not been taken into account in the secondary production found in the literature. Nevertheless, the chitin production due to exuviae is far from negligible. For instance, in *Nyctiphanes australis*, annual tissue production reaches 78.3 mg . m^{-3} . yr^{-1} while the annual production of exuviae is equal to 42.0 mg . m^{-3} . yr^{-1} [150]. In this species, the chitin content reaches 2.7 % in the body and 13.7 % in the exuvia, on a dry mass basis [4]. The body chitin production by this species equal thus 2.1 mg chitin.m^{-3}. yr^{-1} and the exuviae chitin production amounts to 5.8 mg chitin.m^{-3}. yr^{-1}. That is to say that more than 70 % of the total quantity of chitin produced by krill annually corresponds to moults.

Conclusions

Our study highlights the need for further estimations of the crustacean chitin content, on a whole body basis. Some major orders such as amphipods, isopods, cirripeds and mysis must be studied first because of their important role in the freshwater and marine ecosystems. The chitin production associated with the moulting must be also estimated because it represents a significant proportion of the total chitin production.

Considering the high production of chitin by crustaceans, the study of the biodegradation processes of the chitin synthetized is certainly the next step in the analysis of the importance of this polymer in the biogeochemical cycles of carbon and nitrogen.

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TOWARDS TECHNICAL BIOCATALYTIC DEACETYLATION OF CHITIN.

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Abstract
1. Studies aiming at the technical biocatalytic deacetylation of chitin have been carried out using the chitin deacetylase CDA of the fungus Absidia coerulea.
2. Assays to measure enzyme activity on the natural substrate have been improved: a HPLC method to measure the degree of deacetylation of shrimp chitin and a GLC method to assay the amount of acetic acid released during enzyme action.
3. The Absidia CDA has been characterized by its optimal temperature, pH, thermostability and kinetics of deacetylation.
4. The experimental data obtained support current concepts on particle size and particle constitution of the substrate and suggest clues for the appropriate pretreatment of the natural substrate to accomplish technical biocatalytic deacetylation.

Keywords: Chitin, Chitin deacetylase, Shrimp waste bioprocessing.

Introduction

The industrial production of chitin and chitosan from shrimp biowaste in Southeast Asia is a low quality and unsustainable process. Particularly the treatments in hot highly concentrated alkali lead to partial degradation of the end product and to the generation of large amounts of harmful waste. The Bioprocess Technology Program of the Asian Institute of Technology aims at the replacement of the aggressive chemical extractions of chitin and chitosan by biocatalytic processes. The first step, the alkaline deproteination can be replaced by partial fermentation using Lactobacilli (1,2) or protease (3). New data for the scaling up the fermentative deproteination have been reported (4).

The enzymatic deacetylation of chitin in fungi as part of the synthetic route providing the large amount of chitosan in the fungal cell wall, has been studied extensively. Several laboratories have been successful in the purification, characterization and cloning of the enzyme (surveyed in 5).

The enzymological studies reported at present mention problems in the use of natural chitin as substrate, due to its high degree of crystallinity and its insolubility in all common solvents. Alternative artificial substrates to assay for enzyme activity have been used including colloidal chitin, glycol chitin, carboxy methyl chitin and radioactive acetylated chitosan. Although the use of these substrates has lead to highly detailed information on the chitin deacetylase, the available data for the technical use of the enzyme to deacetylate natural chitin are very limited.

As a consequence also the systems to assay for the result of enzymatic deacetylation provide not much information of the efficiency of the process. The assay system should provide reliable data on the degree of deacetylation (DD) of the natural preparation as a whole, or on the release of acetyl groups from the natural substrate. A first step in the technical biocatalytic deacetylation has been therefore the further development of a HPLC assay to measure DD and a convenient method to measure the release of acetic acid using GLC.

Using these assay systems the CDA in Absidia extracts has been investigated for its main enzymological characteristics such as temperature and pH dependence, thermo-stability kinetics and reaction. The nature of the substrate has been studied by studying the effect of the degree of deacetylation on enzyme activity and the effect of particle size. Finally the feasibility of technical biocatalytic deacetylation is discussed.

Materials and Methods

Absidia coerulea strain 5301 was obtained from the collection of the Institute for Fermentation, Osaka and grown on a minimal medium with 2% glucose, 1% peptone and 0.1% yeast extract, temperature 26°C, pH 4.5. The chitin deacetylase was extracted from 0.2g freeze dried mycelium by grinding with 2g of cleaned-sand and 20 mL of phosphate buffer at pH 5.8 for 20 min. The extract was suspended in buffer by gently stirring during 1 hr at 4° C and centrifuged to obtain crude CDA enzyme as a clear supernatant. This supernatant has been used without further purification.

HPLC to assay the DD was carried as described before. Samples were pretreated by acid analysis. In a 5 ml ampule the sample 10-50 mg was mixed with 0.5 mL 12M sulfuric acid and 2 ml of 63mg/L oxalic acid. After gas tight sealing the sample was destructed at 155°C for 60 min. Then the sample was diluted to 50 ml and filtered through 0.45 μm membrane. Analysis of acetic acid was carried out by a Waters HPLC. The analytical system consisted of a ORH-801 column (Interaction, USA), mobile phase 1mM sulfuric acid 0.8 mL/min, pressure 1600 psi, column oven temp 45°C, sample compartment 25°C, injection volume 30mL, detection at 210 nm (7).

