Extremophiles (2000) 4:227–235

ORIGINAL PAPER

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Thermolabile xylanase of the Antarctic yeast *Cryptococcus adeliae*: production and properties

Received: December 10, 1999 / Accepted: March 23, 2000

Abstract Xylanase production by the Antarctic psychrophilic yeast *Cryptococcus adeliae* was increased 4.3 fold by optimizing the culture medium composition using statistical designs. The optimized medium containing 24.2 g l^{-1} xylan and 10.2 g l^{-1} yeast extract and having an initial pH of 7.5 yielded xylanase activity at 400 nkat (nanokatal) ml^{-1} after 168-h shake culture at 4°C. In addition, very little endoglucanase, β-mannanase, β-xylosidase, β-glucosidase, α-L-arabinofuranosidase, and no filter paper cellulase activities were detected. Among 12 carbon sources tested, maximum xylanase activity was induced by xylan, followed by lignocelluloses such as steamed wheat straw and alkali-treated bagasse. The level of enzyme activity produced on other carbon sources appeared to be constitutive. Among the complex organic nitrogen sources tested, the xylanase activity was most enhanced by yeast extract, followed by soymeal, Pharmamedia (cotton seed protein), and Alburex (potato protein). A batch culture at 10°C in a 5-l fermenter (3.5-1 working volume) using the optimized medium gave 385 nkat at 111 h of cultivation. The crude xylanase showed optimal activity at pH 5.0–5.5 and good stability at pH 4–9 (21 h at 4°C). Although the enzyme was maximally active at 45°–50°C, it appeared very thermolabile, showing a half-life of 78 min at 35°C. At 40°–50°C, it lost 71%–95% activity within 5 min. This is the first report on the production as well as on the properties of thermolabile xylanase produced by an Antarctic yeast.

Key words Antarctic yeast · *Cryptococcus adeliae* · Thermolabile xylanase · Medium optimization · Production · Properties

Communicated by K. Horikoshi

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Introduction

Psychrophilic and psychrotolerant microorganisms are most widely distributed in nature where 80% of the biosphere and 90% of the marine environment have temperature below 5°C (Gounot 1991; Margesin and Schinner 1994; Brenchley 1996). Psychrophilic enzymes exhibit high specific activity at low temperatures, low temperature optima, and low thermostability (Feller et al. 1996).

Xylan is the major hemicellulose in hardwoods, whereas in softwoods its abundance is next to galactomannan. Depending on plant sources and extraction procedures, xylan structure varies from linear 1,4-β-linked xylopyranoside chains to highly branched heteropolysaccharides (Aspinal 1980; Puls and Schuseil 1993). The complete degradation of xylan involves the synergistic action of endo-xylanase, β-xylosidase, and several debranching enzymes (e.g., α-L-arabinofuranosidase, esterase, α-glucuronidase (Biely 1985; Bajpai 1997).

Xylanases have been studied extensively from mesophilic and thermophilic fungi and bacteria (Bajpai 1997). On the other hand, reports on xylanases from yeasts and yeastlike microorganisms are few (Lee et al. 1986; Leathers 1986; Biely and Kremnicky 1998). Although the production and properties of a number of psychrophilic enzymes, e.g., β-lactamase, α-amylase, lipase, subtilisin, and proteases from different bacteria have been reported (Margesin and Schinner 1991; Feller et al. 1994; Gerday et al. 1997; Marshall 1997; Turkiewickz et al. 1999), there exists no report on the production and the properties of psychrophilic xylanase, except the K_m and k_{cat} values for the xylanase from an Antarctic yeast *Cryptococcus* TAE85 and *C. albidus* (Gerday et al. 1997).

Compared with the xylanases from mesophiles and thermophiles, nothing is known about the use of cold-active xylanases. However, potential biotechnological applications of cold-active xylanases together with cellulases, lipases, and proteases may be in the digestion of industrial or sewerage wastes and decomposition of agricultural

residues at ambient temperatures in cold and temperate climates (Margesin and Schinner 1994, 1998; Brenchley 1996). Other applications of cold-active enzymes, requiring much lower temperatures, may be in unheated washing machines, in biosensors for on-line environmental monitoring, and in the food and pharmaceutical industries (Margesin and Schinner 1994; Marshall 1997). Moreover, a yeast with the ability to degrade plant polysaccharides and to produce hydrolases may find application as a probiotic inclusion and therapeutic agent in feed or food (Biely and Kremnicky 1998). The present study describes, for the first time, the optimization of a culture medium for the enhanced production of a thermolabile xylanase by the Antarctic yeast *Cryptococcus adeliae* and the partial characterization of the crude enzyme.

