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The Production of Xylanase and β-Xylosidase by *Aspergillus niger* NRC 107

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Aspergillus niger NRC 107에서의 Xylanase와 β-Xylosidase의 생산

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Abstract — The production of xylanase and β-xylosidase was investigated in submerged culture of *Aspergillus niger* NRC 107. The maximum production occurred when the pH was controlled at 6.0 during the fermentation. Among the various carbon sources investigated, corn-cob xylan (1.5%, w/v) yielded maximal production of the enzymes. The $NaNO_3$ was the most favorable nitrogen source for enzyme production and KH_2PO_4 concentration at 0.3%(w/v) was found to be optimum. Incorporation of wheat bran to the culture medium improved xylanase production. Addition of L(−) sorbose to the culture medium promoted the secretion of β-xylosidase. It was possible to increase the production of xylanase (39.43 units/m*l*) and that of β-xylosidase (4.2 units/m*l*) by submerged culturing the *A. niger* NRC 107 in the modified medium.

Hemicellulose is one of the major components of lignocellulosic materials comprising 15 to 35% of agricultural and forestry residues. Xylans (β-1,4-D-xylose polymer) are the major components of hemicellulose (1). Extensive degradation of xylans to monosaccharides is achieved by cooperative action of muti-enzymes; endo-β-xylanase, exo-β-xylanase and β-xylosidase (2). In general, endo-β-xylanase (usually called xylanase, E.C. 3.2.1.8) randomly cleaves the 1,4-β-xylosidic linkages in xylans and exo-β-xylanase and β-glucosidase hydrolyze the 1,4-β-D-xylans or xylooligosaccharides so as to remove succesive D-xylose residue from the non-reducing termini. β-Xylosidase (E.C. 3.2.1.37) prefer to short xylooligosaccharides. Many microbial sources have been reported to produce these enzymes (3-8).

The cost of production and low yield of these enzymes are the major problems for practical application (9). Therefore, the availability of highly active xylanase would be of great significance. This requires the selection of suitable strain and the development of fermentation technology for producing the enzymes in quantity.

This study was undertaken to investigate some factors affecting the production of xylanases and β-xylosidase by a local isolate *Aspergillus niger* NRC 107. From a certain comparison made with other fungi, it is apparant that this culture produces large amount of xylanases. In addition, *A. niger* NRC 107 secretes high amounts of β-xylosidase.

Key words: Hemicellulose, xylanase, β-xylosidase, xylanase fermentation
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Materials and Methods

Microbes and Materials

The microorganisms utilized in the present study were obtained from the fungal collection of the Centre of Cultures of National Research Centre, Cairo, Egypt. They were maintained on potao dextrose agar slants at 25℃ and subcultured twice a month.

Oat splet xylan was obtained from Fluka, Switzerland and *p*-nitrophenol-β-D-xylosidase was purchased from Sigma Chemical Co., MO (U.S.A). Corncob xylan and wheat straw xylan were prepared by the method of Whistler *et al.* (10).

Media and culture conditions

The basal salt medium for the growth of the fungal and the enzyme production was that of Mandels and Stenrnberg (11). The medium contained in each liter: KH_2PO_4, 2.0g; $(NH_4)_2SO_4$, 1.4g; $MgSO_4 \cdot 7H_2O$, 0.3g; $CaCl_2$, 0.3g; urea, 0.3g; Tween-80, 1 m*l*; $FeSO_4 \cdot 7H_2O$, 5.0 mg; $MnSO_4 \cdot H_2O$, 1.6 mg; $ZnSO_4 \cdot 7H_2O$, 1.4 mg; $CoCl_2$, 2.0 mg; and corn-cob xylan, 10g. The pH was adjusted to 5.0. The addition of different substrates to the medium is indicated in each experiment. Cultivation was made in 250 m*l* Erlenmeyer flasks each containing 50 m*l* of sterilized media. One milliliter of spore suspension (8×10^7 spores/m*l*) obtained from 7-day mother culture was used for inoculation. Cultivation was performed at 30℃ on a rotary shaker (180 r.p.m.). The cultures were harvested on the 7th day of growth by filtration through glass wool filter and then centrifuged. The clear supernatants were used for enzyme assays.

The pH control was done by adding either 1 N NaOH or 1 N HCl every 12 hr.