Unless stated otherwise, the assay for the chitin deacetylase using GLC was carried out as follows. Chitosan powder 50 mg from cuttlefish with a DD65% and a size of 60 mesh was mixed with 1 mL of the enzyme preparation to be tested and 5 ml buffer pH 5.8. After incubation of 1 hour at 50°C, the chitosan was spun down for 10 min at 10,000 g. The supernatant was analyzed for acetic acid by GLC by adding 0.2% (v/v) of 0.5M oxalic acid, centrifugation (10 min, 12,500 rpm) and injection of 1 μL into a Shimadzu GLC GC 14B equipped with a glass column packed with PEG 6000 and a flame detector. Nitrogen was used as carrier gas at a flow rate of 40 mL/min. The column was operated isothermally at 165°C and 190°C for both the injector and the detector points. The elution time for acetic acid was 4.55 min. Enzyme activity was expressed in units, one unit being the amount of enzyme that produces 1μmol acetic acid per min under standard conditions.

Results

Improved assay systems for enzyme assay using the natural substrate.
HPLC assay of the degree of deacetylation.

The HPLC method to assay the DD of shrimp chitin and chitosan has been developed (6) and improved (7). The assay is based on destruction of the sample in concentrated sulfuric acid/oxalic acid at 155°C resulting in liberation of acetyl groups in the material as acetic acid, that is analyzed quantitatively by HPLC. The method is highly reproducible and accurate for α and β chitin and for cuttlefish chitosan. It did not satisfy for α chitin due to problems in the destruction method.

In order to make the method usable for chitin treated with protein preparations containing the deacetylase, the destruction conditions have been investigated further. In Table 1 the new conditions are summarized that lead to complete destruction of a chitin and chitosan preparation mixed or coated with protein. This has been accomplished by tripling the amount of sulfuric acid. Adding more sulfuric acid allows to use a lower hydrolysis temperature of 110°C, instead of 155°C and reaction time of 30 min, instead of 60 min. In the new method, the chance for incomplete hydrolysis is low, the chance for ampule tube explosion is negligible, while the precision is about the same. The confirmed range for suitable analysis is 10 - 100% DD for cuttlefish and 30 - 96% for α chitosan.

Table 1 Comparison of previous and new conditions for HPLC sample destruction

| Parameters	Previous Hydrolysis Method	New Hydrolysis Method
Compositions of acids	0.5mL 12M H_2SO_4 + 2 mL 63ppm Oxalic acid	1.5mL 12M H_2SO_4 + 1 mL 126ppm Oxalic acid
Hydrolysis temperature (°C)	155	110
Hydrolysis time (minutes)	60	30
Confirmed suitable analytical range for chitin/chitosan - Cuttlefish - Shrimp	10 ~ 100% 60 ~ 100%	10 ~ 100% 30 ~ 96%
Chance of incomplete hydrolysis	high	low
Chance of ampoule tube explosion	10 ~ 15%	negligible
Precision	1.0	1.3
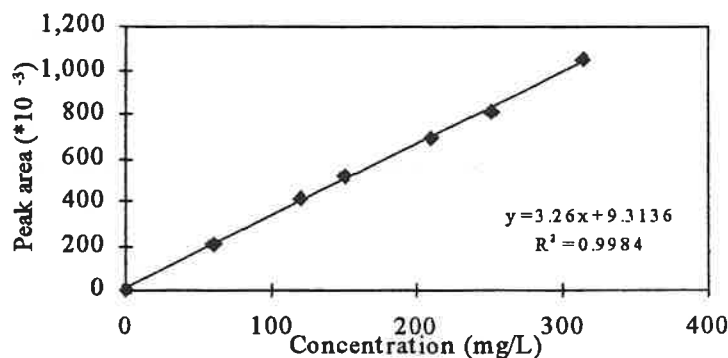
Fig 2 Calibration curve for assay of released acetic acid using GLC

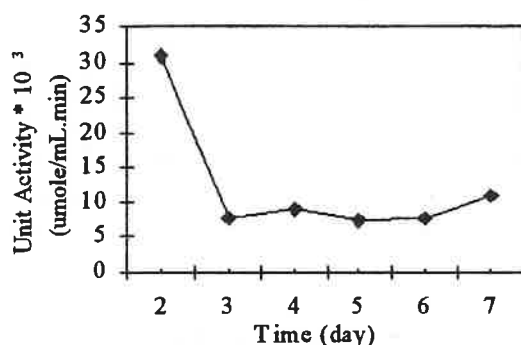$$y = 3.26x + 9.3136$$
$$R^2 = 0.9984$$

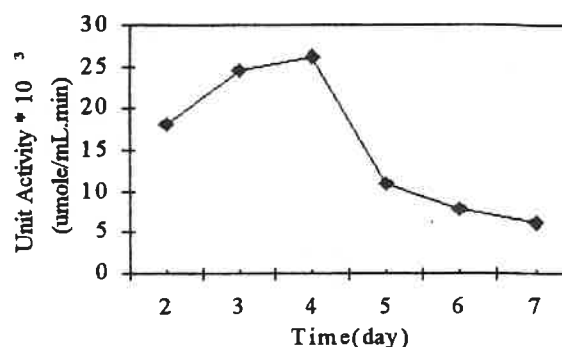Fig 3(a) Extracellular enzyme production (10% mycelium culture)

Fig 3(b) Extracellular enzyme production (spores about 10^8/mL)

Use of GLC to measure activity of chitin deacetylase.

