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Enzymatic Hydrolysis of Pretreated Chitin by *Aspergillus carneus* Chitinase

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Studies of the pretreatment of chitin and its subsequent hydrolysis by *Aspergillus carneus* chitinase are reported. Ball milling was found to be the most effective way among the pretreatment methods tested. Data are presented describing the effect of enzyme and substrate concentrations on the rate and extent of the hydrolysis process. It was found that the successive addition of enzyme improved the saccharification yield. Significant product inhibition of the chitinase was observed when N-acetylglucosamine concentration was 3.6% or higher. Adsorption of enzymes to the substrate occurred during a 24 hr hydrolysis period. An initial rapid and extensive adsorption occurred, followed by gradual desorption which increased during the time of reaction. Intermediate removal of the hydrolyzate and continuation of the hydrolysis by adsorbed enzyme on the residual chitin was also investigated. A total of 75.4 g/l reducing sugars, corresponding to 69.2% saccharificaton yield (as N-acetylglucosamine) was obtained. In addition an increase in the amount of recoverable enzymes was observed under these conditions. Evidence presented here suggests that the technique, whereby the free enzymes in the recovered hydrolyzate are re-adsorbed onto the new substrate, may provide a means of recirculating the dissolved enzymes.

Chitin, β-1,4 link polymer of N-acetylglucosamine, is an abundant biomass structurally related to cellulose which is expected to be widely used for industrial application. Production of N-acetylglucosamine, a basic component of chitin, is very important for an efficient use of chitin. This monosaccharide finds its importance in chemical and pharmaceutical uses, and especially chitin oligomers such as chitobiose and chitotriose are spotlighted in the food industries because of their digestive properties (12).

N-Acetylglusamine can be produced by acid hydrolysis of chitin (11). However, the effects of corrosion and pollution problems have to be considered. Enzymatic hydrolysis has been reported as a best and ideal method for N-acetylglucosamine production from chitin (13); however, there are a few reports on the industrial production of N-acetylglucosamine from chitin (4, 5, 16).

It is well known that enzymatic degradation of chitin to N-acetylglucosamine was accomplished by multi-enzymes of these: chitinase (exo- and endochitinase) that cleaves chitin to chitobiose or chitin-oligomer and N-acetylglucosaminidase [E.C. 3.2.1.30] that yields N-acetylglucosamine (3). The cost of enzyme production, resistance of substrate, low saccharification yield, enzyme inactivation, and inefficient enzyme recovery represent the major economical barriers for practical application.

In a previous work, an active extracellular chitinase [E.C. 3.2.1.14] from *Aspergillus carneus* was produced (14). This enzyme was isolated, purified and characterized as exochitinase (2). This enzyme can be applied to the bioconversion process of chitin.

The aim of our study was to find an optimum pretreatment giving the most effective chitinase hydrolysis. Data of enzyme substrate ratio, multiple addition of enzyme, product inhibition, enzyme adsorption, removal of hydrolyzate during the enzymatic hydrolysis, and enzyme recovery were collected in order to develop economical processes compatible for practical applications.

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Key words: Chitin, chitinase, saccharification, enzymatic hydrolysis

MATERIALS AND METHODS

Microorganism and Cultural Conditions

The fungal culture used for enzyme production was local isolate and identified as *Aspergillus carneus* by the Commonwealth Mycological Institute, Kew, Surrey, England. The organism was maintained at 25℃ on PDA (potato dextrose agar) slants by fortnightly subculture and stored at 5℃. The inoculum preparation and cultural conditions were described earlier (14). The culture filtrate was used for the saccharification experiments as an enzyme source.

Chitinase Activity

One milliliter of appropriately diluted enzyme sample was incubated with 1.0 ml of 1.0% (w/v) pure colloidal chitin (Sigma Co., St. Louis, MO, USA) in 0.05 M acetate buffer, pH 4.5. The reaction mixture was incubated at 45℃ for 1 hr. The released reducing sugars were measured by the Somogyi-Nelson method (8, 15) using standard curve of N-acetylglucosamine (Sigma). Protein concentration was determined according to the method adapted by Lowry *et al.* (6). One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 0.5 μmol of N-acetylglucosamine under the assay conditions.

