

OPTIMIZED PRODUCTION OF XYLANASE FROM FUNGAL STRAINS AND ITS PURIFICATION STRATEGIES

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Abstract: A study to optimize the activity and productivity of xylanase from xylanase-producing strains of fungi *Aspergillus niger* and *Trichoderma reesei* was conducted as well as its purification strategy. In general, solid state fermentation (SSF) in which the ratio of solid wheat bran to water was 1 : 7 (12.5% w/v solid) gave higher enzyme activity than submerged fermentation (1% w/v solid). In all incubation conditions, although cellulase activity was also found in the crude cell extract, its value was much lower than xylanase activity. pH was found to play a very important role in enzyme purification using ion exchange chromatography. CM-Toyopearl, a weak cation exchanger, gave nearly a 100 % adsorption at pH range of 4 to 5, with maximum adsorption at pH 4.5. Using this chromatography column, a 10 times increase of the specific activity of xylanase and a total recovery of 81% were obtained. By SDS-PAGE, the molecular weight was determined to be 27 kDa. No cellulase activity was found in this purified xylanase fraction.

Keywords: Xylanase, fungal strains, optimized production, purification strategy

INTRODUCTION

Process of removing lignin from wood in pulp and paper industry is facing serious problem due to the use of chlorine as a bleaching agent since this compound is very toxic to the environment. Indonesia, one of the largest pulp and paper producing countries, is also facing this problem. An alternative method using xylanase for delignifying lignin is a promising method since, in addition to its environmentally friendly technology, this method is easy to apply which does not require major modification of the existing pulping / bleaching processes.

Production of xylanase has been performed by many researchers as reported in many reviews [1-3]. Xylanases have mainly been produced from fungal and bacterial strains of microorganism. Although bacterial xylanase were reported to have higher value of optimum pH which is beneficial from the view point of applying in biobleaching process, xylanase produced from fungi usually has higher activity [1]. Higher activity of crude enzyme is very important especially when the enzyme is to be applied in its crude form not in pure condition. Type of strains is close related to type of media used for cultivation. Fungal strains usually prefer solid state fermentation media which has low water content. On the contrary, bacterial strains prefer submerged fermentation which has high water content. These media types are one of the most responsible factors for the higher value of crude enzyme activity obtained from fungal strains. Although many reports are available concerning the purification of xylanase, application of xylanase to biobleaching process as well as molecular studies of xylanase, there is no report concerning a stepwise optimization strategy to obtain higher enzymatic activity and productivity from fungal strain of microorganisms in its crude form as well as a stepwise strategy of enzyme purification. All factors responsible for increasing enzyme activity and productivity, especially in its crude form, need be investigated since these are of vital importance from industrial point of view.

The research aims to study the increasing of activity and productivity of xylanase from xylanase-producing strains of microorganism *A. niger* and *T. reesei* as well as study the purification strategy of crude enzyme. Two different fungal strains were selected to make a comparison of the optimization strategy. Several important parameters such as the use and type of inducers, concentration of surfactant, water content of medium, and incubation time were investigated and the results were thoroughly discussed in the perception of obtaining the highest activity and productivity of xylanase in its crude form.

MATERIALS AND METHODS

Microorganisms

Strain of *A. niger* was obtained from Department of Biology, University of Brawijaya, Malang, while strain of *T. reesei* was obtained from Department of Biology, Airlangga University, Surabaya, Indonesia. The strains were maintained on sterilized potato dextrose agar (PDA) slants under room temperature, with subculturing every 3 weeks. PDA slants can be altered by using the following media: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.2 g L^{-1}), $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.4 g L^{-1}), $\text{MnSO}_4 \cdot 5 \text{H}_2\text{O}$ (0.3 g L^{-1}), yeast extract (2 g L^{-1}), and agar powder ($1.5\% \text{ w/v}$). Both can be used to maintain the strains. An actively growing fungal colony (4-5 d old colony in agar slants) in two cm^2 agar block was used to inoculate the growth medium. By using hemocytometer, the number of spores in this two cm^2 agar block was in the range of $1 \times 10^7 - 1 \times 10^8$ spores for both *A. niger* and *T. reesei*.

Substrates

Wheat bran was used as the substrate in all experiments. It was a gift from PT Bogasari Flour, Tbk. Indonesia and Okumoto Flour Milling Co. Ltd., Osaka, Japan. This dried substrate was pulverized to about 40 mesh in size prior to use in the culture medium.

Experimental Condition for Enzyme production

Moistening agent for solid substrate fermentation and liquid medium for submerged fermentation contained the following components: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.2 g L^{-1}), $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.4 g L^{-1}), $\text{MnSO}_4 \cdot 5 \text{H}_2\text{O}$ (0.3 g L^{-1}), yeast extract (2 g L^{-1}),

Tween 80 (0, 0.1, 0.5% and 1%), and inducers (0, 0.5% and 1%). Birchwood xylan (Sigma Aldrich, USA) or xylose was used as the inducer. The pH was adjusted to 5.5 by adding 1 N NaOH. Erlenmeyer flask (500 mL) containing 5 g of wheat bran was moistened by adding moistening agent under substrate to moistening agent ratio (w/v) of 1:7 (\pm 12.5% solid), then autoclaved at 120°C for 15 min. After cooling they were inoculated with the strains and incubated at 35°C for *A. niger* and 30°C for *T. reesei* under no shaking.