HPLC is highly accurate for fully destructed samples and can be applied for larger series of samples. This method does not provide data instantaneously and is only applicable on samples after drying and it is sensitive to protein contaminants (see discussion). A new method for the assay of CDA activity in aqueous suspensions of chitin/chitosan has been developed by using gas liquid chromatography (GLC) to measure the acetic acid released. The method is fast, reliable and sensitive. The calibration curve (Fig 2) shows a linear relationship of acetic acid concentration between 0 and 300 mg acetic acid per liter and the peak area in the chromatogram, and is complementary to the HPLC method.

Characterization of chitin deacetylase from Absidia coerulea
Intra and extracellular enzyme production

In a series of experiments the production of CDA by A. coerulea has been confirmed. The size of the inoculum was 10%. Most of the enzyme is secreted into the medium. Extracellular enzyme concentration is high after 2 days of cultivation but decreases thereafter (Fig 3a). In case, the culture is inoculated with fungal spores, it takes 4 days to reach the maximal extracellular enzyme concentration. Thereafter, the enzyme concentration decreases (Fig 3b). Production patterns show that A. coerulea cultures of 2-4 days cultivation are maximal for extracellular enzyme production.
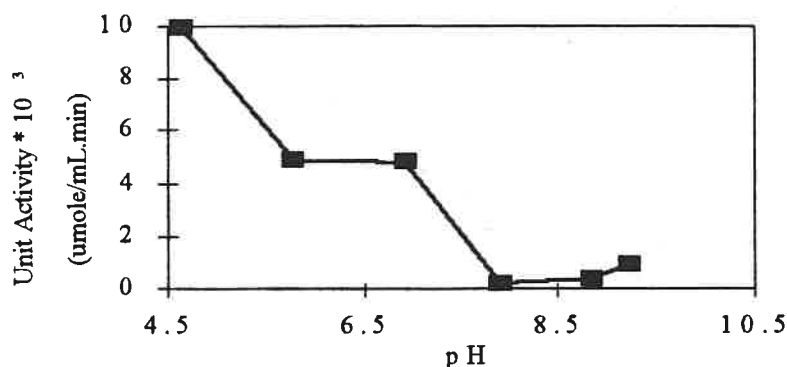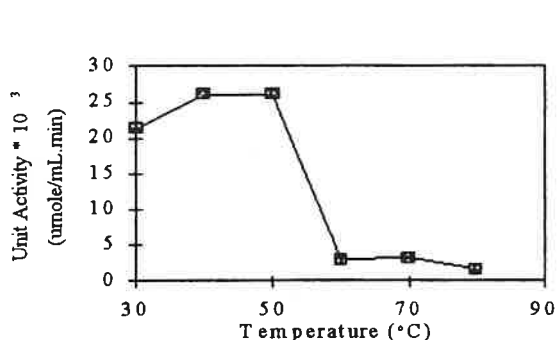
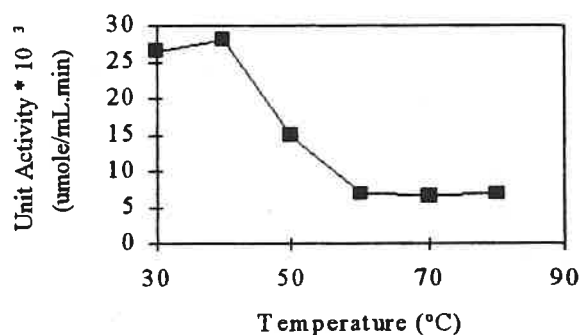Fig 4(a) pH optimum of CDA

Fig 4(b) Temperature optimum of CDA

Fig 4(c) Enzyme stability at various temperatures

Enzyme activity at different pH and temperatures.

In the range of pH 4.5 to pH 7.5, enzyme activity decreased with increasing pH. (Fig 4a). The higher solubility of the enzyme substrate at lower pH as well as the contribution of H^+ catalysis plays a role in higher conversion rates at lower pH. Enzyme activity is maximal at 40-50°C; activity sharply dropped if the temperature is raised to 60°C (Fig 4b). Stability of the enzyme as measured by pre-incubation for 60 min at the indicated temperature followed by enzyme assay at 50°C showed that the enzyme is stable up to 40°C (Fig 4c).

Effect of degree of deacetylation, substrate concentration and mesh size.

