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Xylanase and Ferulic Acid Esterase Production by a Wild Strain of *Aspergillus terreus*

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Summary

The medium composition for the production of endo-xylanase and ferulic acid esterase in shake-flask cultures by ***Aspergillus terreus*** *was optimized using statistical methods. The optimized medium composition for xylanase production was found to be 40 g* L^{-1} *coarse corn cobs (c. 1–5 mm), 4.5 g* L^{-1} *gelitaflex, 12 g* L^{-1} $NaNO_3$, *while that for ferulic acid esterase production was 12 g* L^{-1} *oat spelt xylan, 6.4 g* L^{-1} *soybean meal and 4 g* L^{-1} NH_4NO_3. *The optimized media and culture conditions gave a maximum of 538.3 U/mL i.e. 8973 nkat/ml xylanase and 0.7 U/mL i.e. 11.7 nkat/mL, ferulic acid esterase activities after 5–7 days shake cultures. In addition to xylanase and ferulic acid esterase activities, the culture filtrates exhibited low or appreciable levels of filter-paper cellulase, carboxymethyl cellulase, β-glucosidase, β-xylosidase and acetyl esterase activities. In a laboratory bioreactor (10 L) culture using fine corn cobs (0.25–0.50 mm), xylanase production was slightly better than in the parallel shake-flask cultures. The pH optima of xylanase, β-xylosidase and ferulic acid esterase were 5.0, 4.5 and 5.0–6.0, respectively. The optimum temperature for xylanase, β-xylosidase and ferulic acid esterase was 50 °C. Xylanase and β-xylosidase showed moderate pH and thermal stabilities.*

Keywords: A. terreus; xylanase; ferulic acid esterase; media optimization

Introduction

Cellulose, hemicellulose and lignin are the main components of the plant cell wall. Xylans, the major component of the hemicellulose, are complex heteropolysaccharides of β-1,4-linked D-xylopyranoside residues. They are mostly substituted by pentoses, hexoses and acids and may be linear or branched, depending on their source and extraction procedure (*1–4*). Because of the structural complexity of xylans, concerted action of main-chain-spliting and side-chain-spliting enzymes is necessary for their complete breakdown. The main-chain-spliting enzymes are endo-β-1,4-xylanases and β-xylosidases and the side-chain-spliting enzymes include α-L-arabinofuranosidase, α-glucuronidases, acetylxylan esterase as well as the esterases which can hydrolyse esters of acetic, coumaric and ferulic acids (*1–8*). Many of these enzymes acted synergistically with xylanases after the removal of side-chain substituents to facilitate the action of endo-xylanase (*7,8*) whereas several accessory enzymes removed side-chain groups from oligosaccharides liberated by the prior action of xylanase (*9*). Various types of synergism e.g. homosynergy, heterosynergy, uni- and biproduct synergy and antisynergy have been proposed for the xylanolytic and esterolytic systems (*10*).

The xylan-degrading enzyme systems are produced by many fungi and bacteria (*10–15*). Interest in xylanases and side enzymes including esterases has increased in recent years as they can be used in the bioconversion of lignocellulosics to sugar, fuel and other useful chemicals, in the clarification of juices and wines, in plant oil extraction, in silage and green feed making, in the preparation of clean cellulose and in the biobleaching of pulp (*15–18*).

Although *A. terreus* has been reported to be an efficient producer of xylanolytic, cellulolytic (*12,15,19–23*) and esterolytic (*7*) enzymes there is no thorough report on the optimization of these enzyme productions by this species. The aims of the present work were to investigate the effects of different nutrients on the production of xylanase and ferulic acid esterase and to optimize the me-

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dium composition using cheap carbon and nitrogen sources. Some enzymatic properties of crude xylanase and ferulic acid esterase were studied as well.

Materials and Methods

Chemicals and raw materials

All chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) unless otherwise stated, *p*-nitrophenylglycosides, α-naphthylacetate and carob seed flour were purchased from Sigma (St. Louis, Mo., USA) and oat spelt xylan (OSX) was from Fluka (Buchs, Switzerland). Fish peptone (H0100) was from Protan (Drammen, Norway); bacto-peptone was purchased from Difco (Detroit, Mi., USA); potato protein (Alburex N) was from Roquette Freres (Lestrem, France); collagen hydrolysate (Gelitaflex) was a gift from Deutsche Gelatine Fabriken Stoess (Eberbach, Germany); soybean meal was from Vamo mills (Izegem, Belgium); cotton seed protein (Pharmamedia) and corn steep liquor were from Biochemie (Kundl, Austria); corn cobs, wheat straw and barley husks were collected from the farmers in Upper Austria; wheat bran, wheat flour, peanut shells and spinach leaves were purchased from the local market; rice husks and cassava peels were obtained from India and Nigeria, respectively.

Microorganism

Aspergillus terreus Thom BJ 217 was isolated from decomposed jute stacks /diseased jute stems at the Jute Research Institute, Dhaka, Bangladesh during the rainy season of 1986. The fungus was identified by Centraalbureau voor Schimmelculture, Baarn, The Netherlands. It was grown on potato dextrose agar (PDA) as surface cultures in petri-dishes and as slants in test tubes at 30 °C for 2–4 days and stored at 4 °C. Subcultures were prepared every 6 weeks using only slants.

Media and shake culture conditions

The basic mineral medium used was a modification of that of Mandels and Reese (*24*) containing 0.6 g L^{-1} urea, 0.3 g L^{-1} $MgSO_4$, 0.3 g L^{-1} $CaCl_2 \cdot 2H_2O$, 15 g L^{-1} KH_2PO_4 and 1 mL L^{-1} Vogel's trace element solution (Vogel's solution) in distilled water. Carbon and nitrogen sources were added as indicated in the experiment. The initial pH was adjusted to the indicated level prior to sterilization. Vogel's solution contained (g L^{-1}): $CoCl_2$, 2.0; $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot H_2O$, 1.4; $ZnCl_2$, 1.7 in deionized water. The sterilized culture medium (100 mL) in 300 mL Erlenmeyer flask was inoculated with a 1 cm^2 piece of a 2–4 day-old PDA stock culture of *A. terreus* and shaken at indicated temperature and 150 rpm on a rotary shaker for 5–7 days. An aliquot of the culture was withdrawn, centrifuged at 5000 rpm for 10 min and the enzyme activities and reducing sugars were determined in the clear supernatant.