Enzyme extraction

Extraction of extracellular enzyme was conducted after specified incubation time. For SSF, 100 mL of 50 mmol L⁻¹ sodium acetate buffer, pH 5.5 containing 0.1 % Tween-80 was added to the flask and the incubation was continued for 2 h under continuous shaking of 150 rpm. The suspended slurry was centrifuged at 8,000 rpm for 30 min, and after filtration using Whatman no. 2 filter paper, the clear supernatant was used in enzyme assay. For submerged fermentation, the enzyme extraction was performed by directly centrifuging the culture without adding any buffer. The rest of the procedure was the same as for SSF.

Enzyme Assay

Xylanase assay was conducted by dinitrosalicylic acid (DNS) method as a modification of the method by Gawande and Kamat [4] with xylose as the standard. One gram of birchwood xylan was dissolved with stirring in 100 mL of 50 mM sodium acetate buffer, pH 5.5 at 60°C, boiled for several minutes and continued stirring for 3 h at room temperature. To 1.8 mL of this xylan solution, 200 μ L of enzyme solution was added and incubated at 35°C for 10 min. 3 mL DNS solution (a mixing of 16 g NaOH, 10 g DNS, 300 g sodium potassium tartrate and 8 g sodium metabisulfite in 1 L water) was added to stop the reaction, and the solution was boiled for exactly 5 min and then cooled down rapidly using ice bath until room temperature. Xylose produced was followed by measuring the absorbance at 540 nm. Since xylan or xylose added as inducers during incubation was present in the enzyme solution to be assayed that will obviously affect the measurement, a blank for each sample in which no reaction take place (by immediately stopping the reaction after adding enzyme solution) was carefully conducted in order to obtain accurate data. One unit of xylanase activity was defined as the amount of enzyme that produces 1 μ mol xylose per minute under the assay condition.

Cellulase activity was measured using DNS method with glucose as the standard. The condition was similar to that of xylanase assay except that 1 % carboxymethyl cellulose (Nacalai, Osaka, Japan) replaced xylan as the substrate. One unit of cellulase activity was defined as the amount of enzyme that produces 1 μ mol glucose per minute under the assay condition.

The total extracellular protein concentration was measured by the Bradford method [5] with bovine serum albumin (BSA) as standard.

Purification by Ammonium Sulfate Precipitation

Solid ammonium sulfate was added to the culture supernatant to 80% saturation. The resulting precipitate was collected by centrifugation at 16,000 rpm for 30 min at 4°C. The precipitate was redissolved in a 50 mM sodium acetate buffer (pH 5.5).

Purification by acetone precipitation

Prechilled acetone was added to the culture supernatant at one to one ratio or other (v/v) in an ice-salt bath with stirring. The resulting precipitate was collected by centrifugation at 16,000

rpm for 30 min at 4°C. The precipitate was dissolved in a 50 mM sodium acetate buffer (pH 5.5) and dialyzed against the same buffer prior to enzyme assay.

Purification by ethanol precipitation

Prechilled ethanol was added to the culture supernatant at one to one ratio (v/v) or other in an ice-salt bath with stirring. The resulting precipitate was collected by centrifugation at 16,000 rpm for 30 min at 4°C. The precipitate was dissolved in a 50 mM sodium acetate buffer (pH 5.5) and dialyzed against the same buffer prior to enzyme assay.

Dialysis

Dialysis was performed using a cellophane tubing having a cut-off molecular weight of 14,000 Da (Wako size no. 8). The buffer was altered three times (after 2, 14 and 19 h) to ensure removal of ammonium sulfate which may interfere separation during ion exchange chromatography.

Purification by Ion Exchange Chromatography

To find the best pH condition as well as type of column, purification by ion exchange chromatography was first conducted batchwisely in 1.5 mL microtube. This microtube functioned as a small chromatography column of DEAE-Toyopearl and Carboxymethyl (CM)-Toyopearl.

Larger volume of purification was conducted using a 2.8 × 23 cm CM-Toyopearl column preequilibrated with 10 mM sodium acetate buffer (pH 4.5) and eluted with a linear gradient of 0 – 1 M NaCl (600 mL) in the same buffer.

Electrophoresis and zymogram

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 15% acrylamide gels by the procedure developed by Laemmli [6]. The gels were run at a constant current of 15 mA for approx. 2 h. Duplicate samples were run for simultaneous protein (by Coomassie brilliant blue R-250) and activity staining (by 0.25% Congo red).

RESULTS AND DISCUSSION

Effect of incubation time

Effect of incubation time on xylanase activity from *A. niger* and *T. reesei* in SSF are shown in Figure 1 and 2, respectively. All data in this figure and all other figures are the average of triplicate experiments. For *A. niger*, it can be seen from Figure 1 that xylanase activity increased significantly from 2 to 3 days of incubation and increased slightly at 4 d. Although cellulase activity, which is an undesired enzyme for application in pulp and paper industry, also increased during this period of time, its activity was much lower than xylanase. The xylanase to cellulase ratio also increased from 3.1 to around 6 as the incubation time increased from 2 to 4 d. By considering the activity of xylanase and its ratio to cellulase activity, 4 d can be considered the best incubation time.

