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IMMOBILIZATION OF INDUSTRIAL ENZYMES: IMMOBILIZATION OF CELLULASE COMPLEXES FROM TRICHODERMA VIRIDE ON LOCAL CHARCOAL WOOD SUPPORT

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INTRODUCTION

The renewed worldwide interest in biotechnology and the subsequent necessity for efficient production and application of enzymes in many cases, is associated with the demand for renewable energy sources(1). The bioconversion of cellulose to glucose open routes for the application of microbial enzymatic processes to obtain either organic solvents such as ethanol for energy, or single cell proteins for food. In addition, the conversion of cellulose to simple sugars will help to elleviate the problem of waste disposal and pollution. It is known so far that three enzymes are involved in the degradation of cellulose, namely β-glucosidase(EC 3.2.1.21), endo-β-(1-4)-D-glucanase(EC 3.2.1.4), and exo-β-(1-4)-D-glucanase(EC 3.2.1.91)(2).

The lack of an economical process for saccharification of cellulose by microbial enzymes is one of the most challenging problems still need to be solved. Wilke et al.(3) estimated that the cellulolytic enzymes comprised about 60% of the total cost of the proposed process. Therefore, simple and efficient saccharification process must be developed to minimize the amount and cost of enzyme used. The immobilization of cellulase could make its recovery and reuse possible. If cellulase could be stabilized, recovered and reused, the system would have the economic potential for development into a commercial process(4). Recent developments in enzyme technology have shown that enzymes can be immobilized on an insoluble carrier and still retain significant activity(5). This led to a considerable interest in the use of immobilized enzymes as catalysts in industrial processes and clinical analyses(6,7).

Commonly used carriers derived for enzyme immobilization based on cellulose(8), polyacrylamide(9) or dextran(10) are not ideal for large scale operations in enzymatic reactors because of their low stability and poor resistance to microbial attack. Therefore, the

adsorption of biologically active molecule on relatively inexpensive charcoal is of commercial interest(11). Charcoal is chemically and thermally resistant, and consequently it can not be destroyed by microbial attack.

The present study deals with the immobilization of cellulase complex from Trichoderma viride on local charcoal wood in plug-flow column reactor and optimization of various conditions for saccharification of cellulose.

MATERIALS AND METHODS

Materials

Cellulase type 1V from Trichoderma viride and carboxymethylcellulose(CMC)(sodium salt) low viscosity were obtained from BDH Chemical ltd. Bovine serum albumin and Coomassie Brilliant Blue G-250 were purchased from Fluka AG. All other chemicals were of reagent grade.

Methods

Preparation of support

Fifty grams of charcoal granules(18 mesh) were prepared from local charcoal wood and prior to use was activated in the following manner. It was first treated with dilute HCl (1:1,v/v; 250ml), refluxed for three hours, cooled and filtered. Then,charcoal granules were washed several times with hot distilled water, to remove all traces of hydrochloric acid, and dried at 120°C for 24h.

Enzymatic cellulose hydrolysis in a plug-flow column reactor using immobilized enzyme

The glass column (2.5 x 100cm) equipped with a jacket thermostat and a glass wool supporter at the outlet was used as a reactor. The column contained 10g of activated local charcoal wood (18 mesh) suspended in acetate buffer (0.05M; pH 5.0). At 2-4°C, the enzymes cellulase solution (5 EU,0.02 EU/mg protein) in 0.05 M acetate buffer,

pH 5.0(300ml) was passed through charcoal layer in the column using a preistaltic pump at 6ml/h for 24h. Then, 0.05 M acetate buffer (pH 5.0)(200ml) was fed into the column to remove unadsorbed enzymes. The amount of adsorbed enzymes was determined from the difference between their activities in the initial solution of the cellulase preparation and in the solution of unadsorbed enzymes at the outlet of the column. Carboxymethylcellulose (1%, w/v) as a substrate in 0.05M acetate buffer, pH 5.0 was then fed and the temperature in the column was quickly raised to 40C to start the hydrolysis. The hydrolysate at the reactor outlet was collected and assayed for reducing sugars, protein and cellulase activity.

Enzymatic cellulose hydrolysis using free enzyme

Saccharification of carboxymethylcellulose by free cellulase was done by incubating 20ml of reaction mixture containing 200mg carboxymethylcellulose in 0.05M acetate buffer,pH 5.0, with an average of 15mg of native enzymatic protein at 40°C. Samples of 1.0ml were withdrawn at various time intervals, enzyme activity was terminated by heating in boiling water bath for 10min. and the reducing sugars were determined by the Somogyi-Nelson method(12,13).