Pretreatment of the Chitin Sample

Pretreatment of chitin was achieved as follows: A. Ball milling: the chitin samples were milled (2~180 mesh) for 24 hr and sieved on a shaker (seive opening, 0.25~0.1 mm). B. Hydrochloric acid pretreatment: the chitin samples (10%, w/v) were suspended in HCl (5 N) at 30℃ for 6 to 24 hr and then washed and vaccum dried. C. Phosphoric acid pretreatment: the chitin sample (10%, w/v) was suspended in 50% H_3PO_4 at 30℃ for 6 to 24 hr, and then washed and vaccum dried.

Saccharification of Substrate

After 1.0 g of chitin samples were taken in 250 ml Erlenmeyer flask, the desired quantities of enzyme and acetate buffer (0.05 M, pH 4.5) were added to a final volume of 100 ml. The flasks were incubated at 45℃ on a rotary shaker (150 rpm). Samples were centrifuged and the supernatant were used for sugar analysis. Saccharification (%) defined by the amount of reducing sugars produced by the enzyme for pretreated samples and chitin conversion (%) defined by the amount of released reducing sugar by pretreatment and enzyme for total samples, were calculated by assuming the molecular weight of N-acetylglucosamine to be 221, as shown in the following equations.

$$\text{Saccharification (\%)} = \frac{\text{Amount of reducing sugars} \times 203/221}{\text{Amount of substrate}} \times 100$$

$$\text{Chitin conversion (\%)} = \frac{\text{Amount of released reducing sugar} \times 203/221}{\text{Weight of untreated sample}} \times 100$$

Successive Addition of Enzyme and Product Inhibition

To avoid the sharp deactivation of chitinase during the hydrolysis and to increase the saccharification yield equal portions of enzyme were added successively (every 2 hr) through the course of hydrolysis reaction. Total enzyme concentration for all the samples was 80 U/ml. To minimize the dilution effect, a lyophilized enzyme was used. The experimental layout is outlined in Fig. 1. With the resulting series of samples (A-F), residual enzyme activity as well as reducing sugars were determined after 48 hr.

To test the effect of product (N-acetylglucosamine) inhibition on chitinase activity during the hydrolysis course, different concentrations of N-acetylglucosamine (i.e. 1.0, 2.0, 3.0 and 3.6%) were added to the reaction mixture at the beginning of the reaction and released sugar was determined.

Adsorption Tests

Using 80 U/ml of initial enzyme loaded activity, the adsorption of enzyme to the substrate during the course of 24 hr hydrolysis was estimated by measuring the chitinase activity in the free liquid. The enzyme adsorption to the substrate was investigated by two methods, namely, apparent and actual adsorbed enzyme activity. Apparent adsorbed-enzyme activity: This was determined by subtracting the free chitinase activity in the hydrolyzate from the original amount used. Actual adsorbed-enzyme activity: This was estimated by eluting the adsor-

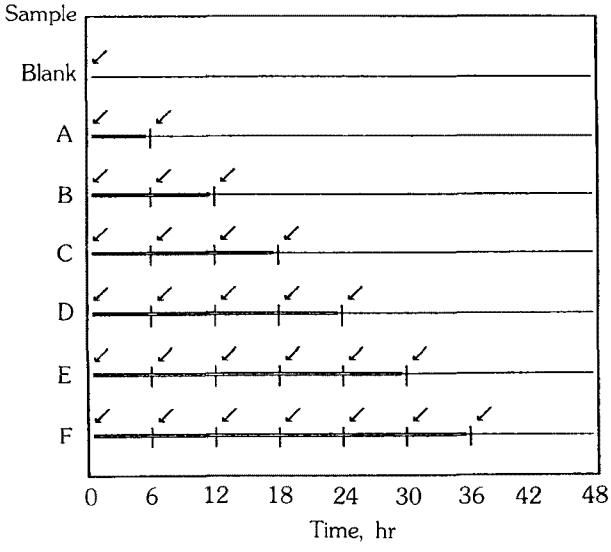

Fig. 1. Experimental layout to study the effect of successive addition of chitinase on the hydrolysis of chitin.

Final enzyme concentration of all samples was 80 U/ml. The enzyme was added at the point of arrows in equal portions every 6 hr.

bed enzyme activity from the residual chitin with 0.5% Tween 80 in 0.1 M phosphate buffer pH 4.5 until the wash was protein free.

For effective recovery of the soluble enzyme in the saccharification process, the 10 ml of enzyme solution from hydrolyzate were adsorbed onto a column in which 1g of newly milled chitin was packed. The enzyme containing liquid used here was the hydrolyzate product after 24 hr reaction. Liquid volumes, sugar concentrations and enzyme activities of hydrolyzate were measured before and after passing through a column of chitin contained in glass tubes (0.5 cm, i.d.).