The effect of substrate constitution has been investigated for substrate with different degree of deacetylation (Fig 5a). Enzyme activity is high at 60% DD but significantly lower at low and high values of deacetylation. At low % DD, this may be caused by the low solubility of the substrate at conditions of the enzymatic test. At high %DD, this may be caused by spontaneous realignment of the chitosan molecules, leading to a lower accessibility of the substrate for the enzyme. In order to assess the optimal substrate concentration, the amount of chitosan (65% DD) in the enzyme test was varied from 25 mg to 300 mg using the same amount of enzyme whereas the chitosan concentration was kept constant (1%) by adding more buffer (Fig 5b). At 300 mg substrate, the total enzymatic liberation of acetic acid was more than twice the conversion at 25 mg substrate. However, it becomes difficult to keep the high amount of substrate homogeneously suspended in the reaction mixture. Therefore, as a standard 50 mg was being used as substrate.
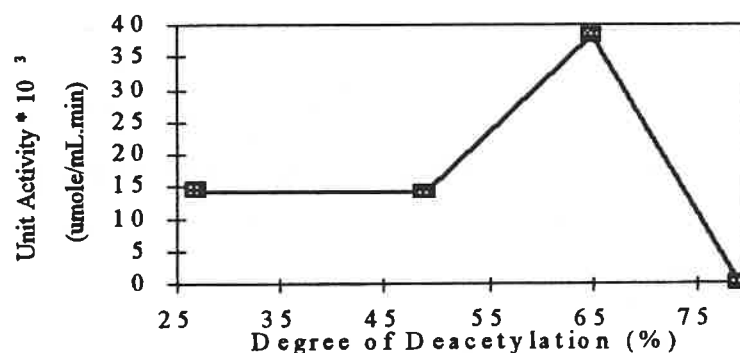
44

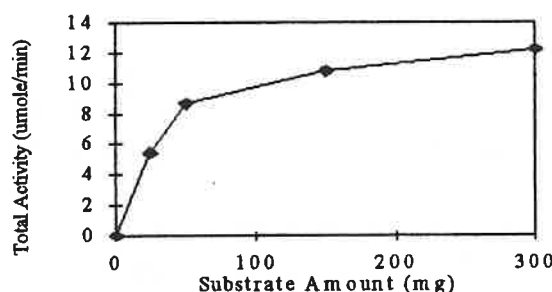Fig 5(a) Effect of deacetylation on enzyme activity

Fig 5(b) Substrate amount and enzymatic
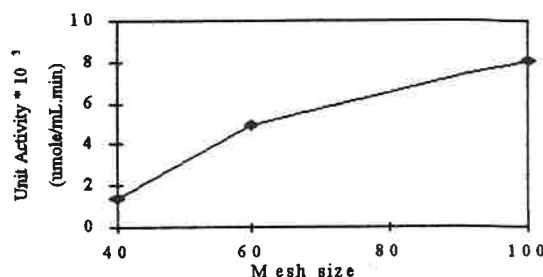deacetylation

Fig 5(c) Effect of mesh size on
enzymatic deacetylation

Substrate having small particle size is more suitable to serve as substrate. In experiments with chitosan 18% DD as substrate but ground to either 40, 60 or 100 mesh, the latter appeared to be a better substrate (Fig 5c).

Kinetics of the enzymatic deacetylation.

Enzymatic deacetylation is fast in the early phase of the assay and much slower later. The experimental data (Fig 6) even suggests that the process is biphasic, firstly very rapid during initial 30 min, followed by slower kinetics during the next 2-3 hours. More data are required to confirm this biphasic phenomenon.
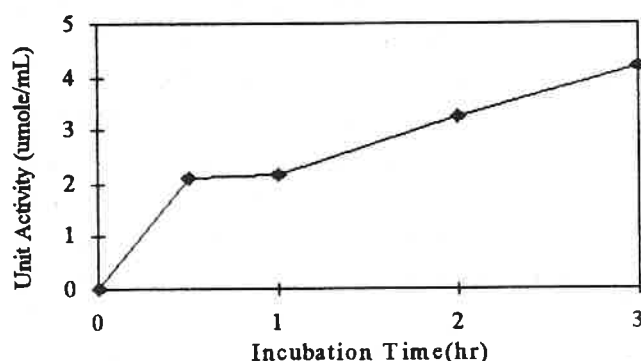

Fig 6 Kinetics of enzymatic deacetylation

Discussion

In the beginning of this research on the chitin deacetylase, the activity of CDA was tried to be shown by incubation of cuttlefish chitosan 55% DD with concentrated extracts of Absidia, followed by HPLC measurement of the DD of the chitosan produced.

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Active enzyme might remove most or all of the acetyl groups. In that case the deacetylated chitosan would not release much acetic acid upon sulfuric acid destruction. In preliminary experiments incubation of cuttlefish chitosan with enzyme, a chitosan product was formed, that did not release acetic acid upon sulfuric acid destruction. Initially this was interpreted in terms of high enzymatic activity until it was observed that cuttlefish chitosan treated with protein solution showed the same incomplete destruction pattern as α chitin. This prompted to reinvestigate the conditions for destruction and lead to the improved method.

The HPLC method has another pitfalls that should be avoided. The method measures the amount of acetyl groups present per mg sample. However, if during enzymatic treatment: (1) non chitinous material sediments adheres on the chitosan, or (2) chitinous material is lost during removal of the supernatant after centrifugation or due to the acidic conditions of the reaction mixture, the resulting DD was too high or too low, respectively. This pitfall does not apply exclusively to HPLC but to any analytical method that requires laboratory handling and is related to the amount of acetyl per unit amount of weight of the material. Experiments have to be designed in such a way that sedimentation or loss of material is avoided.