Enzyme stability

The storage stability of immobilized cellulase was determined by incubation of enzyme samples in 0.05M acetate buffer,pH 5.0, containing 100µg/ml chloramphenicol at 4°C and 25°C. The activity of the immobilized enzyme was assayed as a function of time.

Cellulase assay

Cellulase was assayed using carboxymethylcellulose as a substrate. The reaction mixture contained 0.5ml 1% caboxymethylcellulose, 0.1ml appropriately diluted enzyme and 1.4ml 0.05M acetate buffer,pH 5.0. After 30min. incubation at 40°C, enzyme activity was terminated by

heating in boiling water bath for 10min. and the reducing sugars were determined by the Somogyi-Nelson method(12,13). Protein was estimated by Bradford method using bovine serum albumine as standard(14).

RESULTS AND DISCUSSION

It is well known that the major products of cellulose hydrolysis were D-glucose and cellobiose, and both inhibited the cellulolytic enzymes. However,the process in a plug-flow column reactor used in this study has some special advantages over a batch reactor(15,16). The cellulose hydrolysis is catalysed by the enzymes adsorbed on charcoal surface and the products are continuously removed from the zone of hydrolysis. The removal of products permits a reduction of their negative inhibitory influence on cellulases, which in turn results in an increased substrate conversion. The cellulase from Trichoderma viride were fed into the reactor from the top of the column and adsorbed on charcoal almost completely(see material and methods). Then, after raising the temperature, the hydrolysis is effected. As can be seen in Figure 1, during the initial stages only small amounts of cellulose were converted. With increasing time, say at t= 24hrs, the reducing sugars at the outlet of the reactor reached constant value.

An attempt has been made to determine the effect of charcoal particle size on activity retention of immobilized cellulase complex. The results in Figure 1 showed that immobilized enzyme on charcoal, 18 mesh,preparation retained 95% of the original activity up to five days of operation on 1% carboxymethylcellulose at 40°C. This was followed by gradual loss of cellulase activity and reached stable value with retention of 80% of the original activity after four months of operation. On the other hand, immobilized enzyme on coarse charcoal showed continous steep decreasing in the original activity with retention of 35% of the cellulase activity after 40 days of operation.

This is may be due to a situation wherein substrate is not freely available to the enzyme owing to diffusional restriction. This is in agreement with that reported by Roy et al.(9) using polyacrylamide gel for cellulase immobilization.

It is interesting to note that in the present study and during operation no significant leaching of protein from the support occured and cellulase activity retained 80% of its initial activity after 120 days of operation. No such long periods of operation have been published for cellulase immobilization. This is not the case for the immobilization of cellulase on cellulose using same technique, plug-flow column reactor. Gusakov et al.(8) have reported that the concentration of saccharification products at the reactor outlet reaches a maximum with time and then decreases(nine hours). This is due to the desorption of cellulase from cellulose surface and their removal from the reactor with eluent.

Storage stability

For successful application in saccharification of cellulose it is important that the immobilized enzyme possesses a high retention of activity as well as good storage stability and long operational stability. Samples of cellulase immobilized on local charcoal wood were stored at 4C and 25C suspended in 0.05M acetate buffer,pH 5.0. Aliquots were withdrawn with time and their activity against carboxymethylcellulose assayed and compared with that measured just after immobilization. It is found that there was no loss of immobilized cellulase activity for three months at either 4C or 25C. No such long periods of storage time have been reported for immobilized cellulase.

Effect of flow rate on the degree of cellulose conversion

It has been reported previously (15,16) that flow rate is one of the most important factors affecting the efficiency of enzymatic

cellulase hydrolysis in a column reactor. Deceleration of the flow rate leads to a higher product concentration in the reactor, resulting in the enhancement of the products inhibitory influence on cellulases, which finally leads to a decrease in substrate conversion and in the effeciency of the process. Various flow rates 6,10,20, 30 and 40ml/h have been tried with plug-flow column reactor in the present study. The highest conversion , 46%, of substrate to reducing sugars at the outlet of the reactor has been achieved at flow rate of 6ml/h. This was followed by 29, 22.5, 18 , and 13.5% of substrate conversion for the flow rates 10 , 20 , 30 and 40ml/h respectively. (Figure 2).

pH activity profiles

Figure 3 shows the pH activity profiles obtained for cellulase immobilized on local charcoal wood as compared with the soluble enzyme. There is a marked difference between the pH profiles of native and immobilized cellulase. The latter are more widely spread, i.e. stable over wide range of pH, with retention of its original activity at pH 4.0 and 5.0. These results are similar to those obtained by Rogalski et al. (4) using cellulase from Aspergillus terreus F-413 immobilized on controlled porosity glasses and Karube et al. (17) using cellulase from Trichoderma viride immobilized on a collagen fibril matrix. In general the pH optima for both immobilized and native enzyme were the same (Figure 3).