Materials and methods

Organism, medium, and culture cultivation

Cryptococcus adeliae, isolated from the Antarctic region, was obtained from Prof. Charles Gerday, Institute of Chemistry, University of Liege, Belgium. The yeast was grown as surface culture on PDA (potato dextrose agar) plates at 4°C and maintained at this temperature for further use. Long-term storage was in liquid nitrogen. Unless otherwise stated, the strain was grown at 4°C (on a rotary shaker at 175 rpm) in 300-ml Erlenmeyer flasks containing 100 ml medium. The medium was composed of (in g l^{-1}) 2.14, $Na_2HPO_4{\cdot}12H_2O$; 1.01, KH_2PO_4; 2.2, NH_4NO_3; 0.2, $MgSO_4{\cdot}7H_2O$; 0.1 $CaCl_2{\cdot}2H_2O$; and 1 ml trace element solution (in g l^{-1}): 0.2, $CoCl_2{\cdot}7H_2O$; 0.5, $FeSO_4{\cdot}7H_2O$; 0.16, $MnSO_4{\cdot}H_2O$; 0.14, $ZnSO_4{\cdot}7H_2O$), and supplemented with the proper carbon and nitrogen sources as indicated in the experiments. The initial pH of the medium was adjusted to the desired level before sterilization at 121°C for 20 min. A 72-h-old liquid culture (6%, v/v), grown on xylan and yeast extract medium, was used as inoculum. The culture broth was centrifuged at 4°C to obtain a clear supernatant, which was used for all assays.

Bioprocess experiment

The yeast was cultivated in a 5-l bioreactor (Biostat-V; Braun Melsungen, Melsungen, Germany) with a 3.5-1 working volume. The culture temperature was maintained at 10°C, the aeration rate was 1 vvm, and the stirrer speed was between 200 and 300 rpm. The medium used was identical to that optimized for shake cultures. The inoculum (6%) was a 72-h-old shake-flask preculture, grown on identical medium. The initial pH was adjusted to 7.5 using 5% H_3PO_4 and 5% NaOH and then controlled at this level throughout cultivation.

Medium optimization

Selection of medium components and their approximate concentrations was performed using shake-flask experiments, arranged in a Graeco-Latin square, assuming that the single components acted independently and that their effects were purely additive (Auden et al. 1967; Gomes et al. 1992). The best medium component and the best concentration of a particular ingredient were determined by summing up the enzyme activities obtained with the three respective media. The concentrations of xylan and yeast extract were finally optimized using a central composite design (Retzlaff et al. 1975). Analysis of response surface was done using the computer program Statgraphic Plus, version 3 (Statistical Graphics, Rockville, MD, USA).

Enzyme assays

Xylanase, mannanase, and endoglucanase activities were assayed at 50°C according to the standard fixed time (5 min for xylanase and mannanase; 10 min for endoglucanase) assay procedure of Bailey et al. (1992) using 1% birchwood xylan (Roth, Karlsruhe, Germany), 0.5% locust bean gum (Sigma, St. Louis, MO, USA), and 1% sodium carboxymethylcellulose (Serva, Heidelberg, Germany), respectively, in citrate buffer (25 mM, pH 5.5). Filter paper cellulase activity (reaction time, 1 h) was tested according to IUPAC (1987). The liberated reducing sugars were measured by the DNS (dinitrosalicylic acid) method of Miller (1959). One nanokatal (nkat) of enzyme activity was defined as the amount of enzyme that liberated 1 nmol of xylose or mannose or glucose per second under the assay conditions. β-Xylosidase, β-glucosidase, and α-L-arabinofuranosidase activities were assayed (reaction time, 10 min) at 45°C according to Herr et al. (1978) using the corresponding *p*-nitrophenyl-containing chromogenic substrates (all were from Sigma) in citrate buffer (pH 5.5, 25 mM). One unit (nkat) of enzyme activity was the amount of enzyme that liberated 1 nmol *p*-nitrophenol per second.