Bioreactor cultivation

The bioreactor experiment was performed in a 10 L bioreactor (Biostat-V, Braun Melsungen, BRD) with an 8 L working volume. The culture temperature was maintained at 37 °C. The aeration rate was 4 L min^{-1} during the whole cultivation and the stirred speed was between 100 and 200 rpm and thus the dissolved oxygen level was always maintained above 20% level. The foaming was controlled by using 10% aqueous polypropyleneglycol. The medium composition was (g L^{-1}): corn cobs (fine), 40; gelitaflex, 4.5; $NaNO_3$, 12; urea, 0.6; $MgSO_4$, 0.3; KH_2PO_4, 15; and Vogel's solution, 1.5 mL L^{-1}. The inoculum (10%) was 72 h old shake-flask preculture grown on a similar medium, with the exception that birchwood xylan (soluble) (10 g L^{-1}) was used as carbon source instead of corn cobs. The initial pH of the bioprocess medium was adjusted to 6 and was not regulated during the cultivation.

Medium optimization and experimental designs

Preliminary optimization of the media for the formation of xylanase and feruloyl esterase was carried out using a design like Graeco-Latin square in order to select the nutrients and their approximate concentrations. It was assumed that the single components acted independently and their effects were purely additive (*25,26*). The optima were determined by summing up the results obtained with three respective media for one type or concentration of a particular ingredient. Based on these results, the concentrations of carbon and nitrogen sources were further optimized using a two-level factorial design (*27*).

Enzyme assays

Unless otherwise stated, the enzyme activities were determined at 50 °C and pH = 5 using citrate buffer (50 mM). *Xylanase* activity was determined according to Bailey *et al.* (*28*) using 1% birchwood xylan (Roth, Karlsruhe, Germany) after 5 min reaction time. The liberated reducing sugars were measured by the dinitrosalicylic acid (DNS) method of Miller (*29*). *Filter paper (FPase) and carboxymethyl cellulase (CMCase)* activities were determined by IUPAC method (*30*) using filter paper (Whatman No. 1, Maidstone, UK) and 1% solution of CMC-Na salt (Serva, Heidelberg, Germany), respectively. One unit of enzyme activity was defined as 1 μmol of xylose or glucose equivalents released per minute under the assay conditions. *β-xylosidase* and *β-glucosidase* activites were measured by the method of Herr *et al.* (*31*) using synthetic substrates *p*-nitrophenyl-β-D-xylopyranoside (3 mg/mL) and *p*-nitrophenyl-β-D-glucopyranoside (4 mg/mL), respectively. One unit of activity was expressed as the amount of enzyme which liberated 1 μmol *p*-nitrophenol in one minute. *Acetyl esterase* activity was assayed according to the method of Poutanen and Puls (*32*) using α-naphthylacetate as substrate. One unit of activity was expressed as the amount of enzyme which released 1 μmol of α-naphthol per min. *Ferulic acid (feruloyl) esterase* activity was measured according to the modified method of Johnson *et al.* (*33*). Starch-free wheat bran (100 mg), used as the substrate, was suspended in *c.* 1.5 mL of N-morpholino-sulphonic acid (100 mM, pH = 6) to which the enzyme solution (0.5 mL) was added to bring the final volume to 2 mL. The reaction mixture was incubated at 50 °C for 30 min followed by centrifugation at 10 000 rpm for 2 min. The content of ferulic acid in the supernatant was

estimated by high pressure liquid chromatography (HPLC). The activity was defined as the amount of enzyme releasing 1 μmol of ferulic acid per min.

HPLC

Ferulic acid liberated by feruloyl esterase was determined by HPLC using a 5 μm, 4 × 125 mm 79925 OD-564 Li chrospher 100 RP-18 column. The elution was performed at 1.6 mL min^{-1} at room temperature with 17.5 mM potassium phosphate buffer (pH = 7.0) and 5 mM tetrabutylammonium hydrogen sulphate in methanol (20%) and ferulic acid was detected by a refractive index detector at 315 nm.

Other analyses

Reducing sugars in the culture filtrates were determined by the DNS (29) method. *Soluble protein* in the culture filtrate was determined according to the modified method of Lowry *et al.* (34) using bovine serum albumin as standard. *Total dry matter* (TDM) (lignocellulosic material + mycelia) was determined by filtering a definite volume of culture broth and by drying the extensively washed filter cake in a moisture analyzer at 130 °C to a constant weight.

Enzyme characterization

The pH optima for enzyme activity were determined by measuring the enzyme activities at 50 °C in 50 mM citrate (pH = 3–6) and Tris-HCl (pH = 7–9) buffers. For determination of the temperature optima, the enzyme solutions were incubated with the respective substrates at their optimum pH and at different temperatures (30–60 °C). For determination of long-term thermal stability, the enzymes were incubated in citrate buffer (50 mM, pH = 5.0) containing 10 mg L^{-1} Thimerosol (antimicrobial) at temperatures between 30 and 60 °C for 0–6 days. The samples were withdrawn at different times and the residual activity was assayed using the standard conditions. For determination of pH stability, enzyme preparations were incubated at 30 °C for 0–96 h at different pH values (50 mM citrate buffer, pH = 3–6; 50 mM Tris-HCl buffer, pH = 7–9). The residual activities were then measured at the respective optimum pH and temperature for each enzyme.