Figure 2 and 3 show the effect of incubation time on xylanase activity from *T. reesei* using xylan and xylose as an inducer, respectively. Both figures show that activity of xylanase increased significantly from 4 to 6 d of incubation and increased slightly after 6d. Activity of cellulase also increased during this period of time and the ratio of the xylanase activity to the cellulase activity was practically the same during the incubation period. Based on the increase of

xylanase activity as well as ratio of xylanase to cellulase activity, 7d was considered to be the best incubation time for *T. reesei* whether xylan or xylose was used as the inducer.

Productivity of xylanase can be calculated as the amount or the activity of xylanase produced per unit volume per unit time. From Fig. 1, 2 and 3, their values could be derived and the results are given as bar charts in each figures. For *A. niger*, productivity increased as the incubation time increased, but at the 4th day when the activity of xylanase increase only slightly, the productivity began to decrease. 3rd day gave the highest productivity of xylanase from *A. niger*. Similar results were found for xylanase from *T. reesei*. At first, the productivity of xylanase increase as the incubation time increase up to 6th day. But at the 7th day, when the increase in xylanase activity was not so high compare to increase of the previous days, the productivity decreased. 6 d gave the highest productivity of xylanase from *T. reesei* whether xylan or xylose was used as the inducer.

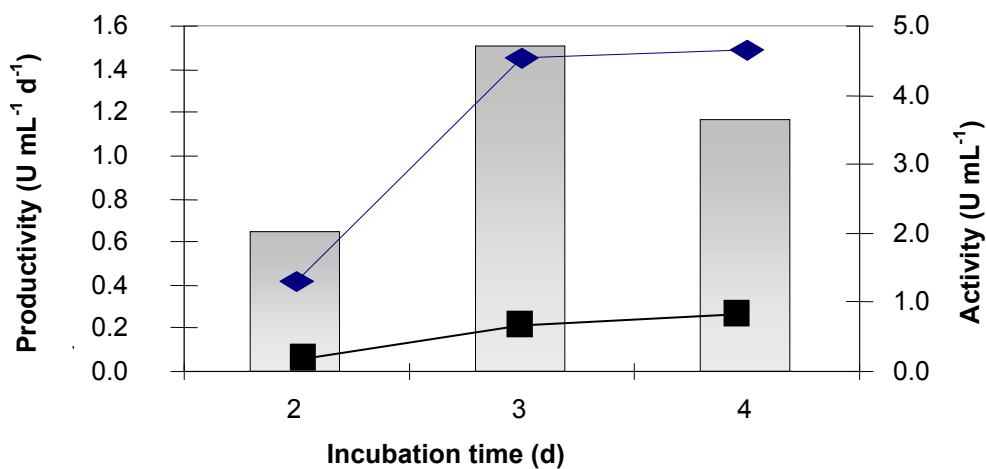


Fig 1: Effect of incubation time on the activity of xylanase (◆) and cellulase (■) from *A. niger* using SSF medium. The corresponding productivities of xylanase are shown using bar chart.

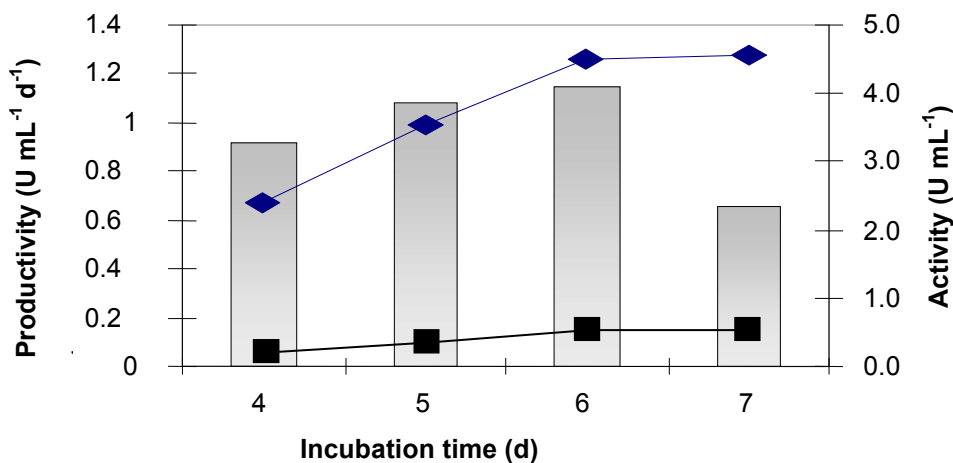


Fig 2: Effect of incubation time on the activity of xylanase (◆) and cellulase (■) from *T. reesei* using xylan as inducer in SSF medium. The corresponding productivities of xylanase are shown using bar chart.