Determination of enzyme activities

Xylanase activity in the culture filtrate was determined from the amount of the reducing sugars formed in terms of xylose according to the method of Somogyi (12). The half milliliter of appropriately diluted culture filtrate was added to 0.5 m*l* of 1.0% (w/v) xylan in 0.05 M phosphate buffer (pH 5.0). The reaction mixture was incubated at 45℃ for 30 min. One unit (U) of xylanase activity was defined as the amount of enzyme liberating one mole of reducing sugars as a xylose per min.

β-Xylosidase activity was also assayed by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl-β-D-xyloside (PNPX) (13). The assay mixture consisted of 0.5 m*l* of 0.1 M phosphate buffer, pH 5.0, 0.5 m*l* of 0.05 M PNPX and 1 m*l* of enzyme solution. The reaction mixture was incubated at 40℃ for 15 min, then 2 m*l* of 1M Na_2CO_3 were added to stop the reaction. The color developed was read at 400 nm with the spectrophotometer. One unit (U) of β-xylosidase activity was defined as the amount of enzyme that produced one mole of *p*-nitrophenol per min.

Protein content of mycelium

The protein content in the mycelium was determined indirectly by estimation of Kjeldahl nitrogen of total solids (14). With determined nitrogen content (N), mycelium protein was calculated as N× 6.25. Soluble protein in culture filtrates was determined by the method of Lowry *et al.* (15). This was sometimes in this paper used as an index of cell growth.

The results shown represent the means of at least three separately undertaken experiments.

Results and Discussion

Screening of some fungal strains for the production of extracellular enzymes

All tested fungi were grown on the basal medium containing corn-cob xylan. The culture filtrates were investigated for extracellular xylanase and β-xylosidase activities on the 7th day of growth (Table 1). *Aspergillus niger* NRC 107 was found to be the most potent fungus for xylanase and β-xylosidase production, followed by *A. oryzae* NRC 13. On the other hand, *Fusarium oxysporum* 3A was comparatively the lowest xylanase producer. Therefore, *A. niger* NRC 107 was selected for further works.

Optimization of *A. niger* NRC 107 culture conditions for xylanase and β-xylosidase

Effect of pH of culture medium: The pH of culture medium was maintained at pH 3.0, 4.0, 5.0, 6.0 and 7.0 through the fermentation process (Fig. 1). Maximal enzyme production was detected at pH 6.0. These results coincide with those reported for xylanase and β-xylosidase production by *A. tereus* IJIRA (16) and *A. terreus* 603 (6). At pH 6.0, xylanase activity was 4.5-fold that at pH 3.0. The most significant effect of low pH was demonstrated by the complete inhibition of β-xylosidase at pH 3.0. The low enz-

Table 1. Survey of fungal cultures for the production of xylanase and β-xylosidase

Microrganisms	Final pH of C.F.*	Protein content of		Enzyme activities (unit/m*l*)	
		mycelium (mg/50 m*l* culture)	C.F. (mg/m*l*)	Xylanase	β-Xylosidase
Aspergillus niger NRC107	7.5	70.3	0.65	6.84	0.54
Aspergillus oryzae NRC13	8.5	84.3	0.54	4.64	0.19
Aspergillus saitoi B22	7.0	60.3	0.49	1.29	0.03
Fusarium oxysporum 3A	7.4	43.2	0.66	0.39	0.00
Penicilium citrinum PY30	6.8	90.3	0.55	2.1	0.12
Penicilium funiculosum WD7	7.6	36.3	0.44	0.88	0.04
Trichoderma viride 250	6.5	55.3	0.74	2.57	0.07
Trichoderma harzianum 27	6.8	64.3	0.39	1.84	0.00

*C.F. is the abbreviation of culture filtrate. This is the same as in this and following Tables and Figures.