Results and Discussion

Some preliminary shake cultures showed that xylanase production and growth of *A. terreus* were markedly influenced by the initial pH of the medium and culture temperature. The fungus synthesized the highest levels of xylanase activity (228 U/mL) in the medium (fine corn cobs, 10 g L^{-1}) having initial pH = 6 (Fig. 1a). The highest levels of xylanase synthesis (117.5 U/mL) and the fungal growth (7.9 mg/mL) occurred at 37 °C on 10 g L^{-1} birchwood xylan (soluble) medium (Fig. 1b). Progress of typical shake-flask cultures for 1–7 days at 37 °C and initial pH = 6 using 10 g L^{-1} birchwood xylan medium showed the highest mycelial (8 mg/mL) and xylanase (120 U/mL) yields after 3 and 5 days, respectively. Therefore, in subsequent studies the fungus was cultivated at 37 °C for 5 days unless otherwise stated.

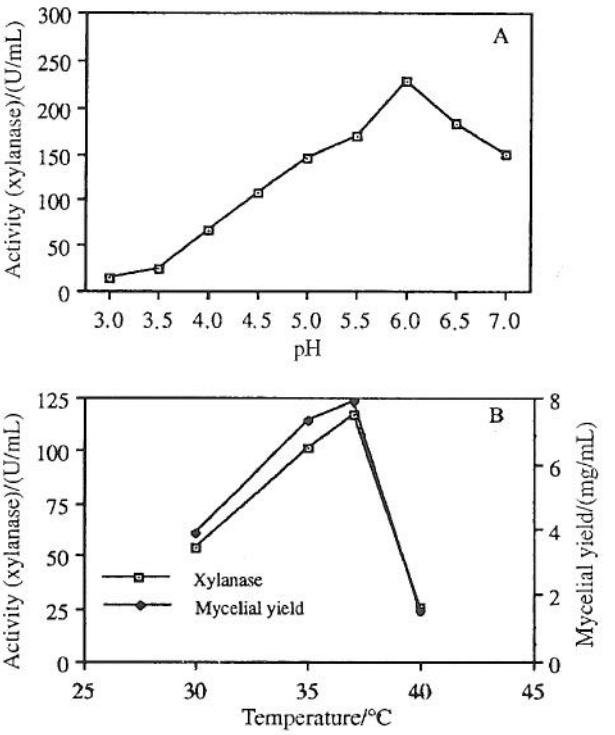

Fig. 1a,b. (a) Effect of initial pH of the medium on xylanase production by *A. terreus*. The mineral medium was supplemented with (g L^{-1}): corn cobs (fine), 10; meat peptone, 5.0; $NaNO_3$, 5. The fungus was shake-cultured for 7 days at 30 °C; (b) Effect of incubation temperature on xylanase production and mycelial yield of *A. terreus*. The mineral medium contained (g L^{-1}): birchwood xylan (soluble), 10; meat peptone, 5: $NaNO_3$, 5. Initial pH was 6. Shake-cultured for 7 days.

Shake culture experiments, arranged in Graeco-Latin square design, were performed in order to select the medium components.

Table 1 shows the experimental design and the xylanase activities (11.6–197.2 U/mL) obtained in 9 different media. Summation of the xylanase activity in three respective media indicated that coarse corn cobs (in media 2, 5 and 8), gelitaflex (in media 4, 5 and 6), $NaNO_3$ (in media 3, 5 and 7) and 1.5 mL Vogel's solution (in media 2, 6 and 7) were the best for maximal production of xylanase.

Table 2 shows the second Latin square design and xylanase activities (120.5–359 U/mL) obtained using three different concentrations each of corn cobs, gelitaflex nitrogen, $NaNO_3$ nitrogen and Vogel's solution. The summarized xylanase activity showed that corn cobs at 30 g L^{-1} (in media 3, 6 and 9), gelitaflex nitrogen at 0.5 g L^{-1} (in media 1, 2 and 3), $NaNO_3$ nitrogen at 2 g L^{-1} (in media 3, 5 and 7) and Vogel's solution at 1.5 mL L^{-1} (in media 3, 4 and 8) were the best concentrations for xylanase production. The highest xylanase yield (359 U/mL) was in medium 3.

The final optimization experiments for xylanase production were carried out using 2^3 factorial designs. The design of the first experiment and xylanase (150.4–431.2 U/mL) and other enzyme activities are shown in Table 3.

The regression coefficients (slope) with respect to xylanase activity for the three factors were as follows:

Table 1. Design for optimization of medium ingredients using the Graeco-Latin square for xylanase production by *A. terreus* and the enzyme activities obtained

Medium[a] ingredients	Medium serial No.								
	1	2	3	4	5	6	7	8	9
γ (wheat bran)/g L^{-1}	10			10			10		
γ (corn cobs (coarse))/g L^{-1}		10			10			10	
γ (carob seed flour)/g L^{-1}			10			10			10
γ (Alburex N nitrogen)/g L^{-1}[b]	0.5	0.5	0.5						
γ (Gelitaflex N)/g L^{-1}				0.5	0.5	0.5			
γ (Pharmamedia N)/g L^{-1}							0.5	0.5	0.5
γ $(NH_4)_2SO_4$ N)/g L^{-1}[b]	2					2		2	
γ (NH_4NO_3 N)/g L^{-1}		2		2					2
γ ($NaNO_3$ N)/g L^{-1}			2		2		2		
φ (Vogel's solution)/mL L^{-1}	10				10				10
φ (Vogel's solution)/mL L^{-1}			5	5				5	
φ (Vogel's solution)/mL L^{-1}		1.5				1.5	1.5		
Enzyme activity/(U/mL)[c]									
Xylanase	50.1	121.1	14.5	70.8	197.2	39.8	76.9	187.3	11.6
FPase	0.02	0.04	0.04	0.03	0.09	0.06	0.02	0.05	0.07

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$ and 15, KH_2PO_4 in tap water. The initial pH was adjusted to pH = 6 prior to sterilization. The fungus was shake-cultured at 37 °C for 5 days.

[b] The organic nitrogen sources were added at equal N concentration. The nitrogen contents of alburex, gelitaflex and pharmamedia were 12.2%, 11.2% and 9.2%, respectively. The inorganic N sources were also added at equal N concentrations.