Removal of Hydrolyzate during Hydrolysis

One method for effective chitin conversion is the removal of enzyme hydrolyzate which contains the product (N-acetylglucosamine) during hydrolysis, if the product inhibits the enzyme activity seriously. Following this concept, the reaction was started with enzyme concentration of 80 U/ml, and 10% chitin. After 24 hr, the reaction was cooled to 5℃ for 30 min. Next the hydrolyzate was filtered off. The solid materials were washed with cooled buffer (5℃) to remove all soluble sugars. Then the buffer was added, and then to maintain the substrate concentration at 10% (w/v), the hydrolysis was continued at 45℃. At the end of each run, the reducing sugars apparent and actual adsorbed enzyme activities were determined after the hydrolysis (24 hr).

All the data reported here are the mean of five individual experiments which rendered values ranging in margin ± 5%.

RESULTS AND DISCUSSION

Evaluation of Pretreatment Techniques

The aim of pretreatment of chitin substrate before enzymatic hydrolysis is to render the material maximally susceptible to the action of chitinase. Therefore, the research for an effective pretreatment merits very high priority. In this study, the efficiency of any pretreatment was judged by some parameters including the extent of weight loss, degree of saccharification, and chitin conversion.

Concerning the pretreatment studies, the efficiency of the pretreatments was judged by chitin conversion, since it represents the general output of the pretreatment process. Table 1 presents the recovery of pretreated chitin and reducing sugars released, as well as chitin conversion. Among three different pretreatment methods (ball milling, HCl and phosphoric acid treatment), ball milling from 140~180 mesh (0.125~0.1 mm seive opening) was found to be the most efficient. In conjugation with enzymatic step, 23.5% of reducing sugar can be liberated (Table 1). The most significant effect of ball milling pretreatment is the reduction of particle size, which may result in the increase of the surface area. This contributes to an increase susceptibility of chitin to enzymatic hydrolysis (10). Ball milled chitin was used successfully for enzymatic hydrolysis of chitin (4, 5, 10).

Influence of Enzyme Concentration on Chitin Hydrolysis

The degree of saccharification of chitin depends on the enzyme substrate ratio (10). To identify a suitable condition for hydrolysis, the effect of enzyme concentrations between 20 and 100 U/ml was studied. In this experiment, the concentration of ball-milled chitin was 10% (w/v). Fig. 2 shows the change of sugar concentration (g/l) in the hydrolyzate after enzymatic hydrolysis (6~48 hr). The data showed that 80 U/ml would be an adequate enzyme concentration under the assay conditions, with 10% chitin concentration, because further

Table 1. Effect of pretreatment on the hydrolysis and saccharification and chitin conversion.

Pretreatment	Recovered chitin (w/w, %)	Enzymatic hydrolysis[a]		
		Released sugars (g/l)	Saccharification (%)	Chitin conversion (%)
Ball milling (24 hr)				
20-60 mesh	99.6	8.2	15.1	15.0
80-100 mesh	99.0	9.5	16.8	16.6
140-180 mesh	98.3	12.8	23.5	23.1
Acid treatment (5 N HCl)				
6 hr	83.2	10.3	17.2	15.7
12 hr	71.6	14.3	20.5	18.8
24 hr	60.3	16.8	20.3	18.2
Phosphoric acid treatment (50% H_3PO_4)				
6 hr	89.4	10.5	19.3	17.2
12 hr	82.6	13.2	24.2	20.0
24 hr	76.0	15.1	27.8	21.1

[a]Hydrolysis was carried out with 80 U/ml chitinase activity and 5% (w/w) chitin for 24 hrs.

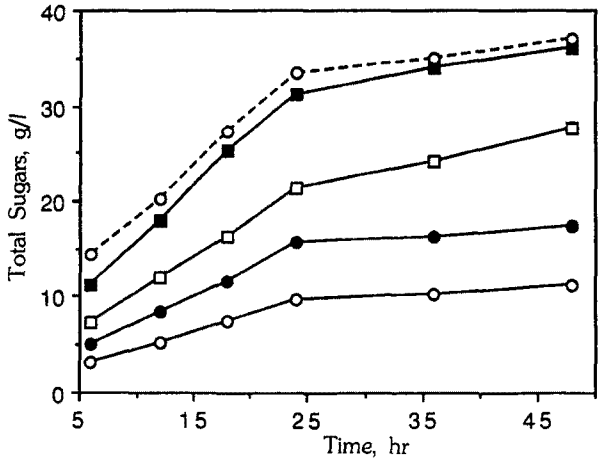

Fig. 2. Effects of enzyme concentration on the rate and extent of chitin hydrolyzate using the 10%(w/v) ball milled chitin.