Protein and reducing sugar determination

Soluble proteins in the culture filtrate (cooled to −4°C) were precipitated with 2 vols chilled (−18°C) acetone, centrifuged, and redissolved in distilled water and then determined by bicinchonic acid protein assay reagent (Pierce, Rockford, Illinois, USA) using bovine serum albumin as standard. The reducing sugars in the culture filtrates and enzymatic hydrolysates were determined according to Miller (1959).

Partial characterization of the crude xylanase

The effect of pH on xylanase activity was determined by measuring the activity at 50°C in citrate (25 mM, pH 3–6), phosphate (25 mM, pH 6–8), and glycine-NaOH (50 mM, pH 9–10) buffers. The effect of temperature on enzyme activity was determined by performing assays at pH 5.5 and at temperatures ranging from 4° to 60°C. For the determination of pH stability, the crude enzyme solution was diluted tenfold with citrate (25 mM, pH 3–6), phosphate

(25 mM, pH 6–8), and glycine-NaOH (50 mM, pH 9–11) buffers and stored for 21 h at 4°C. Thermostability was determined by incubating the enzyme solution (taken in screw-capped glass tubes) at 30° to 50°C for different time periods. The tubes were withdrawn at the indicated times and cooled in ice water. In both cases, the residual activities were determined at pH 5.5 and 50°C.

Enzymatic hydrolysis

The hydrolysis of birchwood xylan was carried out using the crude xylanase solution. The reaction mixture contained 2 g xylan, 75 ml citrate buffer (50 mM, pH 5.5) containing 1 g l^{-1} sodium benzoate, and 25 ml enzyme solution containing 350 nkat ml^{-1} xylanase activity. The mixture was incubated at 25° and 30°C on a rotary shaker (100 rpm). Samples (5 ml) were withdrawn at the indicated times, heated in a boiling water bath for 5 min, cooled, and centrifuged. The supernatant was assayed for reducing sugar (Miller 1959).

Results

Effects of initial medium pH and xylans on growth and xylanase production

To determine the optimum initial medium pH, *C. adeliae* was preliminarily grown on minimal media (10 g l^{-1} of xylan and 0.4 g l^{-1} yeast extract) having initial pH values from 5 to 8. The yeast growth as well as xylanase secretion was maximum in the medium having initial pH 7.5, irrespective of the xylan type used (Fig. 1). However, the highest biomass (OD, 6.6) and xylanase activity (92 nkat ml^{-1}) were produced on birchwood xylan, followed closely by that on oat spelt (OD, 6.4; 88 nkat ml^{-1} xylanase) and beechwood (OD, 5.8; 77 nkat ml^{-1} xylanase) xylans. Henceforth, the yeast was cultivated on birchwood xylan medium with initial pH 7.5.

Medium optimization

Shake-flask experiments, arranged in Graeco-Latin square design, were performed to select the medium components. Table 1 shows the experimental design and the xylanase activities obtained in nine different media. The highest xylanase yield (202 nka ml^{-1}) was on xylan in medium no. 3 whereas the yield (13 nkat ml^{-1}) on xylose in medium no. 1 was the lowest. Summation of xylanase activity in three