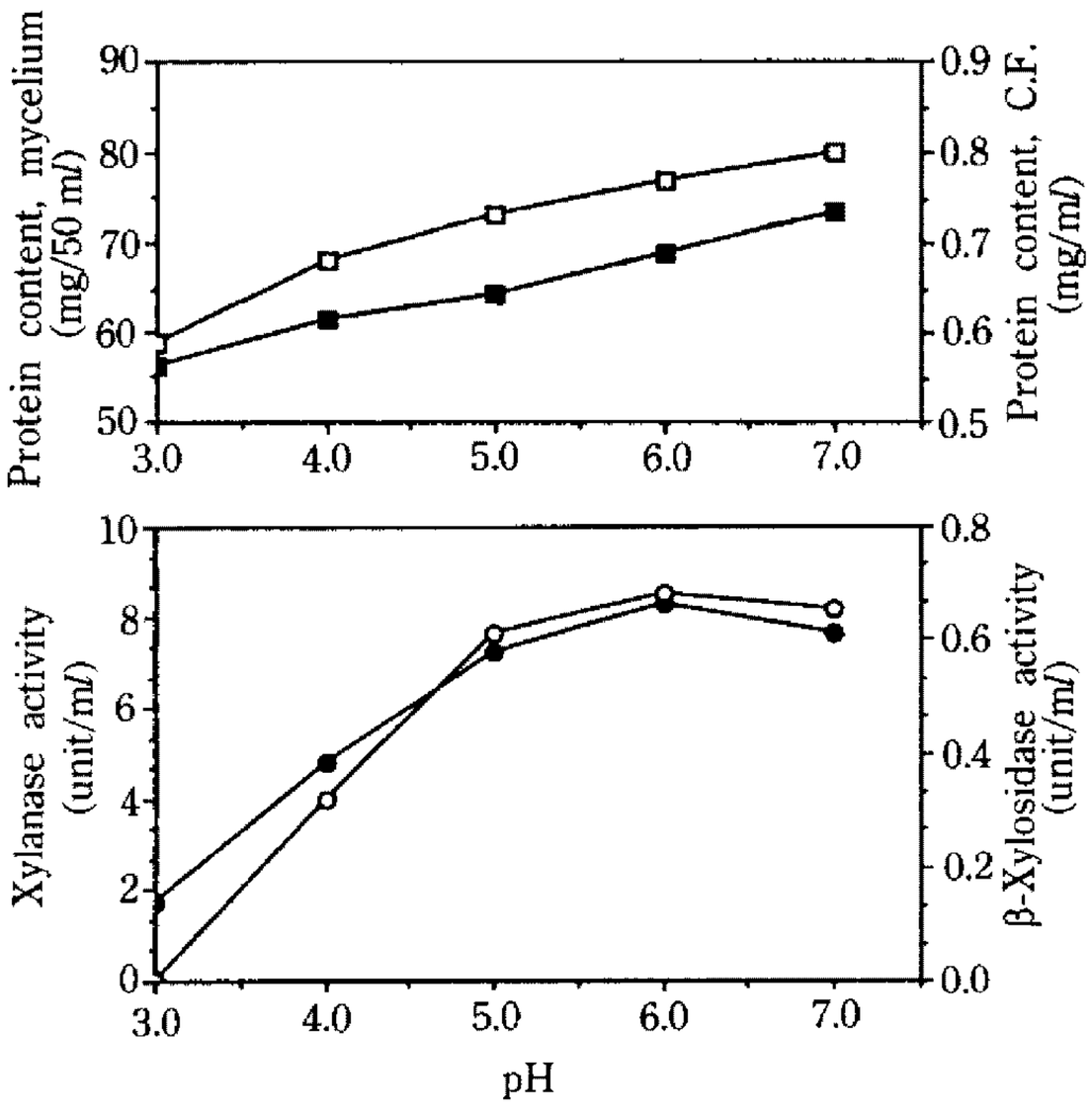

Fig. 1. Effect of pH on the production of xylanase and β-xylosidase by *A. niger* NRC 107.
Protein content of mycelium (■), protein content of culture filtrate (□), xylanase (●), and β-xylosidase (○). Standard medium: corn-cob xylan, 10 g/*l*; $(NH_4)_2SO_4$, 1.4 g/*l*.

yme activity at pH 3.0 may at least partially be due to inactivation of the enzyme at this pH rather than to inhibition of enzyme biosynthesis (17). This view may explain the production of appreciable amounts of extracellular protein at pH 3.0 and the large increase of enzyme yield with limited increase of extracellular protein and mycelial biomass at higher pH's.