Enzyme loading concentrations were 20(○−○), 40(●−●), 60(□−□), 80(■−■) and 100(○---○) U/ml. Total sugars mean the amount of N-acetylglucosamine hydrolyzed by the enzyme in the hydrolyzate (g/l).

increase of the enzyme concentration to 100 U/ml did not significantly increase the hydrolysis yield. The curves show that the bulk of sugar that eventually solubilized is released during the first few hours of hydrolysis course. Furthermore, extension of hydrolysis time from 24 to 48 hr did not significantly increase the degree of hydrolysis. Thus a concentration of about 32~33 g/l reducing sugar in the hydrolyzate (corresponding to about 25.7% chitin conversion) was taken as an index. Based on these results, 80 U/ml enzyme concentration, 10% milled chitin and 24 hr hydrolysis time were chosen as the standard conditions for the enzymatic hydrolysis. Under these conditions, 32.3% of sugars were saccharified. Tom and Carroad (16) reported a yield of about 10g reducing sugars after 24 hr hydrolysis of milled chitin at enzyme substrate ratio of 252 U/g chitin. As shown in Fig. 2, our saccharification values 32.3% (at enzyme substrate ratio, 80 U/g) is much better than those reported by Tom and Carroad (16).

Successive Addition of Enzyme

The data in Fig. 2, show that the rate of chitin hydrolysis was sharply decreased after 24 hr. To investigate if this was related to the enzyme inactivation by the effect of thermal instability and/or shear stress of agitation through long term hydrolysis, the saccharification with successive addition of enzyme was determined.

It is clear that successive addition of enzyme improved the saccharification yield (Table 2). Furthermore, the prolonged time of addition rendered a favorable effect on the formation of reducing sugar as well as the residual

Table 2. Effect of successive addition of the enzyme on the rate and extent of enzymatic hydrolysis[a].

Sample	Released reducing sugars (g/l)	Saccharification (%)	Chitinase activity in the hydrolyzate (%)
Blank[b]	36.0	33.0	50.2
A	38.2	35.1	52.6
B	43.3	39.8	60.4
C	48.2	44.3	65.6
D	51.4	47.2	70.2
E	55.3	50.8	74.0
F	61.4	56.4	78.6

[a] Substrate concentration was 10% (w/v) chitin. Total enzyme concentration for all samples was 80 U/ml.
[b] The total enzyme was added at zero time in blank and Samples A, B, C, D, E, and F are the same as in Fig. 1.

enzyme activity in the hydrolyzate. For example, by the addition of the enzyme through a 18 hr period (Sample C) the saccharification yield was increased by about 34.2 % in comparison with a single addition (Blank). Furthermore a prolonged addition of enzyme up to 36 hr (Sample F) resulted in 27.4% and 70.9% increases in the saccharification yield in comparison with Sample C and Blank, respectively.

The positive effect of successive addition of the enzyme must be partially due to the decrease in the exposure time of bulk enzyme to inactivation conditions, particularly due to the effect of shaking during the long-term hydrolysis. This view is substantiated by the parallel relationship between the increase in residual enzyme activity in the hydrolyzate and the extension of the addition period (Table 2). Similar results were reported for enzymatic hydrolysis of cellulose and hemicellulose (1). On the other hand, Rao *et al.* (9) demonstrated another advantage with multiple addition instead of single addition of enzyme for obtaining a higher recovery of cellulases.

Effect of Product Inhibition

From the results shown in Table 2 it was found that only 29.3% of substrate was hydrolyzed although there was about 50.2% (40.1 U/ml) of chitinase activity in the reaction mixture (in case of Blank). Therefore, it was necessary to investigate the effect of product inhibition on the rate and extent of hydrolysis course. The results (Fig. 3) showed that N-acetylglucosamine significantly inhibited enzymatic hydrolysis of chitin. It is worthy to note that the addition of 3.6% N-acetylglucosamine at the beginning of reaction (this is the equivalent level of maximal hydrolysis yield with single addition, see Table 2) inhibits the enzymatic hydrolysis. Only 14.8 g/l reducing sugar (*i.e.* a decrease of 36~14.8=21.2 g/l)