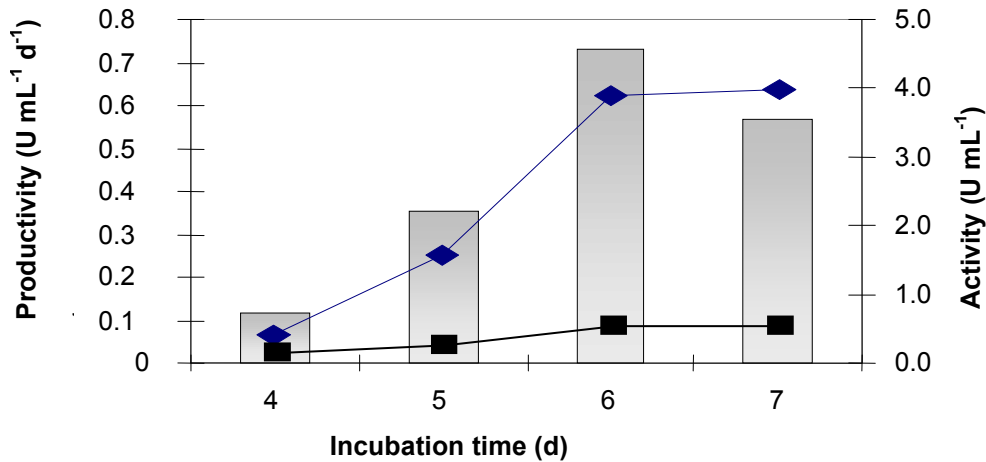


Fig 3: Effect of incubation time on the activity of xylanase (◆) and cellulase (■) from *T. reesei* using xylose as inducer. The corresponding productivities of xylanase are shown using bar chart.

Effect of water content

Submerged fermentation vs. SSF. Comparison of the experimental results of the xylanase activity in submerged fermentation and SSF was given in Figure 4 for xylanase from *A. niger* and in Figure 5 for xylanase from *T. reesei*. As explained in the materials and method, the ratio of solid wheat bran to water in submerged fermentation was 1 : 100, while under SSF the ratio was 1 : 7. As shown in Figure 4, xylanase activity from *A. niger* under SSF was much higher than that under submerged fermentation whether or not inducers were used in the cultivation media. For *T. reesei*, as shown in Figure 5, both condition of water content gave no significant difference when no inducer was used in the medium. However, when xylan or xylose was used as an inducer, xylanase activity in SSF was much higher than the activity in submerged fermentation. The above figures showed that xylanase activity obtained under SSF were generally much higher than that under submerged fermentation. Both fungi preferred solid media than media with too much water for their growth.

Effect of inducers type

Figure 4 also shows the effects of type of inducers on xylanase activity from *A. niger*. Figure 4 (a) showed that the highest xylanase activity in submerged fermentation was obtained using no inducers. Xylan and xylose gave undesired results in which xylanase activity decreased upon adding these chemicals to the media. Furthermore, xylan was found to induce cellulase activity more than xylanase which is also an unexpected result for the application in pulp and paper industry. From Figure 4 (b) it was found that both chemicals gave no significant effect to the activity of xylanase from *A. niger* grown under SSF media.

Effects of type of inducers on xylanase activity from *T. reesei* are shown in Figure 5. Similar to the results of xylanase activity from *A. niger* shown in Fig. 4 (a), Fig. 5 (a) showed that the highest xylanase activities were obtained using no inducers when submerged fermentation media was used. However, as shown in Fig. 5 (b), both xylan and xylose induced xylanase activity from *T. reesei* significantly when the media was SSF. Xylanase activity increased by more than 3 times

than activity without adding any inducers. Although cellulase activities were also found in the crude cell extract, their value were much lower than xylanase activity.

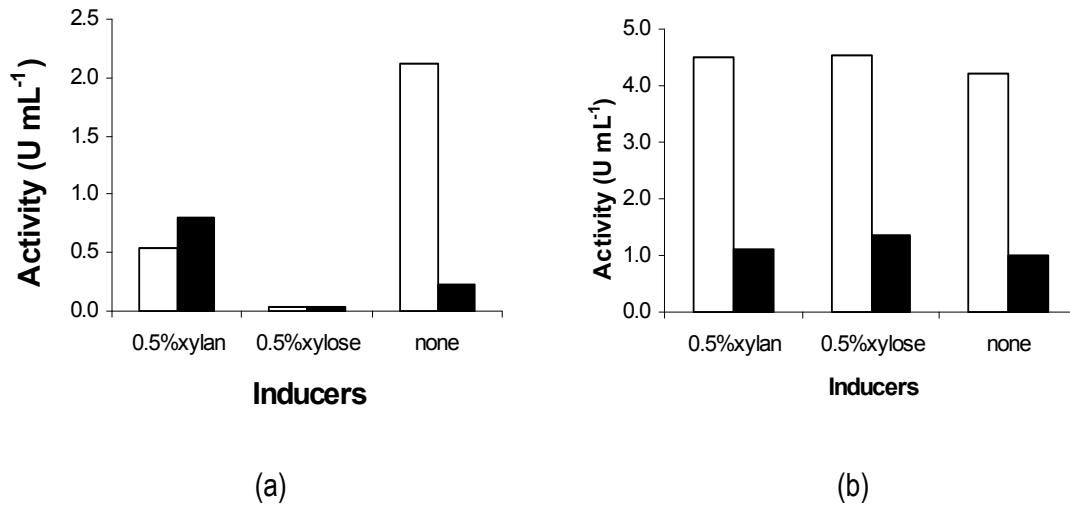


Fig. 4: Effect of different type of inducers on xylanase (□) and cellulase (■) activity from *A. niger* in (a) submerged fermentation and (b) SSF.