Table 2. Effect of different carbon sources on the production of xylanase and β-xylosidase by *A. niger* NRC 107 at pH 6.0

Carbon source*	Enzyme activities(unit/m*l*)	
	Xylanase	β-Xylosidase
Control	8.24	0.68
Cellulose	1.14	0.00
Starch	5.94	0.22
Maltose	3.14	0.06
Sucrose	2.44	0.03
Lactose	6.24	0.39
Glucose	3.69	0.00
Xylose	3.29	0.00
Arabinose	1.89	0.04

*Carbon levels of each samples were kept constant equivalent to the amount of the control: Control; 10 g/*l* corn-cob xylan. Nitrogen levels were fixed to $(NH_4)_2SO_4$, 1.4 g/*l* + urea, 0.3 g/*l*.

Effect of carbon sources: The production of xylanase and β-xylosidase was compared when *A. niger* NRC 107 was grown on the basal media containing different carbon sources (Table 2). The carbon level was kept constant (equivalent to 1.0% corn-cob xylan). The results showed that xylan served as a superior carbon source for xylanase and β-xylosidase production by *A. niger* NRC 107. This results were in agreement with those reported for xylanase and β-xylosidase from *Trichoderma viride* (18), *A.*

terreus (16), *Talaromyces byssochlamydoides* (19) and *T. lignorum* (20). On the other hand, feeble production of xylanase and β-xylosidase were obtained with glucose, maltose, xylose and lactose. The utilization of these sugars in the culture medium indicated good cell growth (data not shown). These results were in accordance with those of reported data(3, 7, 20, 21). Many investigators found a considerable yield of xylanase and β-xylosidase on using a culture medium containing cellulose as a unique carbon source (16, 20, 22). In A. niger NRC 107, however, a feeble yield (15% of that with xylan) of enzyme was observed upon using cellulose as a sole carbon source. This may be partially due to poor biomass production, perhaps because of the lack of cellulase to break down the cellulose to soluble sugars. In fact, protein content of mycelium with cellulose (22.13 mg/50 m*l* culture) was about 30% of that with xylan (68.94 mg/50m*l* culture).

In further studies on the effect of carbon source on enzyme production, different xylan substrates at various concentrations were investigated for production of xylanase and β-xylosidase from *A. niger* NRC 107 (Table 3). Of the substrates investigated corn-cob xylan was the most favorable for enzyme production. Maximal enzyme yield was observed at 15 g/*l* corn-cob xylan.

Effect of nitrogen sources: On equivalent nitrogen basis, the nitrogen sources from the basal medium ($(NH_4)_2SO_4$ + urea) were substituted by several kinds of nitrogen sources (Table 4). Of the nit-

Table 4. Effect of different nitrogen sources on the production of xylanase and β-xylosidase by *A. niger* NRC 107 at pH 6.0.

Nitrogren source*	Enzyme activities(unit/m*l*)	
	Xylanase	β-Xylosidase
Control	12.2	1.07
$NaNO_3$	17.8	1.85
$(NH_4)Cl$	11.3	1.88
$(NH_4)NO_3$	10.9	1.6
$(NH_4)_2SO_4$	13.9	1.03
Urea	7.2	0.44
Meat extract	2.8	0.06
Peptone	4.7	0.74
Yeast extract	3.9	0.54
Casein hydrolyzate	2.9	0.37

*Nitrogen levels of each samples were kept constant equivalent to the amount of the control: Control; $(NH_4)_2SO_4$, 1.4 g/*l* + urea, 0.3 g/*l*. Carbon source (corn-cob xylan) levels of each samples were fixed to concentration: 15 g/*l*, which was optimized from the Table 3.

Table 3. Effect of various xylan substrates on the production of xylanase and β-xylosidase by *A. niger* NRC 107 at pH 6.0*.

