Hydrolysis of Xylan by *Aspergillus niger* Immobilized on Non-woven Fabrics

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*Aspergillus niger*, which produces xylan-degrading enzymes, was immobilized on non-woven fabrics. The maximum xylan hydrolysis activity (15 U/cm²-support) and the highest stability were obtained when the fungus was immobilized on non-woven fabric made of silk. The enzymatic properties of the immobilized preparation were similar to those of the free enzyme. Ten times repeated batch hydrolysis of birch-wood xylan was done over a period of 450 h. Hydrolysis of different xylan substrates such as oat spelt and rice bran by the immobilized mycelia was also investigated.

**Key words:** immobilization; *Aspergillus niger*; xylan; hydrolysis; non-woven fabrics

Xylan is widely distributed in plant cell walls and forms a primary part of the hemicellulose portion. About 20–40% of dry weights of some higher plants and agricultural wastes are composed of xylan. Such materials are potentially good carbon sources and their effective use in enzymatic and microbiological processes is of great interest. Many microorganisms can hydrolyze xylan and many xylanases from various microorganisms have been isolated and characterized. 1–7

From the viewpoint of bioprocess design, it is very important to investigate xylan hydrolysis systems using a bioreactor, and several reports on immobilization of xylanases for xylan hydrolysis8–11 have been published. However, satisfactory results were not been obtained, because of the low reaction rate between immobilized enzymes and water-insoluble xylans. Considering the findings that xylan is water-insoluble and complex in chemical structure, another idea is desired to improve the bioreactor for xylan hydrolysis. As a preliminary experiment, xylan hydrolysis ability of immobilized mycelia from *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma viride* was examined. As a result, it was found that only immobilized *Aspergillus niger* effectively hydrolyzed xylan at an appreciable reaction rate for several repeated operations.

In this paper, we report the immobilization of *Aspergillus niger* and its characterization as a catalyst for xylan hydrolysis.

**Materials and Methods**

*Microorganism and media.* *Aspergillus niger* IFO 6662 was used throughout this study. Medium A used for harvesting the mycelia was composed of (per liter of distilled water): 20 g glucose, 0.12 g MgSO₄·7H₂O, 0.15 g KH₂PO₄, 0.5 g (NH₄)₂HPO₄, 0.2 g peptone (Wako), 4.5 g malt extract (Difco), and 20 g agar. The immobilized mycelia were cultivated in medium B, which contained per liter of distilled water: 15 g peptone, 9 g yeast extract, 9 g malt extract, 3 g corn steep liquor (Sigma), and 20 g birchwood xylan.

*Substrates.* Commercial birchwood xylan (Sigma Chemical Co.), oat spelt xylan (Sigma Chemical Co.), and rice bran xylan (Boso Oil & Fat Co.) were used. These were passed through a sieve and uniform particle sizes of about 180 μm were used.

*Support materials.* The following non-woven fabrics, which are reported to be suitable for mycelia and enzyme immobilization,12 were used as the support materials: A (acrylonitrile), Aₜ (modified acrylonitrile in which 50% of ACN is substituted by COOH), N (nylon), PP (polypropylene), PT (polyester), R (rayon), S (silk). All the non-woven fabrics were obtained from Japan Vilene Co., Ltd.

*Immobilization technique.* Immobilization of mycelia was done by the method described previously.13 A non-woven fabric was cut into a 3 × 3 × 0.15 cm piece and fixed on the surface of a stainless steel wire net (Fig. 1). After autoclaving at 121°C for 10 min, this was soaked for 10 min in medium B containing 10⁻ⁱ⁰⁻¹⁰⁰ spores per ml, then incubated in air in a petri dish at 30°C for 24 h. Once the spores germinated, the mycelia grew rapidly, branched, and entangled itself in the non-woven fabric.