The method to measure the release of acetic acid by GLC is a easy tool for the study of the deacetylase. The method can be applied on the enzyme incubation mixture itself and is insensitive to changes in solubilized or precipitated material during the incubation. It has been used to establish most of the enzymatic properties reported in this paper. Absidia coerulea has been chosen as a source for the enzyme since this fungus has a very high content of chitosan in its cell wall (about 55% in dry weight basis). The enzyme is produced as an intracellular enzyme but a large part of it is secreted into the surrounding medium. The enzyme has a normal thermosensitivity in contrast to the high stability of the Mucor enzyme at higher temperatures (8).

The design of a enzyme test for the natural substrate is highly dependent on the physical ability of the enzyme to reach the acetyl groups in the chitin. In natural chitin (15% DD) this accessibility is a serious problem, that has to be solved in order to accomplish enzymatic deacetylation at the technical level. The data on the suitability of the substrate at various degrees of deacetylation showed that once a partial (enzymatic) deacetylation has been accomplished, the enzyme will be more efficient to proceed. Whether the low activity of chitosan with high DD will be prohibitive to produce enzymatically deacetylated chitin of high DD is difficult to predict. It will depend on the speed at which the chitosan formed enzymatically adapts in aqueous environment, the crystalline nature of highly deacetylated chitosan in dry condition. The data using 55% DD chitosan as a substrate might give some clues for the deacetylation of natural chitin. The experiment using larger mesh size suggested that the larger the effective surface of the chitinous particle, the higher was the enzyme activity. This might be related to the limited penetration of the enzyme into the chitosan particle. The larger the effective surface, the more acetyl groups will be accessible for the enzyme.

A similar suggestion can be derived from the experiments on enzyme kinetics and various amounts of substrate. Reaction kinetics using 50 mg substrate show a sigmoidal curve that start to level of when only a fraction (5%) of the substrate has been consumed. The curve might even be biphasic (Fig 6). This might be explained similarly by assuming that most acetyl groups are in the interior of the particle and not accessible for the enzyme. Only external acetyl groups are being attacked easily in the first phase, whereas in the second phase the deacetylation of the interior acetyl groups proceeds much slower. A

similar phenomenon is observed when the amount of substrate is raised. Although substrate is in excess, more substrate still gives raising the amount of substrate in the enzyme assay leads to more than twofold increase of the amount of acetic acid formed. This might be explained by the preferential (or exclusive) deacetylation of acetyl groups at the outside of the chitosan particle. It would mean that in using 25 mg total acetyl might be in excess but available acetyl groups might be present in limited amounts. The data presented here support the suggestion made by several authors that the accessibility of the acetyl groups in the substrate plays a crucial role. The availability of acetylgroups is presumably the limiting factor for enzymatical deacetylation at the technical scale. Also the presence of chitinase activity in the enzyme preparations could heavily interfere with a technical production and should be controlled (9).

The levels of chitin deacetylase found in Absidea coerulea cultivated in a minimal medium with some common supplements produces about 5 μmol acetic acid per min per 0.2 g mycelium. This enzyme activity as such might allow in principle for a technical application of the enzyme. In industry, it is easy to produce the mycelium in kg amounts. If accessibility of the acetate groups could be solved and a deacetylase could be used that is stable for 10 hours, 5 kg 15% DD chitin could be converted in 65% DD chitin per kg mycelium in 10 hours. An alternative is to use the extracellular enzyme in the supernatant, that has a lower enzyme activity per mL but a 5 fold higher total activity. Common industrial microbial practice could lead to many fold higher production of enzyme. Even further increase in enzyme activity might be accomplished using genetic engineering of the fungal deacetylase (5). In this way a technical biocatalytic conversion of chitin into chitosan might come within reach.

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Chitosan from Absidia sp.

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Abstract

The results of experimental investigations of chitosan separated from some strains of Absidia are reported. The replacement of acetic acid by hydrochloric acid in extraction leads to obtain in some cases unknown chitosan-like biopolymer. The influence of cultivation conditions on acetylation degree, molecular weight and content in cell walls of separated biopolymers is also discussed. The highest chitosan content was 23.4 % of dry cell walls' mass. The molecular weigh of chitosan ranged from 28 000 Da to 194 000 Da and the acetylation degree changed from 26.1 % to 30.9 %.

Key words: chitosan, cell wall biopolymer, *Absidia orchidis*, *Absidia glauca*,
Absidia coerulea

1. Introduction

The idea of chitosan production from the fungi cell walls has been known since 70's. White and coworkers [1] proposed a method for chitosan separation in 1979. From that time many others groups of investigators succeeded in repeating theirs experiments. The goals of these works were to find a proper "producer", to present chitosan quality, to evaluate the yield factors and investigate the influence of cultivation and biopolymer separation methods.
It had been shown that the best chitosan "producers " belong to Mucoraceae (Mucor, Absidia, Rhizopus, Zygorhynchus and others). The fungi chitosans have very low acetylation degrees (from 0.009 to 35 %) and small or middle molecular weights (from 140 000 to 700 000 Da). These properties induce further fungi chitosan investigations.
The aim of presented work was to compare the chitosans separated from different strains of Absidia fungi cultivated at different conditions.