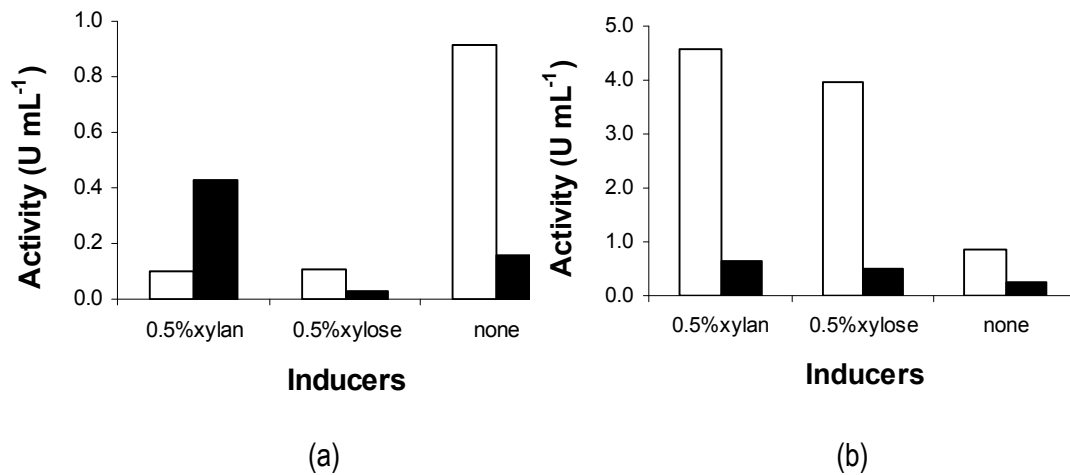


Fig. 5: Effect of different type of inducers on xylanase (□) and cellulase (■) activity from *T. reesei* in (a) submerged fermentation and (b) SSF.

Effect of inducers concentration on xylanase activity from *T. reesei* in SSF media

Figure 6 (a) and 6 (b) show the effect of increasing the concentration of xylan and xylose, respectively, on the activity of xylanase from *T. reesei* grown in SSF media. Both figures show

that xylanase activity increased by increasing the concentration of xylan or xylose. The ratio of xylanase to cellulase also increased slightly by increasing the concentration of inducers.

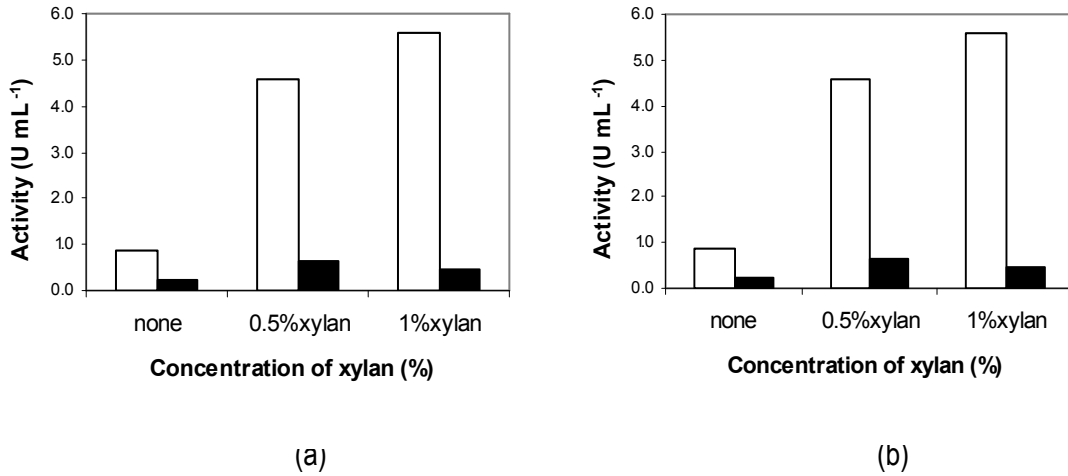


Fig. 6: Effect of (a) xylan and (b) xylose concentration on xylanase (□) and cellulase (■) activity from *T. reesei* in SSF.

Effect of Tween-80 on the activity of xylanase

Effect of Tween-80 on xylanase activity from *A. niger* and *T. reesei* in SSF are given in Figure 7 and 8, respectively. As shown in Fig. 7, Tween-80 gave no significant effect on increasing the xylanase activity from *A. niger*. Higher concentration of Tween-80 gave even a bad effect in which xylanase activity decreased significantly. For *T. reesei*, Fig. 8 clearly shows that any concentration of Tween-80 decreased xylanase activity and its bad effect was more severe in higher Tween-80 concentration. This result was different to some results reported so far [7-10]. In their reports, Tween-80 of up to 1.5% induced xylanase activity quite significantly.

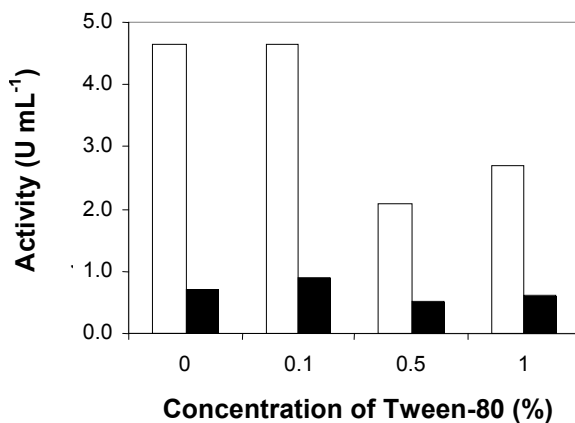


Fig. 7: Effect of Tween-80 concentration in medium on xylanase (□) and cellulase (■) activity from *A. niger* in SSF.