Operational stability

The relatively high cellulolytic activity of immobilized preparation is clearly seen by comparison of native and immobilized enzyme hydrolytic activity (Figure 4). This is due to the continous removal of products from the zone of hydrolysis, which in turn results in an increased substrate conversion and reached stable value after 24h of enzymatic hydrolysis. The degree of cellulose saccharification

was 46% . With native enzyme accumulation of hydrolytic products resulted in an negative inhibitory influence on cellulase activity clearly seen in Figure 4. Cellulose saccharification was reached to 16% after 24h of enzymatic hydrolysis followed by continous steep decreasing in the hydrolytic activity.

In conclusion, the conversion of carboxymethylcellulose to reducing sugars by immobilizing cellulase on inexpensive local charcoal wood in plug-flow column reactor, with retention of 80% of the initial activity over considerable long time of operation at 40°C (four months), eliminate the cost of enzymes and represents a very promising approach for saccharification of cellulosic materials.

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Figure 1. Effect of local charcoal wood bead sizes on the retention activity and operation stability of immobilized cellulase. charcoal , 18 mesh (O) and charcoal,coarse (□).

Figure 2. Effect of flow rate on the degree of cellulose conversion by cellulase immobilized on local charcoal wood.

Figure 3. Effect of pH on the activity of cellulase immobilized on local charcoal wood (O) and on free enzyme (△).

Figure 4. Hydrolysis of cellulose by cellulase immobilized on local charcoal wood (O) and by free cellulase (△).

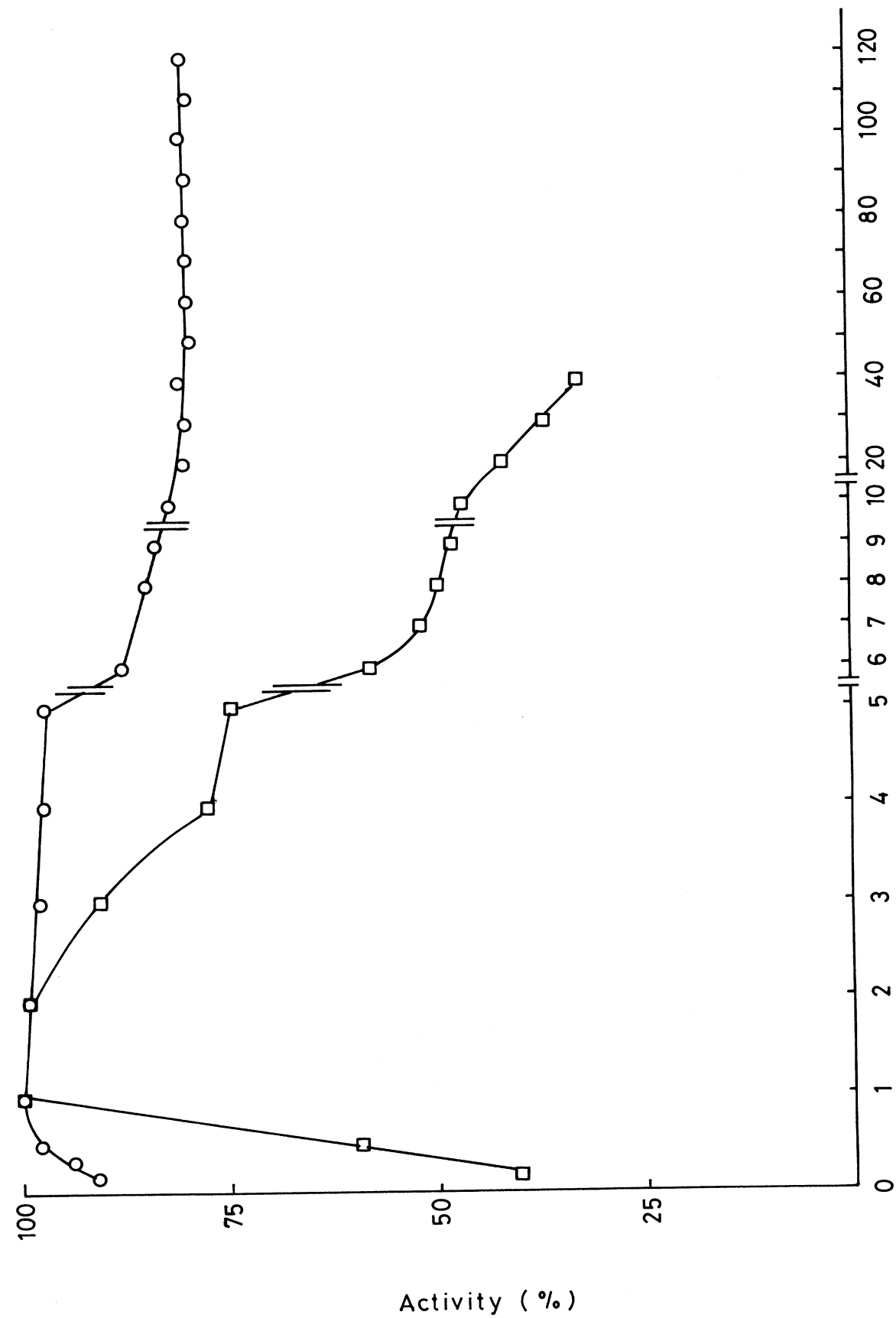
100
75
50
25
Activity (%)
0
1
2
3
4
5
6
7
8
9
10
20
40
60
80
100
120
Time (day)