[c] All results are averages of two replicate shake cultures and assays.

Table 2. Design for optimization of medium ingredient concentration using Graeco-Latin square for xylanase production by *A. terreus* and the enzyme activities obtained

Medium[a] ingredients	Medium serial No.								
	1	2	3	4	5	6	7	8	9
γ (corn cobs, coarse)/g L^{-1}	10	20	30	10	20	30	10	20	30
γ (Gelitaflex N)/g L^{-1}	0.5	0.5	0.5	1.5	1.5	1.5	1.0	1.0	1.0
γ ($NaNO_3$ N)/g L^{-1}	1.5	2.5	2.0	2.5	2.0	1.5	2.0	1.5	2.5
φ (Vogel's solution)/mL L^{-1}	1.0	5.0	1.5	1.5	1.0	5.0	5.0	1.5	1.0
Enzyme activity/(U/mL)[b]									
Xylanase	162.7	296.4	359.0	120.5	195.5	173.8	135.3	160.7	177.4
FPase	0.06	0.09	0.11	0.06	0.08	0.09	0.05	0.08	0.14

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$ and 15, KH_2PO_4 in tap water. The initial pH was adjusted to pH = 6 prior to sterilization. The fungus was shake-cultured at 37 °C for 5 days.

[b] All results are averages of two replicate shake cultures and assays.

Table 3. Design for optimization of ingredient concentration using a 2^3 factorial design for xylanase production by *A. terreus* and the results obtained

Medium[a] ingredients	Medium serial No.							
	1	2	3	4	5	6	7	8
γ (coarse corn cobs)/g L^{-1}	35	35	35	35	45	45	45	45
γ (Gelitaflex N)/g L^{-1}	0.25	0.25	0.75	0.75	0.25	0.25	0.75	0.75
γ ($NaNO_3$ N)/g L^{-1}	1.50	2.50	1.50	2.50	1.50	2.50	1.50	2.50
Enzyme activity/(U/mL)[b]								
Xylanase	394.2	279.6	425.0	302.2	150.4	431.2	370.4	390.2
β-Xylosidase	0.15	0.18	0.18	0.14	0.16	0.21	0.21	0.18
FPase	0.10	0.12	0.10	0.09	0.08	0.09	0.09	0.08
CMCase	1.38	1.90	1.14	1.10	1.06	1.10	1.10	1.15
β-Glucosidase	1.45	1.54	2.10	2.50	1.30	1.45	2.10	2.60

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$; 15, KH_2PO_4 and 1.5 mL L^{-1} Vogel's solution. Initial pH of the medium, prepared in tap water, was adjusted to 6. The fungus was cultivated at 37 °C for 5 days.

[b] All results are averages of two replicate shake cultures and assays.

–58.8 for corn cobs, +232.6 for gelitaflex nitrogen, and –137.1 for $NaNO_3$ nitrogen. From these values the new variables for the second experiment to obtain the 'ascent' were calculated (Table 4).

In this experimental design, corn cobs concentration was serially decreased by 0.5 g L^{-1} and correspondingly, gelitaflex nitrogen was increased serially by 0.05 g L^{-1} and $NaNO_3$ nitrogen was decreased serially by 0.06 g L^{-1}. The yield plateau with respect to xylanase yield (432.8 U/mL) was reached at medium 1 containing 40 g L^{-1} corn cobs, 0.5 g L^{-1} gelitaflex nitrogen and 2 g L^{-1} $NaNO_3$ nitrogen.

Similarly, the medium for feruloyl esterase production was preliminarily optimized using Graeco-Latin square design.

Table 5 shows the design and the yields of feruloyl esterase. Summation of enzyme activities indicated that OSX at 10 g L^{-1} (in media 1, 4 and 7), soybean meal nitrogen at 0.5 g L^{-1} (in media 2, 4 and 9) and NH_4NO_4 nitrogen at 1.5 g L^{-1} (in media 1, 2 and 3) were the best for obtaining maximal feruloyl esterase production.

The medium was further optimized using 2^3 factorial design for obtaining maximal feruloyl esterase production. Table 6 shows the design and feruloyl esterase yields. The regression coefficients (slope) for OSX, soybean meal nitrogen and NH_4NO_3 nitrogen were: +0.14, –0.14 and –0.14, respectively. Based on these values the concentration of OSX was increased serially by 1 g L^{-1} while those of soybean meal and NH_4NO_3 nitrogen were decreased serially by 0.025 g L^{-1} and 0.05 g L^{-1}, respectively (Table 7).

The highest feruloyl esterase activity (0.69 U/mL) was obtained in medium 3 containing 12 g L^{-1} OSX, 0.45 g L^{-1} soybean meal nitrogen and 1.4 g L^{-1} NH_4NO_3 nitrogen.

The optimum culture temperature and initial pH of the medium for the enzyme production were again examined using the optimized medium (corn cobs and OSX media for xylanase and feruloyl esterase, respectively). Cultivation of the fungus at 30 °C for 5 days in the media having initial pH = 3–7 yielded the highest xylanase activity (406.6 U/mL) in the medium with initial pH = 6, while the maximum feruloyl esterase activity (0.45 U/mL) was formed in a medium with slightly lower initial pH (5.0–5.5). At initial pH values of 3 and 7 very little enzyme activities were produced. Ghosh and

Table 4. A 2^3 factorial experimental design for obtaining the ascent and enzyme activities produced by *A. terreus*

Medium[a] ingredients	Medium serial No.							
	1	2	3	4	5	6	7	8
γ (coarse corn cobs)/g L^{-1}	40.0	39.5	39.0	38.5	38.0	37.5	37.0	36.5
γ (Gelitaflex N)/g L^{-1}	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85
γ ($NaNO_3$ N)/g L^{-1}	2.00	1.94	1.88	1.82	1.76	1.70	1.64	1.58
Enzyme activity/(U/mL)[b]								
Xylanase	432.8	426.0	366.2	330.7	395.9	331.9	287.3	236.9
β-Xylosidase	0.23	0.22	0.22	0.21	0.19	0.17	0.16	0.12
FPase	0.11	0.10	0.08	0.11	0.12	0.09	0.09	0.07
CMCase	0.75	0.68	0.63	0.64	0.67	0.62	0.61	0.56
β-Glucosidase	3.68	3.76	3.49	3.88	3.82	3.65	3.96	2.88

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$; 15, KH_2PO_4 and 1.5 mL L^{-1}, Vogel's solution and tap water at an initial pH = 6. The fungus was shake-cultured for 5 days at 37 °C.