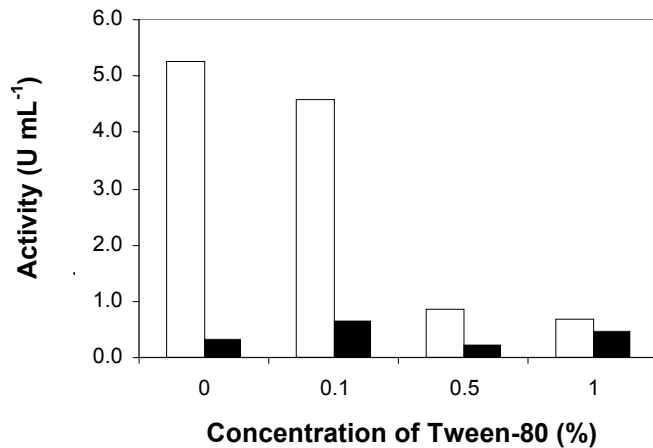


Fig. 8: Effect of Tween-80 concentration in medium on xylanase (□) and cellulase (■) activity from *T. reesei* in SSF using 0.5% xylan as inducer.

Best culture conditions

From the results of the study of the effect of several important parameters responsible for obtaining high xylanase activity as given above, best culture conditions were obtained for both fungi. Using 1% xylan as the inducer and without adding Tween-80, the highest xylanase activity of about 7 U/mL was obtained from *T. reesei*. Higher concentration of xylan may give even better result, but considering the price of xylan, higher xylan concentration may be economically insufficient for xylanase production especially when larger production scale is to be considered. For comparison, using much cheaper inducer, xylose under 1% concentration, also gave very high xylanase activity of more than 6 U/mL. The highest xylanase activity from *A. niger* of 4.7 U/mL was obtained under no inducer and no Tween-80 in the medium. Although the highest xylanase activity from *A. niger* was less than that obtained from *T. reesei*, since this best condition was obtained without the necessity of adding any inducers, production of xylanase from *A. niger* is still interesting from the view point of the whole economical consideration of xylanase production.

Purification by precipitation

The next experiment was conducted to purify the crude enzyme having the highest activity obtained from the above procedure. Since *T. reesei* gave higher activity as well as higher xylanase to cellulase ratio, crude xylanase from *T. reesei* was chosen as the model for conducting a stepwise purification strategy. By ammonium sulfate precipitation method, specific activity increased upon adding 80% ammonium sulfate and increased further after dialyzed. Recovery of 87% was obtained as shown in Table 1. Precipitation by acetone and ethanol were also conducted but both methods gave worse results in comparison to precipitation by ammonium sulfate (data not shown).

Purification by ion exchange chromatography

Figure 9 shows the effect of pH on the % adsorption using DEAE-Toyopearl, a weak anion-exchange resin. As shown in this figure, the highest adsorption using DEAE-Toyopearl was only

30 % obtained at pH 5. The amount of enzyme adsorbed under higher and lower pH values than this optimal value tended to decrease.

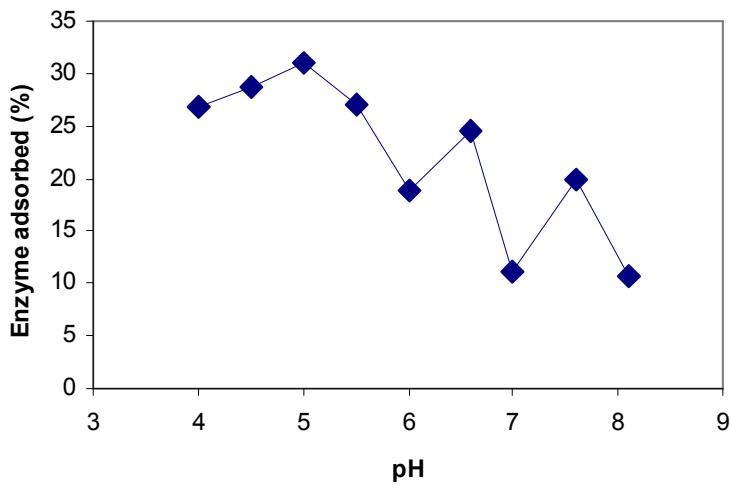


Fig. 9: Effect of pH on degree of adsorption of xylanase on DEAE-Toyopearl column chromatography conducted batchwisely in a microtube.

To clarify the phenomena in Figure 9, experiments to see the pH optimum and pH stability were conducted and the results are shown in Figure 10 and 11, respectively. Both Figures 10 and 11 show that the enzyme was active and stable under pH range of 4 – 8.1. Therefore, it is clear that the decrease in adsorption in Fig. 9 was not due to inactivation of enzyme but it was due to less enzyme adsorbed onto DEAE-Toyopearl resin.