Figure (1)

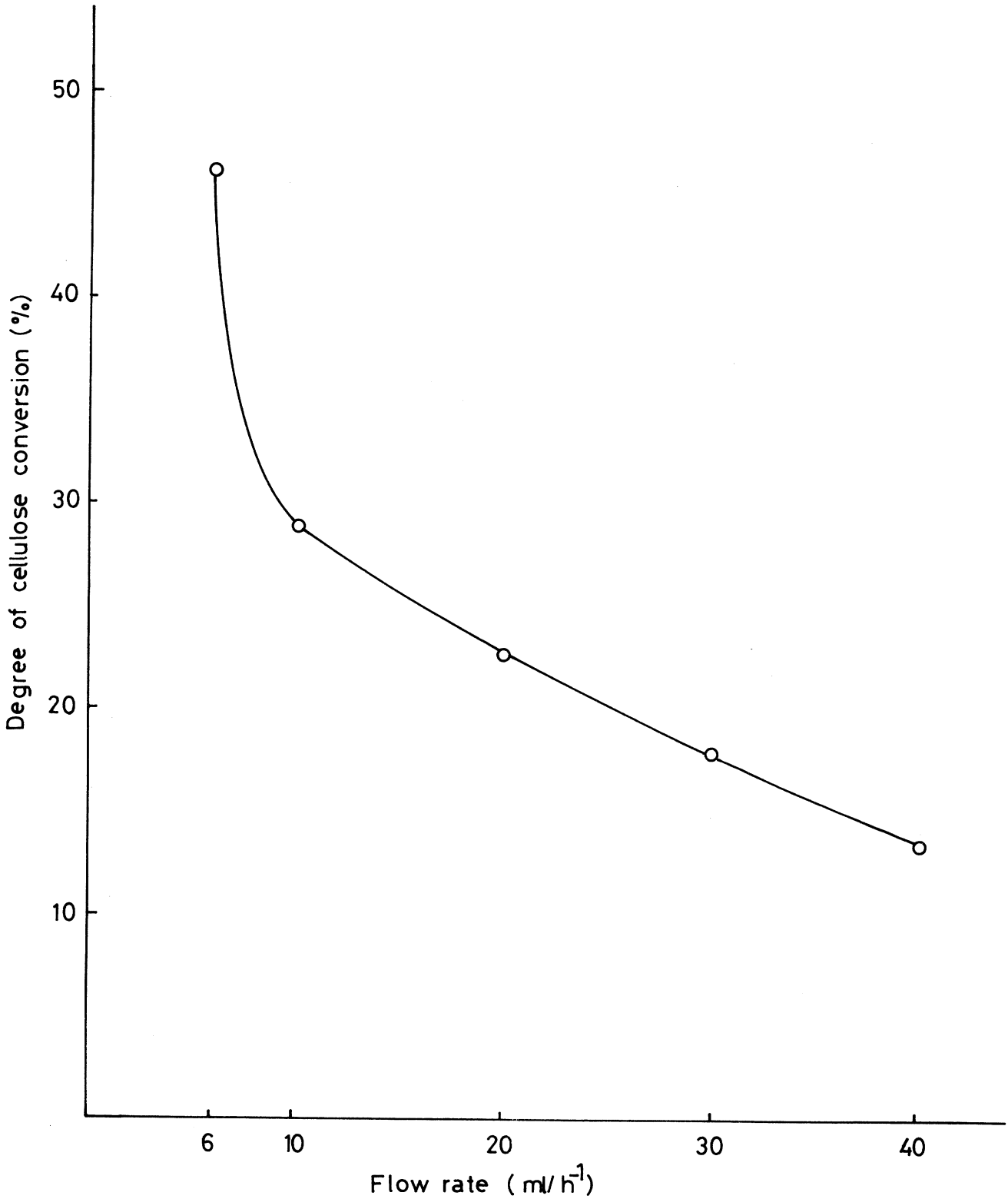
50
40
30
20
10
Degree of cellulose conversion (%)
6
10
20
30
40
Flow rate (ml/ h^{-1})