[b] All results are averages of two replicate shake cultures and assays.

Table 5. Graeco-Latin square design for optimization of ingredient concentrations of the culture medium for feruloyl esterase production by *A. terreus* and the enzyme activities obtained

Medium[a] ingredients	Medium serial No.								
	1	2	3	4	5	6	7	8	9
γ (oat spelt xylan)/g L^{-1}	10			10			10		
γ (birchwood xylan)/g L^{-1}		10			10			10	
γ (wheat flour)/g L^{-1}			10			10			10
γ (NH_4NO_3 N)/g L^{-1}[b]	1.5	1.5	1.5						
γ (NH_4NO_3 N)/g L^{-1}				2.0	2.0	2.0			
γ (NH_4NO_3 N/g L^{-1}							2.5	2.5	2.5
γ (meat peptone N)/g L^{-1}[b]	0.5					0.5		0.5	
γ (soybean meal N)/g L^{-1}		0.5		0.5					0.5
γ (Gelitaflex N)/g L^{-1}			0.5		0.5		0.5		
Enzyme activity/(U/mL)[c]									
Feruloyl esterase	0.38	0.31	0.16	0.26	0.23	0.12	0.23	0.17	0.20

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$; 15, KH_2PO_4, 1.5 mL L^{-1}, Vogel's solution and tap water at an initial pH = 6. The fungus was shake-cultured at 37 °C for 5 days.

[b] The nitrogen sources were added at equal N concentration. The nitrogen contents of meat peptone, soybean meal and gelitaflex were 11.8%, 7.0% and 11.2%, respectively.

[c] All results are averages of two replicate shake cultures and assays.

Table 6. A 2^3 factorial design for optimization of concentrations of medium ingredients for feruloyl esterase production by *A. terreus* and the results obtained

Medium[a] ingredients	Medium serial No.							
	1	2	3	4	5	6	7	8
γ (oat spelt xylan)/g L^{-1}	5	5	5	5	15	15	15	15
γ (soybean meal N)/g L^{-1}	0.25	0.25	0.75	0.75	0.25	0.25	0.75	0.75
γ (NH_4NO_3 N)/g L^{-1}	1	2	1	2	1	2	1	2
Enzyme activity/(U/mL)[b] Feruloyl esterase	0.52	0.50	0.48	0.50	0.58	0.54	0.56	0.46

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$; 15, KH_2PO_4 in tap water at initial pH = 6. The fungus was shake-cultured at 37 °C for 5 days.

[b] All results are averages of two replicate shake cultures and assays.

Table 7. A 2^3 factorial design for obtaining the 'ascent' and the enzyme activities produced by *A. terreus*

Medium[a] ingredients	Medium serial No.							
	1	2	3	4	5	6	7	8
γ (oat spelt xylan)/g L^{-1}	10	11	12	13	14	15	16	17
γ (soybean meal N)/g L^{-1}	0.50	0.475	0.45	0.425	0.40	0.375	0.35	0.325
γ (NH_4NO_3 N)/g L^{-1}	1.50	1.45	1.40	1.35	1.30	1.25	1.20	1.15
Enzyme activity/(U/mL)[b] Feruloyl esterase	0.63	0.64	0.69	0.68	0.64	0.62	0.61	0.55

[a] In addition, each medium contained (g L^{-1}): 0.6, urea; 0.3, $MgSO_4$; 15, KH_2PO_4 in tap water at initial pH = 6. The fungus was shake-cultured at 37 °C for 5 days.

[b] All results are averages of two replicate shake cultures and assays.

Kundu (*19*) also obtained a pH optimum of 6 for the production of cellulase and xylanase by *A. terreus* IJIRA, but Garg and Neelakantan (*20*) reported that an initial of pH = 4 was optimum for the productions of these enzymes by another strain of *A. terreus* GNI. These differences were perhaps due to the use of different culture media or to the difference in the genetic constitution of the strains isolated at different times at different places. Cultivation of the fungus between 30 and 45 °C in the respective optimized medium with initial pH = 6 (for xylanase) and 5.5 (for feruloyl esterase) exhibited the maximum xylanase (450 U/mL) and feruloyl esterase (0.63 U/mL) yields at 37 °C. Lower levels of enzyme activities (325 U/mL xylanase and 0.42 U/mL feruloyl esterase) were produced at 30 °C although the fungal growth was good. Very low levels of enzyme activities were produced at 40 °C due to poor fungal growth at this temperature. At 45 °C the fungus did not grow at all. These results support our initial results obtained with the unoptimized medium.

A confirmatory shake-flask culture, performed for 7 days using the medium and conditions optimized for xylanase, produced good levels of xylanase (538.3 U/mL), β-glucosidase (7.1 U/mL), β-xylosidase (0.3 U/mL), acetyl esterase (2.2 U/mL) and low levels of FPase (0.11 U/mL) and CMCase (0.75 U/mL) activities. The activities of xylanase, β-glucosidase and β-xylosidase were much higher than those reported by Gomes *et al.* (*12*) for the same strain of *A. terreus* grown on 10 g L^{-1} Avicel which induced higher levels of FPase (0.3 U/mL) and CMCase (3.25 U/mL). The yield of xylanase activity was increased by a factor of *c.* 2.4 compared to the first result obtained in the screening experiments. The highest yield of xylanase activity of this strain using a cheap medium is also significantly higher than the values reported for other *A. terreus* strains (*15,19–22*). These results indicate that optimum concentrations of coarse corn cobs used in this study induced high level of xylanolytic and β-glucosidase activities suppressing cellulase (exo and endo) activities. The yield of feruloyl esterase in the optimized medium under optimized conditions was 0.7 U/mL which was 1.8 fold higher than the value obtained in the initial optimization experiments. Based on these and previous results it may be concluded that by proper optimization of the medium components and culture conditions the synthesis of high levels of cellulolytic and/or xylanolytic enzymes by *A. terreus* may be directed.