*Hydrolysis of xylan by the immobilized mycelia.* Xylan hydrolysis using the immobilized mycelia was done in a cylindrical glass vessel that had an inner diameter of 6.5 cm. A piece of fabric containing the immobilized mycelia was placed in the reactor with 100 ml of autoclaved xylan suspension (McIlvaine buffer, pH 4.5), and the suspension was agitated slowly by a magnetic stirrer. The xylan suspension was sterilized by autoclaving at 121°C for 10 min. The reaction was done at 30°C.

In the case of repeated hydrolysis of xylan, 100 ml of 1% (w/v) xylan suspension was used. When the concentration of the reducing sugar in the reaction was over 5.5 g/liter, the broth was replaced with a fresh xylan suspension for the next run. Heat sterilization of the reactor was done only for the first batch reaction.

*Enzymatic properties.* The Michaelis constant (K_m) was calculated from a Lineweaver-Burk plot. Thermal and pH stabilities were defined by measuring the residual activity after incubating enzyme for 30 min at various temperatures and pHs.

*Enzyme activity.* Immobilized mycelia (1.0 × 1.0 cm) or 1 ml of the crude enzyme were used for measurement of enzyme activity.

Xylanase, CMCase, and amylase activity were measured by measuring the increase in reducing sugar formed by enzymatic hydrolysis of 1% xylan (McIlvaine buffer, pH 4.5), 2% CMC (acetate buffer, pH 4.5) and 2% starch (phosphate buffer, pH 5.2), respectively. The volumes of the substrate to measure the activities of immobilized mycelia and crude enzyme were 10 ml and 9 ml, respectively. Hydrolysis reactions were done at 45°C, 45°C, and 30°C, respectively. The unit of xylanase activity was a micromole of xylene equivalent produced per min. One unit of CMCase and amylase activity were defined as the amount of enzyme that releases 1 mg of glucose per min from the substrate.

Xylosidase, glucosidase, galactosidase, and arabinosidase activities of the immobilized mycelia were measured at 45°C according to the method of Shinoyama.14 Two ml of M/100 phenyl-β-D-Xyloside, M/100 phenyl-β-D-glucoside, M/100 phenyl-β-D-galactoside, and M/100 phenyl-β-D-
arabinoside (McIlvaine buffer, pH 4.5) were used as the substrate, respectively.

Analysis. Reducing sugar concentration was measured by a modification of Somogyi method.\textsuperscript{13} Xylooligosaccharides were assayed using HPLC (LC-9A, Shimadzu Co., Ltd., Japan) with an RI monitor. A gel filtration chromatography column for HPLC (Shim-pack SCR-101N, Shimadzu Co., Ltd., Japan) was used with a mobile distilled water phase. The weight of mycelia immobilized was measured by drying the mycelia at 80°C to a constant weight.

Results and Discussion
Selection of the most suitable non-woven fabric for immobilization
Selection of proper non-woven fabric in which not only mycelia but also enzyme can be efficiently immobilized is very important to obtain high xylan hydrolysis activity of the immobilized mycelia. The effectiveness of seven non-woven fabrics of various materials, both synthetic to natural fibers, as support materials for mycelia immobilization was investigated. Figure 1(c) and Fig. 2 are microphotographs of mycelia immobilized on non-woven fabric. Mycelia grew inside the non-woven fabric and was entangled with the fiber. There were thick layers of the mycelia near the surfaces of the non-woven fabric, and a vacant space was observed at the center of the non-woven fabric. The void fraction and the water holding capacity of the non-woven fabric\textsuperscript{12} represent how much space the non-woven fabric has for the growth of mycelia and how much medium the non-woven fabric can absorb, respectively. These two parameters can, therefore, be considered as the major factors that affect mycelia growth in the non-woven fabric. And it was assumed

\begin{figure}[h]
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\includegraphics[width=0.8\textwidth]{fig1.jpg}
\caption{Photograph of the Non-woven Fabric Support.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig2.jpg}
\caption{A Cross-section of the Non-woven Fabric Showing the Immobilized Mycelia.}
\end{figure}

Arrows indicate the surface of the non-woven fabric.
that the larger these values were, the higher were the mycelia growth and xylan hydrolysis activity. The highest xylan hydrolysis activities of each immobilized preparation were obtained by cultivating the mycelia for 24 h (Fig. 3), and after that they decreased rapidly. But these results were not obtained in case of amylase of the immobilized Aspergillus oryzae. The causes of the decline of the xylanase activity may be that xylanase was hydrolyzed by a protease produced by the mycelia, and that xylanase was incorporated by the mycelia, but detailed investigations were not done.