Xylan substrates	Xylan concentration (g/*l*)	Protein content of		Enzyme activities (unit/m*l*)	
		mycelium (mg/50 m*l* culture)	C.F. (mg/m*l*)	Xylanase	β-Xylosidase
Corn-cob	5	60.3	0.68	4.39	0.45
	10	68.9	0.77	8.24	0.68
	15	82.2	1.05	12.24	1.07
	20	94.7	1.24	10.24	0.84
Wheat straw	5	53.2	0.74	3.69	0.39
	10	60.8	0.79	7.74	0.7
	15	71.2	0.84	9.28	0.84
	20	78.5	0.93	8.88	0.9
Oat splet	5	66.4	0.82	6.69	0.44
	10	73.2	0.91	10.33	0.63
	15	88.2	0.97	9.24	0.71
	20	94.3	1.11	8.21	0.71

*Nitrogen levels of each samples were kept constant equivalent to the amount of the control: $(NH_4)_2SO_4$, 1.4 g/*l* + urea, 0.3 g/*l*.

rogen sources investigated $NaNO_3$ was the most favorable for the production of active xylanase and β-xylosidase from *A. niger* NRC 107. This result was similar to that reproted by Ismail *et al.* (6). Ammonium compounds such as NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl showed moderate levels of xylanase. On the other hand, organic nitrogen sources (meat extract, peptone, yeast extract, casein hydrolyzate and urea) were relatively unsuitable for xylanase and β-xylosidase production from *A. niger* NRC 107. These result was in contrast to that reported by Ghash and Kundu (16).

Effect of phosphate level: The effect of phosphate level on the production of xylanase and β-xylosidase was investigated using different concentrations of KH_2PO_4 (Fig. 2a). The results showed that KH_2PO_4 concentration at 0.3%(w/v) was the most favorable concentration for the production of both extracellular enzymes. Higher levels had an adverse effect on xylanase production.

Effect of surfactants: The addition of surfactants was found to increase extracellular enzyme activity for a variety of microorganisms (23). The effect of surfactants on production of xylanase and β-xylosidase from *A. niger* NRC 107 was investigated using different concentration of Tween-80 (Fig. 2b). As shown in the results, the increase of Tween-80 concentration from 0.1% to 0.3% led to activity increase in xylanase (by 16.5%) and β-xylosidase (by 13.0%) relative to control. Higher concentrations, However, had an adverse effect on the enzyme production. These results were consistent with those of Szczodrak *et al.* (24). On the other hand, Ghash and Kundu (16) found no significant effect of Tween-80 and Triton X-100 on xylanase and β-xylosidase production *A. terreus*.