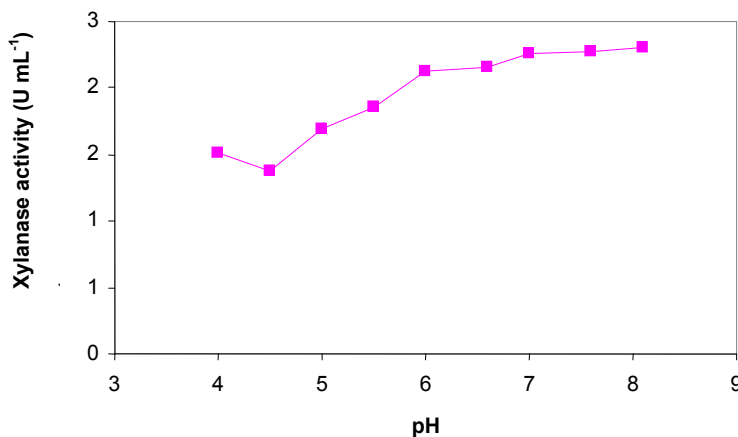


Fig. 10: pH optimum for xylanase

The next experiment was conducting a batchwise adsorption using Carboxymethyl (CM)-Toyopearl, a weak cation-exchange resin, and the results are shown in Figure 12. At a pH range of 4 to 5, a nearly 100 % adsorption was obtained using this cation exchanger. Higher pH values gave less xylanase adsorption.

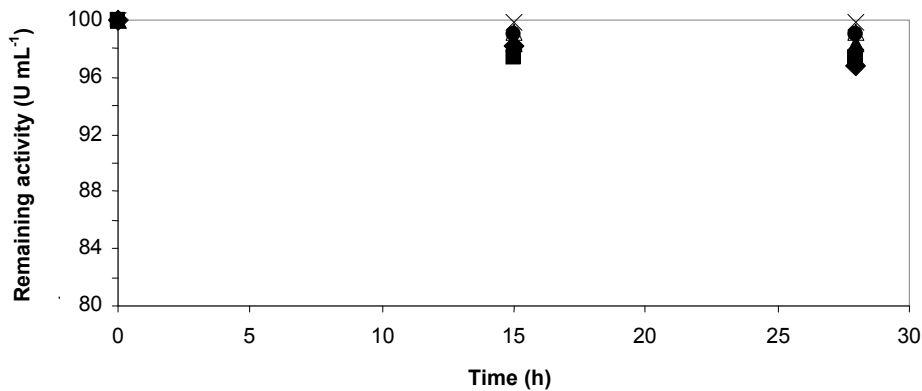


Fig. 11: Enzyme stability experiments conducted at 30°C under various pH values of (◆) 4, (◇) 4.5, (▲) 5, (△) 5.5, (■) 6, (□) 6.6, (●) 7, (○) 7.6, and (×) 8.1.

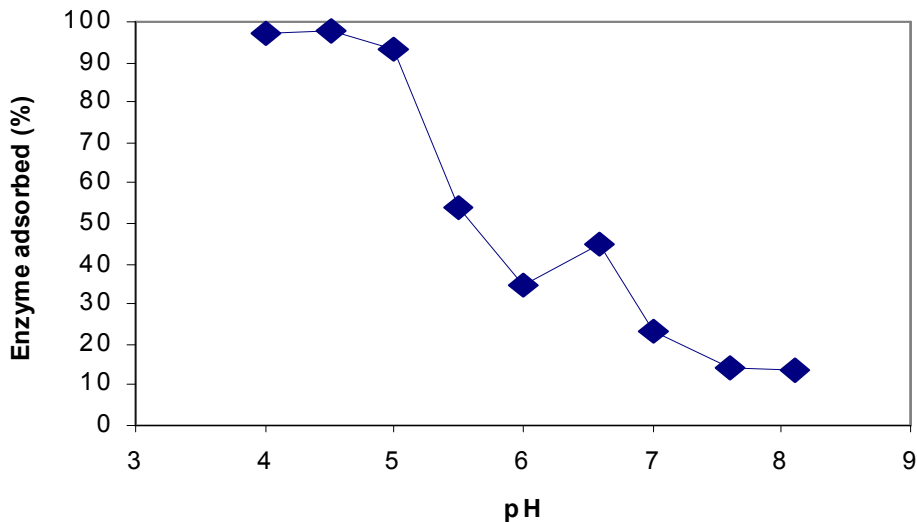


Fig. 12: Effect of pH on degree of adsorption of xylanase on CM-Toyopearl column chromatography conducted batchwisely in a microtube.

From Fig. 10, optimum pH of the enzyme was around 8. The pI of the enzyme is usually around its optimum pH. Thus by applying buffer with pH less than pI, the enzyme will function as a basic protein or a positively charged protein. This could be the reason why cation exchanger exhibited a much superior adsorption of xylanase at low pH value.

The results of further experiment using a 2.8×23 cm CM-Toyopearl at pH 4.5 are shown in Figure 13. This method gave nearly a 100 % xylanase recovery relative to xylanase activity applied to the column. Results of all purification steps are shown in Table 1. It can be shown from the table that crude xylanase was purified 10 times from a specific activity of 3.5 to 36 U mg⁻¹. The table also shows that the activity of the purified xylanase was reduced by less than 20 % of the crude enzyme activity, i.e. from 93 to 75 U. This resulted in a high purification yield of 81 %.