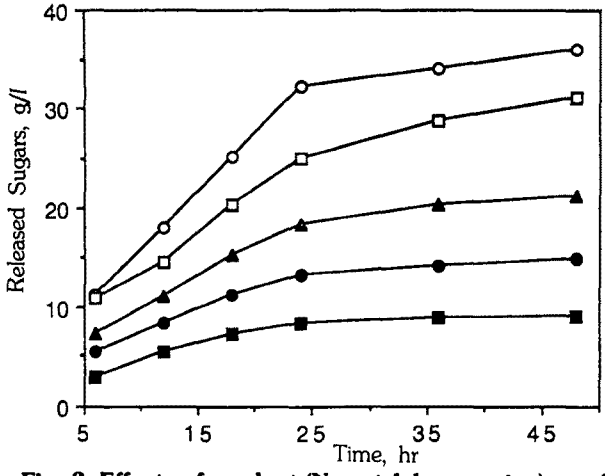

Fig. 3. Effects of product (N-acetylglucosamine) on chitinase acitivity during the course of hydrolysis. Released sugar (N-acetylglucosamine) was determined for the purpose.

Four different concentrations (1.0, 2.0, 3.0 and 3.6 %) of N-acetylglucosamine were added at the beginning of reaction. Symbols: ○, blank; □, 1%; ▲, 2%; ●, 3%; ■, 4% N-acetylglucosamine concentration.

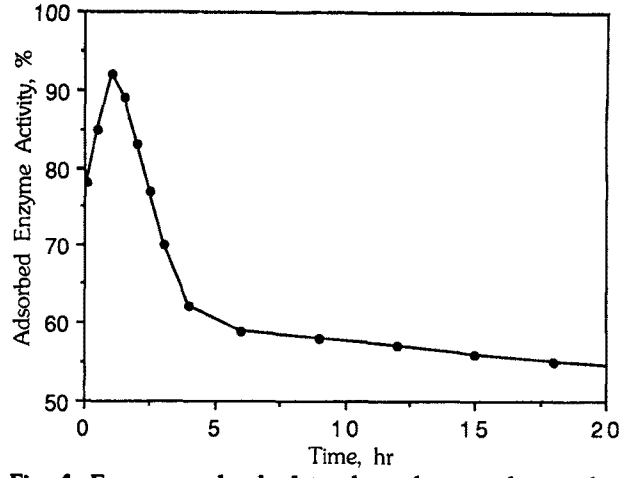

Fig. 4. Enzymes adsorbed to the substrate during the course of hydrolysis. Initial enzyme activity loaded was 80 U/ml.

was produced which is equivalent to a 58.9% decrease in yield of hydrolysis.

Due to the inhibitory effect of hydrolyzate products, removal of hydrolyzate and continuation of hydrolysis by the adsorbed enzyme may provide a way of improving the saccharification yield. This can be applied usefully provided that the variation of enzyme adsorption to the substrate during the hydrolysis course is known.

Enzyme Adsorption

For economical purposes it is necessary to recirculate a very high proportion of the used enzyme. In order to determine the time when the enzyme can be recovered most easily, the variaton in their adsorption to the substrate during the course of hydrolysis (24 hr) was studied. It was found that about 91% (72 U/ml) of total activity (80 U/ml) was adsorbed on chitin particles (10%, w/v) after only one hour of the reaction (Fig. 4). This was followed by gradual desorption of the enzyme as the substrate was digested. After 24 hr, about 50.2% of total activity was found in the hydrolyzate.

Readsorption of the dissolved enzyme can be controlled by adjusting the temperature, enzyme concentration, substrate concentration, and ionic strength of the buffer (17). Our investigations of maximal readsorption of dissolved enzyme showed that the maximal enzyme adsorption was detected at 5℃ after an incubation for 30 min in 0.05 M acetate buffer, pH 4,5. These results are in agreement with those reported by Young (M.E. Young. 1983. Ph.D. Thesis, University of California, Davis) and Young *et al.* (18) for the adsorption of chitinase on chitin particles.

Applying these conditions on the enzymatic hydrolyzate (after 24 hr) by incubating the reaction mixture for 30 min at 5℃, only 17.6% of the total activity (14.1 U/ml) was detected in the hydrolyzate.

Removal of Hydrolyzate during Enzymatic Hydrolysis

Due to the significant inhibitory effect of the hydrolyzate products (Fig. 3) and the general appearance of enzyme adsorption, further studies on the enzymatic hydrolysis with adsorbed enzyme after removal of the hydrolyzate have been carried out. Table 3 showed that removal of hydrolyzate and continuation of the hydrolysis by adsorbed enzyme resulted in 69.2% of saccharification yield (about 76.9% increase in comparison with Blank).