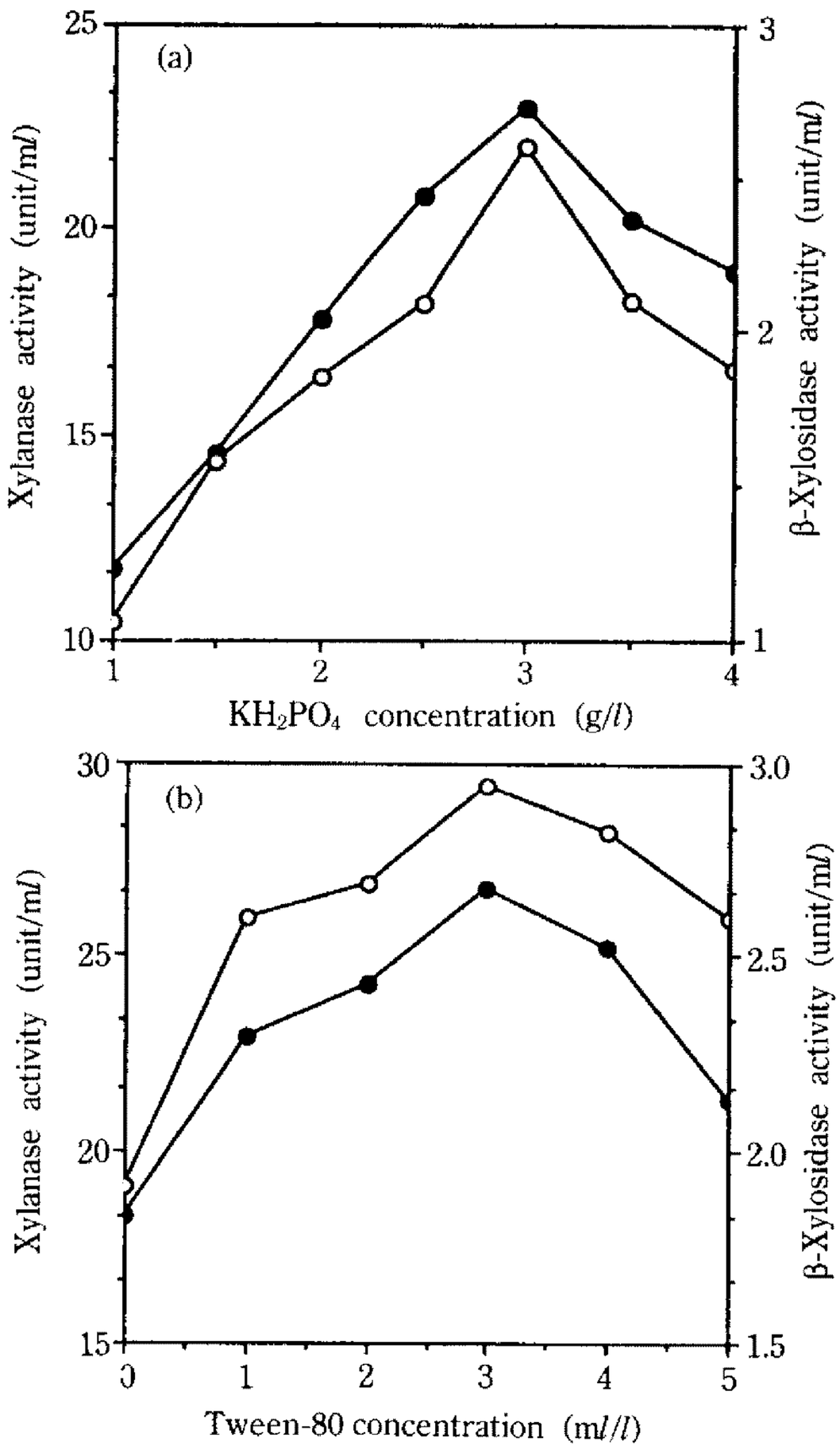

Fig. 2. Effect of phosphate (a) and Tween-80 (b) concentration on the production of xylanase and β-xylosidase by *A. niger* NRC 107 at pH 6.0.
Standard medium: Corn-cob xylan, 15 g/*l*; $NaNO_3$, 2.67 g/*l* was used. The concentration of Tween-80 in the phosphate effect testing (Fig. 2a) and the concentration of KH_2PO_4 in Fig. 2b were 1 ml/*l*, and 3.0 g/*l*, respectively. Symbols: xylanase (●), and β-xylosidase (○).

Effect of addition of organic substrates: The effect of various organic substrates and inducers on the production of xylanase and β-xylosidase from *A. niger* NRC 107 was investigated as shown in Table 5. Corn steep had an adverse effect on xylanase production. On the orther hand, remarkable increase (74%) in β-xylosidase was shown by L(−) sorbose at 0.3%(w/v). Bisaria *et al.* (25) found that L(−) sorbose at 0.5%(w/v) improved β-glucosidase yield from *Trichoderma reesei* QM 9414. Table 5 shows that rice bran at 1.0%(w/v) provided a slight increase in xylanase activity while wheat bran was the best for xylanase. Thus addition of 1.5%(w/v) wheat bran to the culture medium resulted in 55.3 % and 36.0% increase in xylanase and β-xylosidase. This result coincide with those reported by other investigators (18, 19, 26 and 27). The highest yield of xylanase (39.43 unit/ml) and a high one β-xylosidase (4.2 units/ml) were achieved upon addition of both wheat bran (at 1.5%, w/v) which is the best

Table 5. Effect of some organic substrates on the production of xylanase and β-xylosidase by *A. niger* NRC 107 at pH 6.0

Organic substrates	Substrate concentration (g/*l*)	Protein content of		Enzyme activites (unit/m*l*)	
		mycelium (mg/50 m*l* culture)	C.F. (mg/m*l*)	Xylanase	β-Xylosidase
None*	0	98.7	1.39	26.7	2.94
Corn-steep	0.5	156.7	1.89	20.4	2.2
	1.0	192.5	2.56	18.7	2.08
	1.5	214.3	2.56	18.7	1.89
Rice bran	5	110.3	1.58	25.8	2.7
	10	139.7	1.69	27.7	2.78
	15	156.8	1.84	26.6	2.16
Wheat bran	10	102.3	1.47	34.6	3.14
	15	118.9	1.66	38.7	3.54
	20	134.3	1.73	37.7	3.5
L(−) sorbose	1	109.3	1.42	28.9	3.41
	3	121.3	1.54	29.6	4.52
	5	133.7	1.63	26.3	4.31
Wheat bran+	15				
L(−) sorbose	3	144.7	1.72	39.4	4.2

*For the experimentals, major medium composition of control is as follows: corn-cob xylan, 15 g/*l*; $NaNO_3$, 2.67 g/*l*; KH_2PO_4, 3.0 g/*l*, Tween-80, 0.3 (%,v/v)

for xylanase production, and L(−) sorbose (at 0.3%, w/v) which is the best for β-xylosidase (Table 5).