2. Materials and Methods

2.1. Fungi

Absidia orchidis, obtained from NCAIM F 00642 (Hungary), *Absidia glauca*, obtained from AR - Poznań (Poland) and *Absidia coerulea*, IMI

202719 (Great Britain) were used in the investigations.

2.2. Culture medium

The complex medium [2] was used for the fungi cultivation. It consisted of: 2.0g glucose, 1.0g peptone, 0.1g yeast extract, 0.5g $(NH_4)_2SO_4$, 0.1g K_2PO_4, 0.1g NaCl, 0.5g $MgSO_4*7H_2O$, 0.1g CaCl, 100 ml H_2O. pH of the medium was 6.3.

2.3. Culture conditions

Bath culture without pH stabilization The bath culture was performed at bioreactor BIOFLO III (Braun, Germany). The culture medium (4.5 dm^3) was added to the bioreactor, sterilized (121^0C, 20 min) and inoculated with 500 ml of 2-day old shaken culture (total liquid volume was 5 dm^3). The fungi were incubated at 26^0C, aerated (7 l/min) and mixed (200 rpm). The time of cultivation was 48 h.

Bath culture with pH stabilization   The fungi were cultivated at constant pH = 5.5 [3]. Bath cultures with pH stabilization were performed as above described bath culture. To stabilize the pH the 1N NaOH and 1N HCl solutions were used.

2.3. Biopolymer separation

The biopolymer separation consisted of the following steps:

1. Biomass separation   Fungi biomass was homogenized in the bioreactor (900 rpm, 30 min). Fungi biomass was then centrifugalized (6 000 rpm, 20 min) and washed twice with deionized water.

2. Cell wall separation   The fungi biomass was treated with 1 N NaOH solution, in the ratio 1 g biomass : 20 cm^3 NaOH solution, at 121^0C, (20 min). In this way the proteins were removed. The alkali insoluble fraction (cell walls) was then centrifuged (6 000 rpm, 20 min), washed twice with deionized water and dried 24 h at 60^0C. Finally dry cell walls were ground down.

3. Biopolymer separation   The separation was done according to White chitosan separation method [1]. The fungi cell walls were treated with 1 N HCl solution in the ratio 1 g dcw : 100 ml HCl and were kept 12 h at 95^0C. The solution was centrifuged and an acid soluble fraction was collected. The liquid was mixed and alkalized to pH 10.0 with 1 N NaOH - biopolymer was precipitated. Biopolymer was separated from the liquid by centrifugation (20 000 rpm, 20 min), washed twice with deionized water and dried 24 h at 50^0C. The biopolymer can be stored at room temperature in a closed vessel.

2.4. Chitosan and chitin

Commercial chitosan and chitin were used as reference materials. Chitosans of different molecular weight (MW = 70 000, 750 000, 2 000 000) were purchased from Fluka (Germany) and used without further purifications. Cryl chitin was supplied by Sea Fisheries Institute (Poland). The chitin was

additionally purified by extraction of residual calcium carbonate with 2N hydrochloric acid [4].

2.5. Analytical methods

IR measurements   IR spectra of all samples were run on Perkin-Elmer System 2000 spectrometer. The resolution was 4 cm^{-1}. The average of 32 scans for each spectrum were used. Samples of obtained biopolymers and reference chitosans were used in the form of KBr disc (2mg / 250mg). The chitin was prepared as a film.

Degree of acetylation Degree of acetylation (AD) was determined based on biopolymers infrared spectra (IR) according to the direct method of Domszy and Roberts [5]. The method involved uses of the amide I band at 1655 cm-1 as a measure of N-acetyl group content and the hydroxyl band at 3450 cm-1 as an internal standard to correct for differences in chitosan concentration in KBr disc. The direct method of calculation of the degree of N-acetylation was used. The method is based on the relationship:

$$\% \text{ N-acetyl} = (A_{1655} / A_{3450}) \times 100 / 1.33$$

where: A_{1655} and A_{3450} denote the value of absorption at appropriate wave numbers, respectively.

Molecular weight   The biopolymer molecular weight was determined using Hewlett - Packard GPC chromatography with PL-GFC 4000A and PL-GFC 300A columns, differential refractometer detector HP 1047A and acetic eluent (0.3 M acetic acid + 0.2 M sodium acetate). For the calibration the narrow pullulan standards were used.

3. Results

The microorganisms were cultivated in batch cultures with and without pH stabilization. Biopolymers were separated according to White's method [1] with modification that acetic acid was replaced with hydrochloric acid. This modification gives lower chitosan acetylation degrees [1, 6].

The biopolymers obtained from Absidia strains were identified on the base of IR spectrum and compared with commercial chitosan and chitin IR spectra; then the acetylation degree, molecular weight and mass fraction in the cell wall were determined.

3.1. Biopolymer identification

The infrared spectra of biopolymers separated from the cell walls of all cultivated fungi were compared with the spectra of commercial chitosan and

chitin. This comparison was the base for chitosan identification.

Obtained biopolymers could be divided into two groups:

1. biopolymers with IR spectra very close to commercial chitosan spectrum (*Absidia orchidis, Absidia glauca*); they were recognized to be the chitosan,

2. biopolymers with IR spectrum similar neither to chitosan nor to chitin (*Absidia coerulea*); they were recognized not to be the chitosan (Ch-L, chitosanlike biopolymer).