Table 8 shows the effects of different carbon and nitrogen sources on xylanase and feruloyl esterase production in shake-flask cultures under optimized conditions. The enzyme productions in these experiments were much less compared to those obtained in the confirmatory shake cultures due to the use of carbon sources at 10 g L^{-1} concentration. The optimized concentration (40 g L^{-1}) of coarse corn cobs was not used here in order to avoid the possible negative effects of high concentration of widely differing substrates on enzyme production. The fungus grew well almost on all substrates and produced xylanase, cellulase and feruloyl esterase activities irrespective of the carbon sources used. The highest xylanase yield (187.3 U/mL) was obtained on coarse corn cobs, whereas fine corn supported low enzyme production (88.0 U/mL). Although the fungus grew profusely on cassava peel low enzyme yield was perhaps due to the presence of easily metabolizable starch. Peanut shells supported little fungal growth as well as enzyme production. Avicel, a pure cellulose, induced mainly cellu-

Table 8. Effect of different carbon and nitrogen sources on xylanase and feruloyl esterase production by *A. terreus*

Nutrients	Enzyme yields/(U/mL)[d]		
	Xylanase	FPase	Feruloyl esterase
Carbon sources[a]			
Corn cobs (coarse, 1–5 mm)	187.3	0.05	ND
Corn cobs (fine, 0.25–0.5 mm)	88.0	0.05	ND
Wheat straw	170.3	0.06	0.39
Wheat bran	83.9	0.10	0.46
Wheat flour	ND	ND	0.18
Xylan (birchwood)	114.3	0.21	0.36
Oat spelt xylan	ND	ND	0.70
Barley husks	76.9	0.23	0.33
Avicel (microcrystalline cellulose)	72.4	0.50	ND
Carob seed flour	52.8	0.15	ND
Rice husks	39.8	0.06	0.14
Cassava peel	22.6	0.01	ND
Peanut shell	15.2	0.01	ND
Spinach leaves	ND	ND	0.14
Organic nitrogen sources[b]			
Gelitaflex	197.2	0.05	0.30
Pharmamedia	187.3	0.09	ND
Urea	152.7	0.05	ND
Alburex N (potato protein)	121.0	0.04	ND
Fish peptone	115.5	0.19	0.34
Yeast extract	112.6	0.21	0.31
Corn steep liquor	89.5	0.10	0.34
Meat peptone	72.9	0.05	0.40
Soybean meal	62.8	0.05	0.48
Bacto-peptone	54.1	0.05	ND
Inorganic nitrogen sources[c]			
$NaNO_3$	214.8	0.09	0.17
NH_4NO_3	98.7	0.05	0.25
$(NH_4)_2SO_4$	84.7	0.04	0.12

[a] In addition to 10 g L^{-1} carbon sources, each medium contained (g L^{-1}): 0.5, gelitaflex nitrogen; 2, $NaNO_3$ nitrogen; 15, KH_2PO_4; 0.3, $MgSO_4$ and 1.5 mL L^{-1} Vogel's solution for xylanase production. For feruloyl esterase production each medium contained (g L^{-1}): 0.45, soybean meal nitrogen; 1.4, NH_4NO_3 nitrogen and 1.5 ml L^{-1} Vogel's solution. All lignocellulosics were dried and finally ground.

[b] In addition to organic nitrogen sources (0.5 g L^{-1}), each medium contained (g L^{-1}): 10, coarse corn cobs; 2, $NaNO_3$ nitrogen (10 g L^{-1} OSX, 0.45 g L^{-1} soybean meal nitrogen and 1.4 g L^{-1} NH_4NO_3 nitrogen for feruloyl esterase production); 15, KH_2PO_4; 0.3, $MgSO_4$ and 1.5 mL L^{-1} Vogel's solution.

[c] In addition to inorganic nitrogen sources each medium contained (g L^{-1}): 10, corn cobs; 0.5, gelitaflex nitrogen for xylanase (10, OSX; 0.45, soybean meal nitrogen for feruloyl esterase); 15, KH_2PO_4; 0.3, $MgSO_4$ and 1.5 mL L^{-1} Vogel's solution. Initial pH of each medium was 6 and the fungus was cultured for 5 days at 37 °C.

[d] All results are averages of two replicate shake-cultures and assays.

ND = Not determined

lase production (0.5 U/mL) together with lower level of xylanase (72.4 U/mL). Pure xylan (birchwood), containing only 0.5% glucan as contaminant, induced the formation of mainly xylanase (114.3 U/mL) with appreciable level of cellulase (0.21 U/mL). The formation of both cellulase and xylanase on cellulose has been described for several organisms e.g. *Trichoderma reesei, T. harzianum, T. viridae, A. terresu, A. awamori, A. fumigatus, Schizophyllum commune* (*10–14,21,26,35*). In some *Trichoderma* and *Aspergillus* species, xylan has been reported to specifically induce the formation of xylanase with no or low levels of cellulase (*13,21,22*). The highest (0.7 U/mL) and the lowest (0.14 U/mL) levels of feruloyl esterase activities were produced on OSX and rice husks/spinach leaves, respectively. The maximum yield (0.7 U/mL) of feruloyl esterase by our strain of *A. terreus* was far higher than that reported for another strain of *A. terreus* which produced only 0.06 U/mL (*7*). Much higher productions of feruloyl esterase by *Streptomyces olivochromogenes* (2.56 U/mL) using 10 g L^{-1} OSX (*33*) and by *A. niger* (10.58 U/mL) using 10 g L^{-1} wheat bran (*36*) have been reported. The difference observed in the yields of this enzyme could not be related to the amounts of ferulic and *p*-coumaric acids in these substrates. For example, oat spelt and birchwood xylans lacking the ester bonds linking ferulic and *p*-coumaric acids to arabinoxylan induced more feruloyl esterase activity than the tested lignocellulosic materials which have been reported to contain these substituents