Production of xylanase and β-xylosidase in optimized medium

According to the above mentioned results a medium was formulated taking into consideration the most favorable conditions reached. The each liter of medium consisted of: corn-cob xylan, 15g; $NaNO_3$, 2.67g; KH_2PO_4, 3.0g; $CaCl_2$, 0.3g; $MgSO_4 \cdot 7H_2O$, 0.3 g; wheat bran, 15g; L(−) sorbose, 3.0g; Tween 80, 3 m*l*; $FeSO_4 \cdot 7H_2O$, 5.0 mg; $MnSO_4 \cdot H_2O$, 1.6 mg; $ZnSO_4 \cdot H_2O$, 1.4 mg; and $CoCl_2$, 2.0 mg. The pH was controlled through the fermentation processes of 7 days at pH 6.0.

With this optimized media, the course of production of xylanase, β-xylosidase, extracellular protein and mycelial biomass were investigated as shown in Fig. 4. After inoculation there was a lag of 36 hr before extracellular protein and enzymes reached detectable levels. Extracellular xylanase appeared earlier on the second day of growth. The appearance of β-xylosidase activity was lagged app-

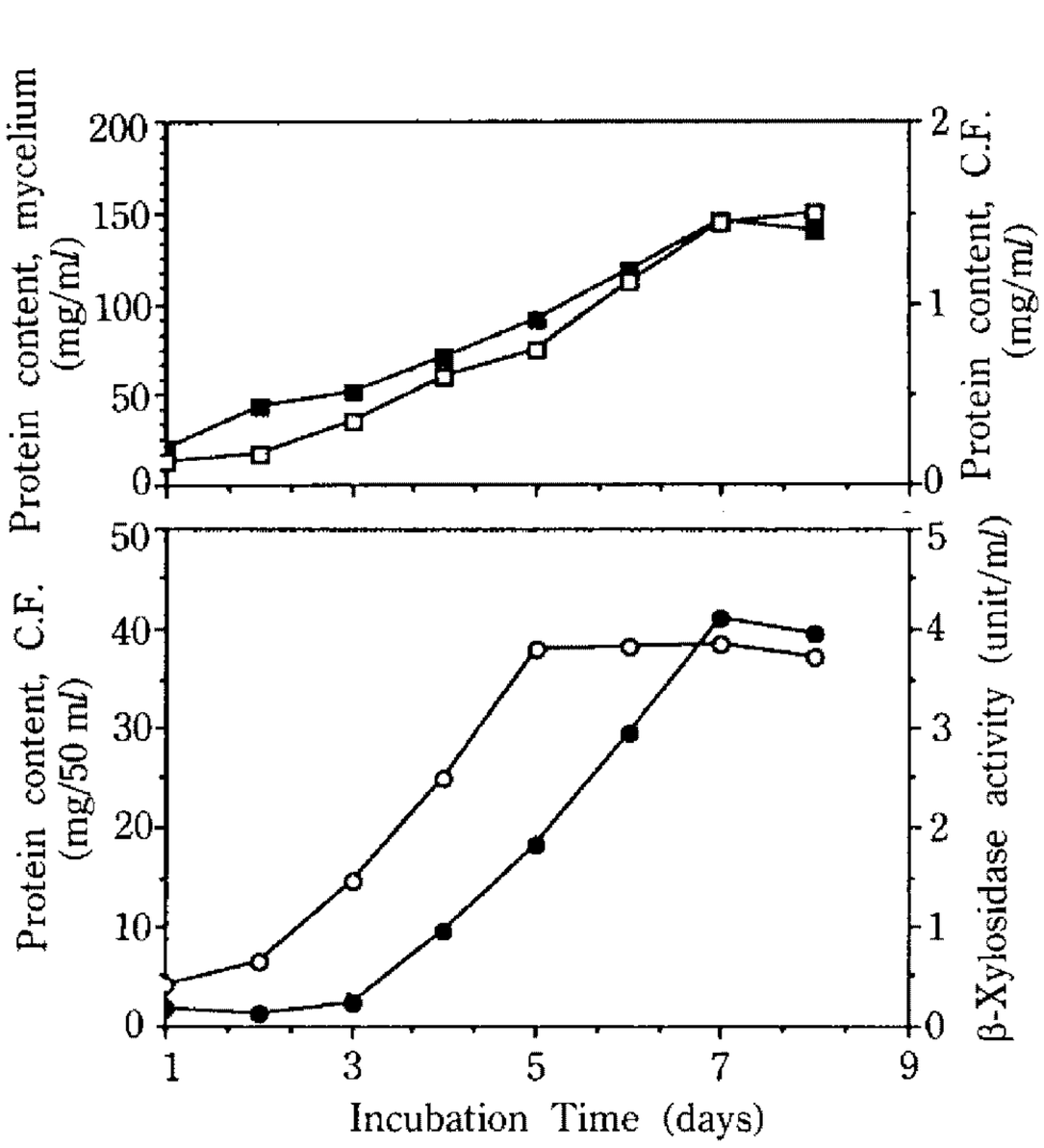

Fig. 3. Fermentation profile for xylanase and β-xylosidase production by *A. niger* NRC 107 at pH 6.0 in optimized medium.
Corn-cob xylan, 15 g/*l*; $NaNO_3$, 2.67 g/*l*; KH_2PO_4, 3.0 g/*l*; Tween 80, 3 m*l*/*l*; wheat bran, 15.0 g/*l*; L(−) sorbose, 3.0 g/*l*. Symbols: protein content of mycelium (■), protein content of culture filtrate (□), xylanase (●), and β-xylosidase (○).