Figure (2)

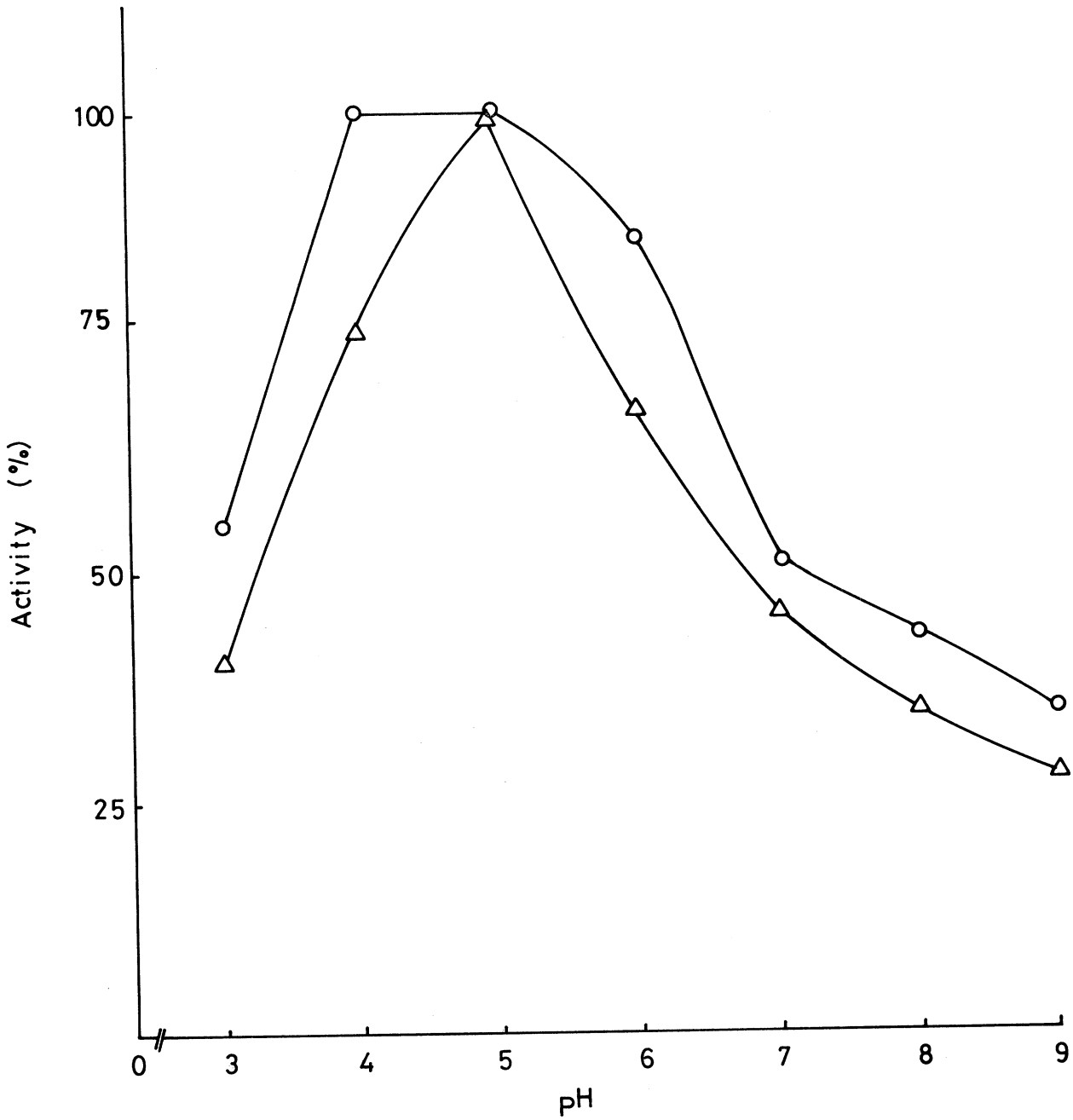

Figure (3)