Table 1: Purification steps of xylanase from *T. reesei*

Purification steps	Xylanase activity (U)	Cellulase activity (U)	Proteins (mg)	Specific activity of xylanase (U mg ⁻¹)	Purification fold	Yield
Crude xylanase	93	9.1	26	3.5	1	100
(NH ₄) ₂ SO ₄ precipitation	81	7.6	10	8.1	2	87
CM-Toyopearl	75	0	2	36	10	81

Result of SDS-PAGE is shown in Figure 14. The molecular weight of purified xylanase was determined to be 27 kDa. No cellulase activity was found in this purified xylanase fraction. This is a very important point from the view point of applying this to industry as well as in conducting further experiment which requires high degree of purification of xylanase.

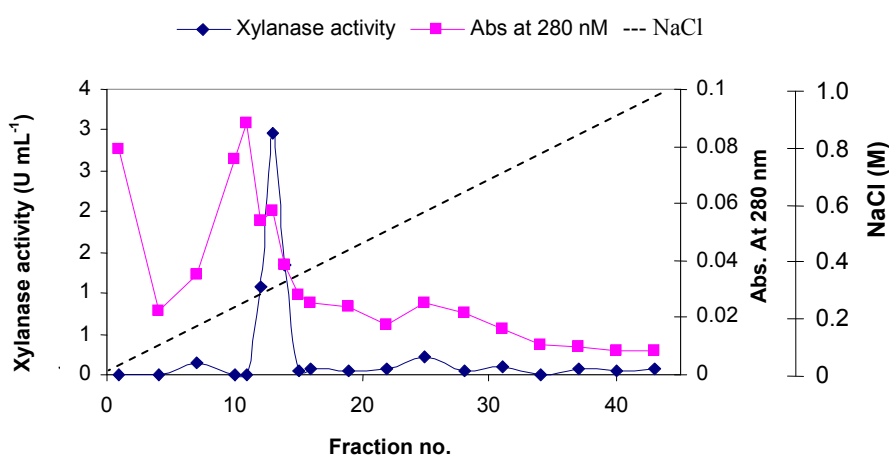


Fig. 13: Results of CM-Toyopearl ion exchange chromatography

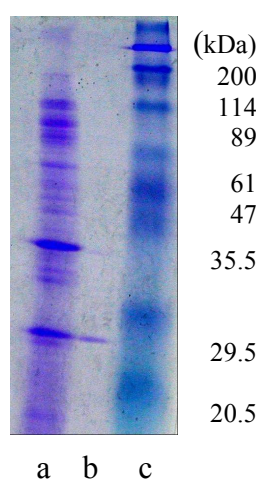


Fig. 14: SDS-PAGE analysis of (a) crude and (b) purified xylanase performed on 15% acrylamide, (c) is protein marker.

CONCLUSION

Based on the increase of xylanase activity and the ratio of xylanase to cellulase activity, 4 d and 7 d were considered the best incubation time for *A. niger* and *T. reesei*, respectively. However, 3 d and 6 d gave the highest productivity of xylanase from *A. niger* and *T. reesei*, respectively. Xylanase activity from *A. niger* under solid state fermentation (SSF) was much higher than that under submerged fermentation. For *T. reesei*, by using no inducers, both conditions gave no significant difference. Under submerged fermentation, the highest xylanase activity of 2.0 U mL⁻¹ and 0.9 U mL⁻¹ from *A. niger* and *T. reesei*, respectively, was obtained. Both strains gave the highest activity using no inducers if cultivated under submerged fermentation. For SSF, it was found that both xylan and xylose gave no significant effect to the activity of xylanase from *A. niger*. However, these two chemicals induced xylanase activity from *T. reesei* significantly, in which xylan induced xylanase activity better than xylose. Xylanase activity increased by more than 3 times than activity without adding any inducers. Increasing xylan or xylose concentration increased xylanase activity. In all incubation conditions, although cellulase activity was also found in the crude cell extract, its value was much lower than xylanase activity. This is a good result from the view point of industrial application of the enzyme in pulp and paper industries. *T. reesei* gave higher xylanase to cellulase ratio than *A. niger*. The highest xylanase activity was around 7.0 U mL⁻¹ obtained from *T. reesei* using 1% xylan. This corresponded to a productivity of 1200 U L⁻¹ d⁻¹. Using xylose as the inducer, the highest xylanase activity was about 6.2 U mL⁻¹ obtained at 1% xylose corresponded to a productivity of 1000 U L⁻¹ d⁻¹. The highest xylanase activity from *A. niger* was 4.7 U mL⁻¹ obtained by using no inducers. This corresponded to a productivity of 1570 U L⁻¹ d⁻¹. In general the activity obtained from *T. reesei* was higher but due to the shorter incubation time, *A. niger* gave higher productivity.

Purification of crude xylanase was conducted by ammonium sulfate precipitation followed by ion exchange chromatography. It was found that the highest adsorption using DEAE-Toyopearl, a weak anion exchanger, was only 30 % obtained at pH 5, while CM-Toyopearl, a weak cation exchanger, gave nearly 100 % adsorption at pH range of 4 to 5, with maximum adsorption at pH 4.5. The optimum pH was around 8 and the enzyme was stable at pH range of 4 - 8.1 and almost no loss of activity was found until 28 h of incubation at 30°C under this wide pH range. From the experiment using a 2.8 × 23 cm CM-Toyopearl column at pH 4.5, an increase in the specific activity from 3.5 U mg⁻¹ to 36 U mg⁻¹ and a total recovery of 81% was obtained. By SDS-PAGE, the molecular weight was determined to be 27 kDa. No cellulase activity was found in this purified xylanase fraction.

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