(*13*). Induction of more feruloyl and *p*-coumaroyl esterases in several mesophilic and thermophilic fungi by meadow fescue grass than by OSX was assumed to be due to the presence of ferulic and p-coumaric acids in the grass (*13*). To the contrary, feruloyl esterase production in *A. oryze* was found to be unrelated to the phenolic acid content of lignocellulosic materials (*7*). Even pure cellulose was reported to induce several-fold more ferulic acid esterase activity in several *Aspergillus* species, *S. commune*, and *S. olivochromogenes* (*6,7,33,36*). However, the differences in enzyme yields with different substrates indicate that the yield of xylanase, cellulase and ferulic acid esterase is not primarily determined by the composition (sugar, lignin, acids, etc.) of these substrates. The nature of substrates and their pretreatments, presence or absence of activators or inhibitors in the substrates, surface area and pore size of the substrates, and favorable degradability of the substrates by the microorganism have great influence on the extracellular enzyme production.

Table 8 shows the effects of various organic and inorganic nitrogen sources on the enzyme production. Remarkable differences in the influences of these nitrogen sources were observed. Gelitaflex appeared to be the best organic nitrogen source yielding a xylanase activity of 197.4 U/mL, while soybean meal was better than other organic nitrogen sources for feruloyl esterase production. The lowest xylanase activity (54.1 U/mL) was produced on bacto-peptone. The differences in the levels of enzyme production may be due to the presence of varying amounts of essential amino acids, peptides, vitamins, trace elements and mineral salts in different organic nitrogen sources. Similar differences in the effects of organic nitrogen sources on xylanase and cellulase production by other fungi including *A. terreus* have been reported (*12,14,20*). Garg and Neelakantan (*20*) reported that *A. terreus* was able to utilize corns steep liquor best for cellulase and xylanase production. Among the three inorganic nitrogen sources tested $NaNO_3$ yielded the highest xylanase activity (214.8 U/mL), whereas NH_4NO_3 yielded the highest feruloyl esterase activity. This result for xylanase agrees with the report of Garg and Neelakantan (*20*) who found that *A. terreus* was able to utilize $NaNO_3$ most efficiently compared with ammonium salts for the production of cellulase and xylanase.

In the bioreactor culture, essentially the optimized culture medium and conditions were used. Only coarse corn cobs had to be replaced by fine corn cobs because coarse corn cobs blocked aeration tube and hindered efficient stirring as the big particles got stuck between the wall and the vertical metal plates of the fermenter. A typical batch cultivation time-course, performed under uncontrolled pH, is shown in Fig. 2.

The synthesis of xylanase started within 24 h (18 U/mL) after which the rate of synthesis increased rapidly reaching the peak (220 U/mL) at 120 h. In addition to xylanase, low levels of β-xylosidase (0.03 U/mL), FPase (0.11 U/mL), CMCase (0.5 U/mL), β-glucosidase (1.5 U/mL), acetyl (0.21 U/mL) and feruloyl (0.02 U/mL) esterase activities were detected. Parallel shake-flask culture using the fine corn cobs yielded similar levels of xylanase (195 U/mL) and other enzyme activities. These values are considerably lower than those obtained in the

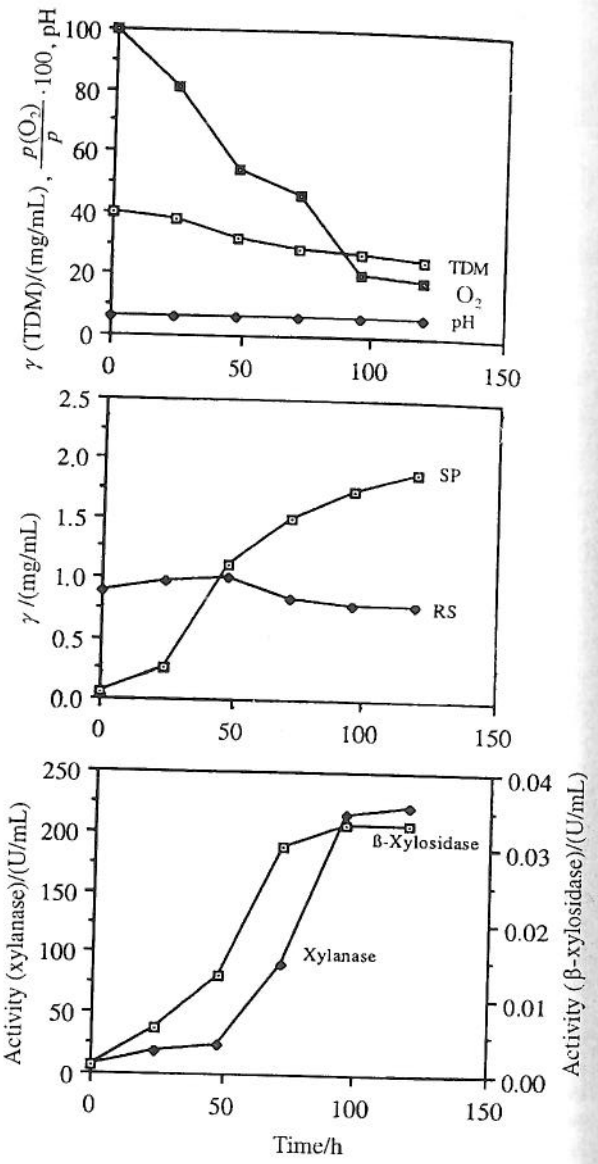

Fig. 2. Time course of a typical batch cultivation of *A. terreus* in a 10 L laboratory bioreactor
TDM = total dry matter, SP = soluble protein, RS = reducing sugar