Two different techniques, apparent and actual adsorbed enzyme activities, were used for evaluating the adsorbed chitinase activity (Table 3). The difference between the values of two activity must be partially due to the values of inactivated enzyme by adsorption. In our results the initial enzyme activity at each step was calculated on the basis of actual adsorbed enzyme activity.

Removal of hydrolyzate and continuation of the reaction with adsorbed enzyme improved the saccharification yield. Thus after four trials the final saccharification reached to 69.2%, which was equivalent to 76.9% increase to the yield of Blank. The decrease in the rate of hydrolysis (*i.e.* the reducing sugars produced per unit chitinase activity) must have been partially due to incomplete adsorption of all chitinase components in the enzyme complex. Similar results were reported for the hydrolysis of cellulose (7, 17).

Table 3. Enzymatic hydrolysis of chitin by adsorbed enzyme after removal of hydrolysate[a].

No. of trials	Initial enzyme concentration (U/g)	Apparent adsorbed enzyme activity[a,b] (U/g)	Actual adsorbed enzyme activity[c] (U/g)	Released reducing sugars (g/l)	Saccharification[d] (%)
Blank[e]	80.0	68.0	21.7	42.6	39.1
1	80.0	65.2	58.2	32.3	29.7
2	58.2	46.2	39.6	21.6	19.8
3	39.6	32.7	27.2	13.7	12.3
4	27.2	18.4	12.2	8.4	7.4
Total (1-4)				76.0	69.2

[a]Apparent and actual enzyme activity was determined after the hydrolysis course of each run (24 hr). Starting reaction mixture was 10% (w/v) chitin.
[b]Apparent absorbed-enzyme activity was calculated as follows: Initial enzyme activity−enzyme activity in the hydrolyzate.
[c]Actual adsorbed enzyme activity (see materials and methods for further details) was considered as an initial enzyme concentration for the next run.
[d]Saccharification (%) was calculated based on the initial chitin concentration as 10% (w/v).
[e]Enzymatic hydrolysis of Blank was carried out for 96 hr.

Table 4. Reutilization of dissolved enzyme in the hydrolyzate by adsorption to the new substrate.

Enzyme concentration (U/ml)	Enzyme state	Adsorbed enzyme activity[a] (%)	Saccharification[b] (%)	Chitin conversion[c]
60	Recovered[c]	48.6	13.1	12.9
60	Fresh	53.2	18.4	18.1
40	Recovered	28.2	8.2	8.1
40	Fresh	32.2	13.4	13.2
20	Recovered	12.2	4.3	4.2
20	Fresh	15.1	7.9	7.8

[a]Adsorbed enzyme was calculated as follows; initial enzyme activity−enzyme activity in free liquid after passing through the column.
[b]Enzymatic hydrolysis after the adsorption was carried out for 24 hr at standard conditions.
[c]From enzymatic hydrolyzate after 24 hr.

Reutilization of Dissolved Enzyme in Hydrolyzate by Adsorption to New Substrate

Another approach for maximal recovery of a high amount of the enzyme in the saccharification process is the re-adsorption of enzyme from hydrolyzate (10 ml) onto a column of milled chitin (1g). In this experiment, the passage through the column took about 30 min at 5℃. The chitin with adsorbed enzyme was washed with cold water to remove the free sugars and unadsorbed enzymes. A parallel experiment was conducted using fresh enzyme at the same concentration.

The efficiency of this technique could be judged by how much enzyme would be adsorbed during the contact time (about 30 min). Table 4 showed that the adsorption of recovered enzyme was decreased from 81% (in case of 60 U/ml) to about 60% (in case of 20 U/ml). This was consistent with findings reported by Young *et al.* (18), who reported that the rate and extent of chitinase adsorption to chitin substrate depended on the bulk enzyme concentration. Thus, at low enzyme concentration it was difficult to rull more enzyme from the dilute solution to be adsorbed to the substrate.

A general feature is evident; the degree of adsorption of fresh enzyme was more than that of the recovered enzyme (Table 4). This may be attributed to the absence of some enzyme components in the hydrolyzate, which still strongly bonded to the residual chitin in the previous hydrolysis and/or the denaturation of some enzyme components during the first hydrolysis.

In general we concluded that this technique may offer a possibility of recovering the major part of dissolved chitinase.

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