Chitosans from *Absidia orchidis* and *Absidia glauca* have the IR spectra similar to the chitosan spectrum; they have peaks at the range of wave numbers 3500 - 2500 cm^{-1} as chitosan has, and in the range 1750 -750 cm^{-1} These peaks are slightly allocated but their amount is the same.

The biopolymers from *Absidia coerulea* have only a few peaks. At the range of the wave numbers 3450 - 2800 cm^{-1} the peaks are smaller and more "sharp" in comparison to chitosan; the peak at the range 3400 - 2300 cm^{-1} is single while chitin has a double peak there. In comparison to both, chitin and chitosan, at the range 1650 - 750 cm^{-1} there are only a few peaks; they are smaller and much wider. At the range 1450 - 1200 cm^{-1} the biopolymers have no peaks which appear in chitin and chitosan spectra. At the range 1200 - 800 cm^{-1} the biopolymers have only one peak instead of few in chitin and chitosans.

As it was shown, the biopolymers obtained from *Absidia coerulea* (no matter how they were cultivated) have the IR spectra which are not similar neither to chitin nor to chitosan. In all spectra one can, however, find the amide (1655 cm^{-1}) and hydroxyl group (3450 cm^{-1}) peaks necessary to calculate the acetylation degree as it is done for chitin and chitosan. Generally the spectra of biopolymers are similar to that one published by Miyoshi et al. [7].

3.2. Degree of acetylation.

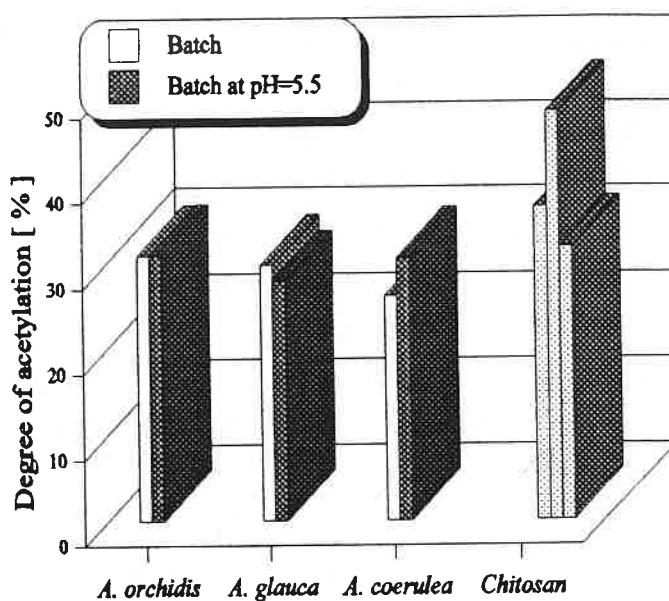The degrees of acetylation of fungi chitosans, fungi biopolymers and commercial chitosans (respectively with low,

Figure 1. The degree of acetylation of fungi chitosan (*Absidia orchidis, Absidia glauca*), fungi biopolymer (*Absidia coerulea*) and commercial chitosans (Fluka).

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middle and high molecular weights) are presented on Fgure 1. It can be observed that the fungi chitosans and biopolymers acetylation degrees (AD) are smaller or similar to the commercial chitosans (high molecular weight chitosan has the lowest acetylation degree - 31.8%).

There are no significant differences in acetylation degrees of chitosan extracted from the fungi cultivated in different way: *Absidia orchidis* - 30.9% and 31.0%, *Absidia glauca* - 29.7% and 27.9% (batch culture and batch culture at constant pH respectively). The method of cultivation influenced slightly AD of the fungi biopolymer (26.1% and 30.5%).

3.3. Molecular weight

The molecular weights of fungi chitosans and fungi biopolymers are presented on Figure 2:

The molecular weights of chitosans and biopolymers depended strongly on the method of cultivation. It was observed that the chitosans obtained from the fungi cultivated without pH stabilization had much lower molecular weights (28 000 and 54 800 Da - *Absidia orchidis* and *Absidia glauca* respectively) then when the fungi were cultivated at constant pH (192 900 and 194 300 Da). For the fungi biopolymers we observed the opposite relation: one's molecular weight extracted from the fungi cultivated at the bath conditions was lower (44 500 Da) then the one's obtained from the batch culture at the constant pH (87 800 Da).
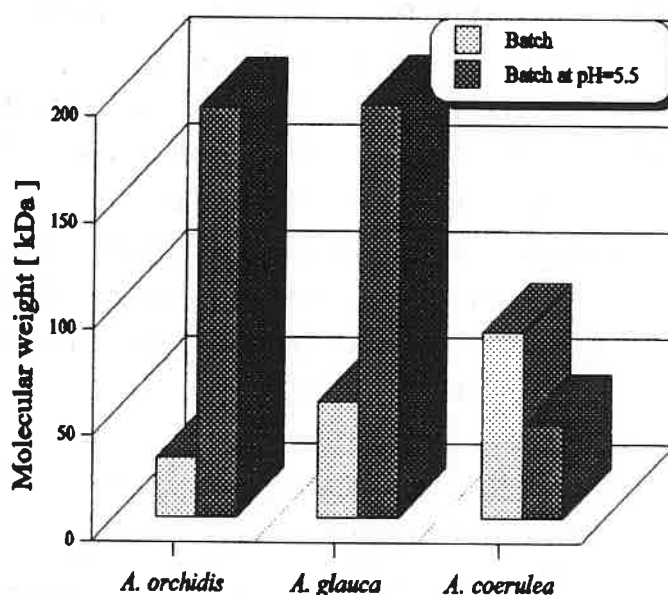

Figure 2. The molecular weight of fungi chitosans (*Absidia orchidis, Absidia glauca*) and fungi biopolymers (*Absidia coerulea*).