During repeated batch xylan hydrolysis by the immobilized mycelia, the xylan hydrolysis activity and the operational stability of mycelia immobilized on silk and rayon fabrics were higher than those immobilized on other fabrics (Fig. 4). These results indicate that the type of fiber material has a significant influence on the xylan hydrolysis activity of the immobilized mycelia. In general, it is said that fibers with functional groups such as \(-\text{NH}_2\) and/or \(-\text{SO}_3\text{H}\) are good for immobilization of enzymes. Since these fibers have neither \(-\text{NH}_2\) nor \(-\text{SO}_3\text{H}\) groups, it is considered that the xylan hydrolyzing enzymes are immobilized on the surface of the non-woven fabric and mycelia by electrostatic or electrodynamic forces, or by physical adsorption. However, these forces seem to be weak, because some of the enzymes released from the immobilized mycelia were detected in the reaction mixture during the xylan hydrolysis. The fact that water-insoluble xylan was successfully hydrolyzed by the immobilized mycelia is considered to depend on the existence of immobilized enzymes and free enzymes in the reaction mixture. On the other hand, the mycelia immobilized on the non-woven fabrics is probably able to produce xylanases during the hydrolysis reaction. These results, that immobilized enzymes, released enzymes, and produced enzymes are closely related to the xylan hydrolysis makes it difficult to discuss the effectiveness of silk and rayon fabrics. To clarify this aspect, further investigation is required.

**Hydrolysis of various xylan**

In general, when the immobilized mycelia are cultivated in a liquid medium, most of the enzymes produced by the mycelia are released into the medium and are, therefore, not immobilized on the support. On the contrary, when the immobilized mycelia are cultivated in air, almost all the enzymes produced are immobilized on the support. In our study, when Aspergillus niger immobilized on non-woven fabric (S) are cultivated in air for 24 h, various enzymes, such as xylanase (8.55 U/cm\(^3\)-support), xylases (2.42 U/cm\(^3\)-support), amylase (0.68 U/cm\(^3\)-support), CMCCase (0.98 U/cm\(^3\)-support), glucosidase (1.46 U/cm\(^3\)-support), arabinosidase (0.50 U/cm\(^3\)-support), and galactosidase (1.02 U/cm\(^3\)-support) were produced. Since the immobilized mycelia showed various enzyme activities, its application for hydrolysis of various kinds of xylans was investigated. As shown in Fig. 5, when 1% (w/v) of birchwood xylan, which is composed of xylene (87% (w/w)) was used as a substrate, xyllose was generated at the beginning of the hydrolysis. However, the amount of xyllose decreased with the reaction time, and the end-product was xylene (percentage of liquefaction (= total liquefied sugar)/(xylene equivalent of the xylan) equals about 80%). On the other hand, oat spelt xylan, which consisted of xylene (77% (w/w)), glucose (15% (w/w)), and arabinose (8% (w/w)) was hydrolyzed to xylose, glucose, arabinose, and xyllose with the liquefaction degree of about 80%. In the case of rice bran xylan, which was made up of glucose (36% (w/w)), xylene (18% (w/w)), and arabinose (18% (w/w)), the degree of hydrolysis was more than 90% and the final product contained glucose, xylose, and arabinose. The differences in the degree of hydrolysis and in the composition of final product depend on the enzyme affinity for each xylan and on the differences in the chemical structure of the xylan.