roximately by 24 hr behind xylanase production. Xylanase and β-xylosidase were apparently produced during active growth and there is a close linear relationship between enzyme formation and the increase of extracellular protein. These results were similar to those reported on the course of xylanase and β-xylosidase from other microbial sources (7, 13, 19, 24 and 28). In addition, the culture filtrate of *A. niger* 107 showed no cellulase activity (data not shown).

A. niger NRC 107 in its optimum medium afforded 39.43 unit/m*l* of xylanase and 4.2 unit/m*l* of β-xylosidase. The optimum culture medium provided 5.76-fold and 7.7-fold increases in xylanase and β-xylosidase, respectively, relative to the original basal medium (Table 1). *A. niger* NRC 107 xylanase activity was higher than those reported for *A. terreus* 603 (6), *A. wentii* Pt 2804 (29), *Talaromyces byssochloamydoides* YH-50 (19), and *Trichoderma reesei* D 1-6 (28). At the same time, the levels of β-xylosidase obtained from *A. niger* NRC 107 are higher than those obtained from *A. terreus* (16), *Trichoderma reesei* QM 9414 (13), *Penicilium funiculosum* (30) and *Streptomyces* sp. (7).

요 약

Hemicellulose를 효소에 의하여 분해시키기 위하여 *Aspergillus niger* NRC 107로부터 xylanase와 β-xylosidase의 생산 조건에 대하여 조사하였다. 이들 효소 생산 최적 pH는 6.0이었으며 탄소원 중 corn-cob xylan과 질소원 중 Na_2NO_3이 효소 생산에 제일 좋은 기질이었으며 이들의 최적농도는 각각 15 g/*l*와 2.67 g/*l*이다. 이들 효소는 인산염(KH_2PO_4)과 Tween-80의 농도를 조절하므로 수율을 높일 수 있으며 wheat bran은 xylanase의 생산에 L(−) sorbose는 β-xylosidase의 생산에 좋은 영향을 주었다. 이와같이 이 *Aspergillus niger* NRC 107의 회분식 배양에서 이들 효소에 영향을 미치는 생산조건을 최적화한 결과 xylanase는 39.43 units/m*l*, β-xylosidase는 4.2 units/m*l*까지 생산할 수 있었다.

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