3.4. Content of biopolymer in cell walls

The content of the fungi chitosans and the fungi biopolymers in the cell walls are presented on the Figure 3.

This can be clearly observed that the content of chitosan in cell wall was much higher then the content of chitosanlike biopolymer. The highest chitosan

content was equal 23.4% while the biopolymer content didn't reach 7%.

There was no observed clear relationship between the cultivation method and chitosan content in cell wall. This relation depended on microorganism used in experiment: for *Absidia orchidis* the amount of chitosan in cell wall was higher when the fungi were cultivated at batch condition (23.3% and 12.9%) while for *Absidia glauca* (12.9% and 15,2%) and *Absidia coerulea* (5.5% and 6.9%) there was opposite relation.
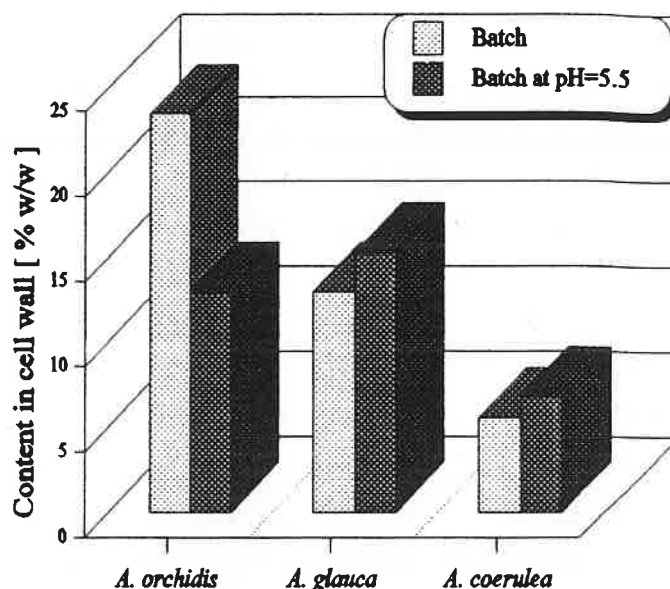

Figure 3. The content of fungi chitosans (*Absidia orchidis, Absidia glauca*) and fungi biopolymers (*Absidia coerulea*) in cell walls.

4. Discussion

Method of separation proposed by White et al. [1] was, in original, used for chitosan separation from fungi cell walls. A modification of this method proposed also by White et al [1] and next by Rane and Hoover [6] led to obtain a chitosan with lower acetylation degree; in this modification hydrochloric acid was used instead of acetic acid. In our investigation hydrochloric acid for chitosan extraction from cell walls was used and we separated not only chitosan but also other, unknown biopolymer (Ch-L). The IR spectra of Ch-L were closed to that presented by Miyoshi and his team [7] and to that obtained previously [8] from Mucor sp. and Rhizopus sp.

Miyoshi et al. extracted this biopolymer from *Mucor rouxii* IFO 5773 cell wall using acetic acid. In this circumstances it is hardly to say that the modification of extraction method may influence the kind of biopolymer extracted (chitosan or unknown one).

The IR spectrum of chitosanlike biopolymer (Ch-L) has the peaks used in acetylation degree calculation so it can be easily mistaken for a chitosan. His acetylation degrees was lower than the Fluka's chitosan and depended on cultivation method; in batch culture a biopolymer with lower acetylation degree was obtained.

The biopolymers had low molecular weights, which also depended on cultivation

method: in batch culture higher one was extracted.

Its content in cell wall of *Absidia coerulea* didn't exceed 7%.

All chitosans separated from *Absidia orchidis* and *Absidia glauca* has degrees of acetylation lower than degrees of commercial chitosan offered by Fluka (Germany). The AD doesn't depend on cultivation method.

Values of the molecular weights are below 200 000 Da and depend not only on cultivation method but also on the microorganism used in experiments. There was clear relation between the chitosan molecular weight and way of cultivation. The chitosan content in cell walls of fungi was much higher than the biopolymers; the lower content was equal 12.9% (*Absidia orchidis* in batch culture at pH=5.5 and *Absidia glauca in batch culture*) but the higher was over 23% (*Absidia orchidis* in batch culture).

Investigation concerning the increase of chitosan content in fungi cell walls and lowering the chitosan acetylation degree are the aims of our further works.

List of symbols

dcw   - dry cell walls,

AD   - acetylation degree, [%],

Ch-L   - chitosanlike biopolymer,

IR   - infrared,

MW   - molecular weigh, [Da].

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