In any case, these results have shown that the immobilized mycelia can be used for efficient hydrolysis of various xylans.
Enzymatic properties of the immobilized Asp. niger

The non-woven fabric (S) made of silk was used as the support. As shown in Fig. 6, the optimal pH and temperature for this immobilized mycelia were 4.5 and 45°C, respectively. The xylan hydrolysis activity of the immobilized mycelia was stable under 50°C at pH 4-9. When xylan hydrolyzing enzymes were immobilized on the support by covalent binding, the shifts of optimum pH to the acidic range and the increase in the optimal temperature in comparison to those of the free enzymes have been reported. However, in this study, the optimal pH and temperature were similar to those of the free enzyme.

It has also been reported that the $K_m$ and the rate of reaction by the enzymes immobilized by crosslinking are higher and lower, respectively than those of the free enzymes. These are attributed to structural changes in the enzyme caused by the immobilization procedure, diffusional resistance of the matrix, and steric hindrance in the vicinity of the enzyme. In this study, the $K_m$ of the immobilized Asp. niger for birchwood xylan was the same as that of the free enzyme (4.2 mg/ml). This implies that the xylan-hydrolyzing enzymes are immobilized on the surface of both mycelia and fiber by adsorption (no structural changes). The binding strength of the enzymes to the support and mycelia is likely to be weak and thus some of the enzymes were released into the reaction mixture. In such a case, both the released and the immobilized enzymes catalyzed the xylan hydrolysis and consequently no decrease in the reaction rate was observed. These reaction rates are thus the sum of the reaction rates by the immobilized and released enzymes (data not shown).

Rate of xylan hydrolysis

Although 20% of xylan was used as the substrate for free xylanase, only 1 to 2% of xylan is usually used in experiments with immobilized enzyme. There is no report yet on the effects of both the immobilized xylan hydrolyzing enzymes and substrate concentrations on the rate of the xylan hydrolysis. This is partly due to the difficulty of getting enough purified xylan and partly to the low reactivity of the immobilized enzyme for xylan. However, information on the effects of enzyme and substrate concentrations on the reaction rate is very useful for process optimization. In this study, it was observed that the rate of hydrolysis increased as the number of sheets of the immobilized mycelia (amount of enzyme) increased (Fig. 7). Furthermore, with xylan concentrations less than 5%, the reaction rate was proportional to the xylan concentration. At xylan concentrations above 7%, the increase in the reaction rate was not proportional to the increase in the xylan concentration (Fig. 8), while a small quantity of xyloboise was detected in the final product (data not shown).
Repeated xylan hydrolysis by the immobilized mycelia

For practical application, immobilization of enzymes on solid materials offer several advantages, including repeated usage of enzyme, ease of product separation, and improvement of enzyme stability. In this study, the stability of immobilized mycelia was tested by repeated batch hydrolysis of 1% (w/v) birchwood xylan using a cylindrical glass reactor. The result is presented in Fig. 9. It was found that the hydrolysis reaction was successfully repeated until the 10th batch in a period of more than 450 h. As mentioned above, it is considered that some of the immobilized xylan hydrolyzing enzymes are released from the surface of the non-woven fabrics and mycelia and subsequently lost from the reactor system with every replacement of reaction mixture. However, repeated xylan hydrolysis by the immobilized mycelia was possible without a definitive decrease in the hydrolysis rate. This implies that the xylan hydrolyzing enzymes are produced by the immobilized mycelia during the xylan hydrolysis reaction. From these results, effectiveness of immobilized \textit{Aspergillus niger} for water insoluble xylan hydrolysis seems to be attributed to the findings that immobilized enzymes, released enzymes, and produced enzymes take part in a series of the reaction. Further work is needed to measure the abundance ratio of these enzymes and to study this aspect.

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References


\[\text{Fig. 9. Repeated Hydrolysis of Birchwood Xylan by Immobilized} \textit{Aspergillus niger}.\]

One percent (w/v) xylan was used.