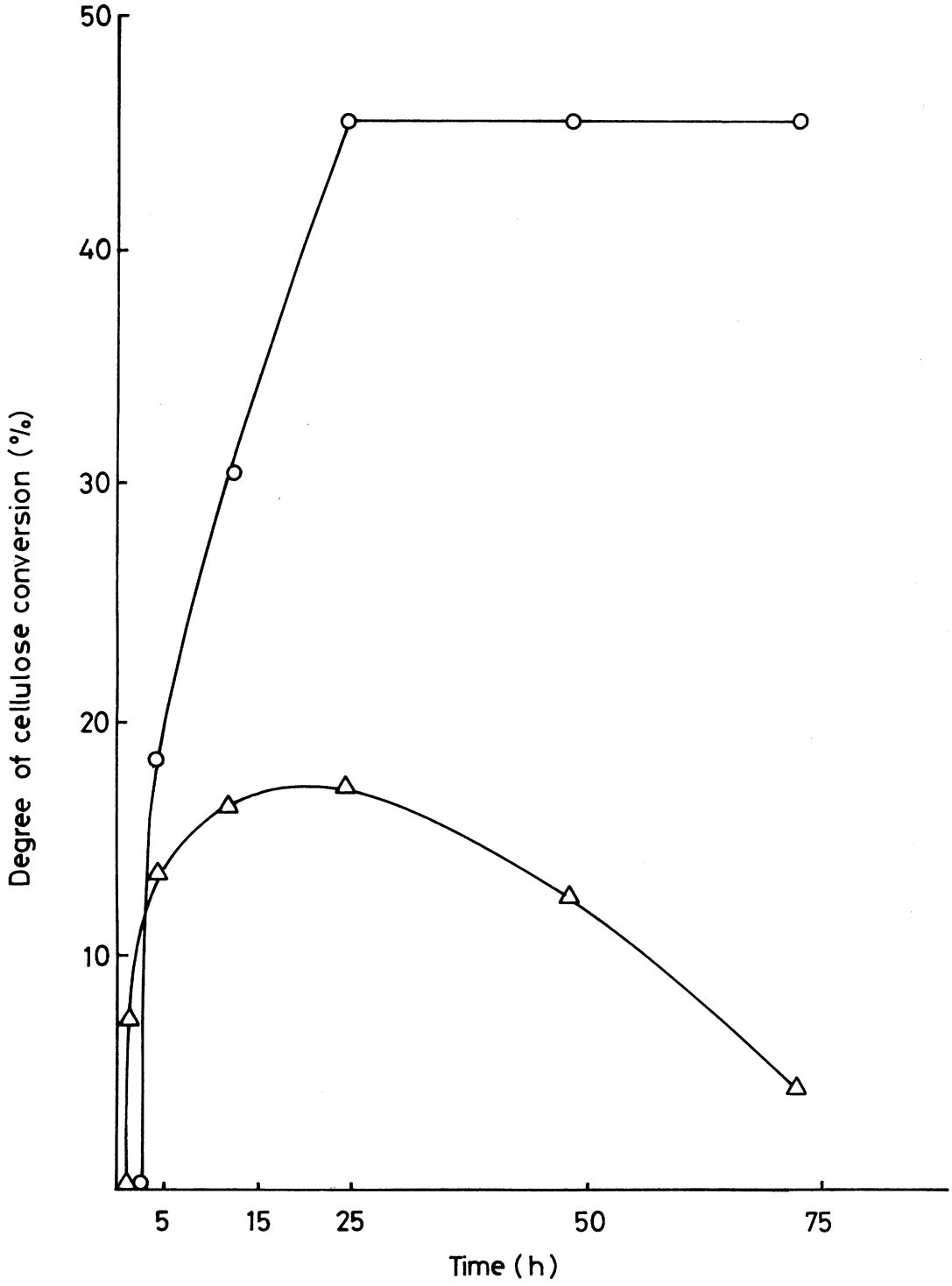
50
40
30
20
10
Degree of cellulose conversion (%)
5
15
25
50
75
Time (h)

Figure (4)