confirmatory shake-flasks (538.3 U/mL xylanase) using the medium optimized with coarse corn cobs. The reason for the lower productions in bioreactor and parallel shake-flasks is mainly due to the use of fine corn cobs. The time course of enzyme production agreed well with the growth of the fungus. In both shake cultures and bioreactor the fungus grew as diffusive mycelia. There was an increase in reducing sugar level from 0.88 to 1.0 mg up to 48 h after which it decreased slowly. The TDM (lignocellulose + mycelia) decreased steadily from an initial value of 40 g L^{-1} to 28 g L^{-1} at 72 h after which the decrease was very slow indicating the stationary phase of the fungal growth and slow utilization of lignocellulose. The pH of the medium, adjusted initially to 6, was almost stable throughout the cultivation process due to the buffering effect of KH_2PO_4 (15 g L^{-1}). Up to 72 h the pH dropped slowly to 5.8, followed by a slight increase to 5.9 at 96 h and then a slight decrease to 5.8 at 120 h.

This indicates that a good level of xylanase may be produced by this fungus without pH regulation. We believe that the productions of xylanase and other accessory enzymes may be improved by performing the bioprocess in a modified bioreactor that will allow the use of coarse corn cobs and by further optimization of the overall batch fermentation profiles, such as pH- and temperature-shifting, inoculum size and age, aeration capacity and stirring.

The pH optima for xylanase, β-xylosidase and feruloyl esterase were 5.0, 4.5 and 5.0–5.5, respectively. These optima for *A. terreus* xylanases are comparable to those for other strains of *A. terreus* and many other mesophilic fungi (*7,11,15,20,23,26*). Xylanase, β-xylosidase and feruloyl esterase were most active at 50 °C. This value compares well to the reports for other organisms (*7,11,15,20, 23,26*).

Apart from pH and temperature optima of the enzymes their long-term stabilities (e.g. pH and thermal) are important properties for practical applications. At 30 °C xylanase and β-xylosidase were almost stable for 24 h after which they lost activities slowly retaining 80–93% activities after 6 days. With the increase of temperature to 37 °C or above the initial inactivation rate of the enzymes increased. At 37, 40 and 50 °C xylanase retained 95, 92 and 85% activity, respectively after 24 h heating. After 6 days heating at the respective temperatures it retained 78, 65 and 51% activity. β-xylosidase retained 83, 71 and 62% activity after 24 h heating at 37, 40 and 50 °C, respectively. After 6 days heating at the respective temperatures it still retained 72, 63 and 53% activity. At 60 °C both enzymes were rapidly inactivated retaining 22% (xylanase) and 56% (β-xylosidase) activity after 24 h and only 13% (xylanase) and 17% (β-xylosidase) after 6 days. Xylanase and β-xylosidase were most stable at pH = 5.0 and 5.0–5.5, respectively, retaining more than 95% of their total activity. The pH values lower than 4 or greater than 7 led to rapid inactivation of the enzymes. However, at pH = 3–4 and 7–9 about 40–45% of the activity was still retained after 96 h. These observations are comparable to those reported for many other mesophilic fungi (*10,11,15,23, 26*). It was not possible to study the pH and thermal stabilities of feruloyl esterase. However, it is assumed that its stabilities would be similar to those of xylanase and β-xylosidase.

Conclusions

The xylanase and feruloyl esterase yields of the wild *A. terreus* were improved 2.4 and 1.8 fold, respectively compared with the initial values by optimizing the economic nutrient media and the culture conditions. In addition to xylanolytic enzymes, this fungus is able to produce cellulolytic and other accessory enzymes. Using proper carbon and nitrogen sources at appropriate concentration either xylanolytic and/or cellulolytic enzymes may be produced in remarkable amounts by this fungus. This makes this organism desirable for different applications like biobleaching of pulp and/or saccharification of biomass for different purposes.

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Nastajanje ksilanaze i esteraze ferula kiseline u divljem soju *Aspergillus terreus*

Sažetak

Sastav podloge za proizvodnju endoksilanaze i esteraze ferula kiseline, uzgojem plijesni **A. terreus** *na tresilici, optimiran je primjenom statističkih postupaka. Utvrđeno je da 1 litra optimirane podloge za proizvodnju ksilanaze sadržava 40 g grubo rezanih kukuruznih klipova (oko 1–5 mm), 4,5 g gelitafleksa, 12 g $NaNO_3$ dok je za proizvodnju esteraze ferula kiseline 1 litra podloge imala 12 g ksilana zobenih pahuljica, 6,4 g sojina brašna i 4 g NH_4NO_3. U optimiranim podlogama nakon 5–7 dana uzgoja plijesni na tresilici postignute su maksimalne aktivnosti od 538,3 U/mL, tj. 8973 nkat/mL za ksilanazu i 0,7 U/mL, tj. 11,7 nkat/mL za esterazu ferula kiseline.*

Osim ksilanazne i esterazne aktivnosti filtrati kultura sadržavali su i male ili znatnije količine celulaze filtrirnog papira, karboksimetil-celulaze, β-glukozidaze, β-ksilozidaze i acetil-esteraze. Uzgojem plijesni u laboratorijskome bioreaktoru (10 L) u podlozi koja je sadržavala fino mljevene kukuruzne klipove (0,25–0,50 mm) proizvodnja ksilanaze bila je nešto bolja nego u usporednoj kulturi na tresilici. Optimalna pH-vrijednost za ksilanazu iznosila je 5,0, za β-ksilozidazu 4,5, a za esterazu ferula kiseline 5,0–6,0. Optimalna temperatura za ksilazanazu, β-ksilozidazu i esterazu ferula kiseline iznosila je 50 °C. Ksilanaza i β-ksilozidaza bile su stabilne pri pH = 5,0–5,5 i umjerenoj temperaturi.