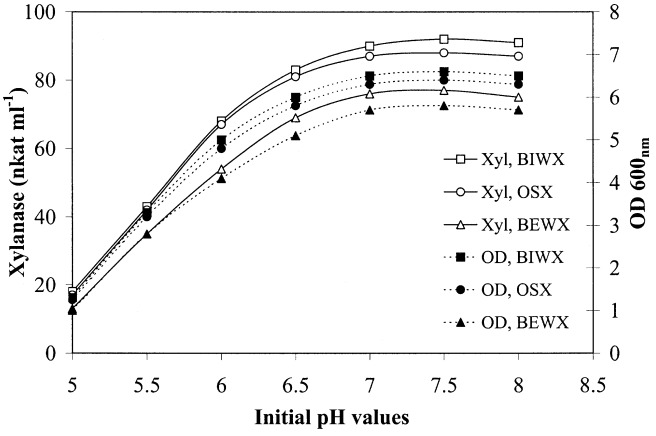

Fig. 1. Effect of initial pH of the medium on the growth and xylanase production of *Cryptococcus adeliae*. The mineral medium (g l^{-1}: 2.14, $Na_2HPO_4 \cdot 12H_2O$; 1.01, KH_2PO_4; 2, $(NH_4)_2SO_4$; 0.2, $MgSO_4 \cdot 7H_2O$; 0.1 $CaCl_2 \cdot 2H_2O$; and 0.5 ml trace element solution) was supplemented with 10 g l^{-1} of a xylan and 0.4 g l^{-1} of yeast extract. The yeast was shake-cultured for 8 days at 4°C. *Xyl*, xylanase; *OD*, optical density; *BIWX*, birchwood xylan; *OSX*, oat spelt xylan; *BEWX*, beechwood xylan

Table 1. Greaco-Latin square design for the selection of culture medium ingredients for xylanase production by *Cryptococcus adeliae* and the results obtained

Medium components[a]	Medium serial no.								
	Med 1	Med 2	Med 3	Med 4	Med 5	Med 6	Med 7	Med 8	Med 9
Xylose (g l^{-1})	10			10			10		
Locus bean gum (g l^{-1})		10			10			10	
Xylan (g l^{-1})			10			10			10
Yeast extract (g N l^{-1})	0.2	0.2	0.2						
Bactopeptone (g N l^{-1})				0.2	0.2	0.2			
Meat peptone (g N l^{-1})							0.2	0.2	0.2
$(NH_4)_2SO_4$ (g N l^{-1})	0.4					0.4		0.4	
$(NH_4)H_2PO_4$ (g N l^{-1})		0.4		0.4					0.4
NH_4NO_3 (g N l^{-1})			0.4		0.4		0.4		
Na_2HPO_4/KH_2PO_4 (mM)	5				5				5
Na_2HPO_4/KH_2PO_4 (mM)			10	10				10	
Na_2HPO_4/KH_2PO_4 (mM)		15				15	15		
Results[b]									
Xylanase (nkat ml^{-1})	13	36	202	36	23	121	37	38	153
Final pH	5.11	7.93	7.97	6.10	7.79	7.07	5.3	7.94	7.60

[a] In addition, each medium contained $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and trace element solution as stated in Materials and methods; the yeast was cultured for 9 days at 4°C

[b] Values reported are averages from duplicate shake cultures and assays with SD within 10% of the mean values

Table 2. Graeco-Latin square design for the determination of approximate concentrations of culture medium ingredients for the production of xylanase by *C. adeliae* and the results obtained

Medium components[a]	Medium serial No.								
	Med 1	Med 2	Med 3	Med 4	Med 5	Med 6	Med 7	Med 8	Med 9
Xylan ($g l^{-1}$)	5			5			5		
Xylan ($g l^{-1}$)		10			10			10	
Xylan ($g l^{-1}$)			20			20			20
Yeast extract ($g l^{-1}$)	2.5	2.5	2.5						
Yeast extract ($g l^{-1}$)				5	5	5			
Yeast extract ($g l^{-1}$)							7.5	7.5	7.5
NH_4NO_3 ($g l^{-1}$)	1.1					1.1		1.1	
NH_4NO_3 ($g l^{-1}$)		2.2		2.2					2.2
NH_4NO_3 ($g l^{-1}$)			3.3		3.3		3.3		
Results[b]									
Xylanase (nkat ml^{-1})	125	188	268	142	205	321	185	215	345
Final pH	7.25	7.20	7.30	8.07	7.99	8.01	8.19	8.15	8.11

[a] In addition, each medium contained 10 mM $Na_2HPO_4 \cdot 12H_2O/KH_2PO_4$ buffer and other ingredients as stated for Table 1; the yeast was cultured for 9 days at 4°C
[b] Values reported are averages from duplicate shake cultures and assays with SD within 10% of the mean values

Table 3. Central composite design for optimization of xylan and yeast extract concentrations of the culture medium for xylanase production by *C. adeliae* and the results obtained

Medium components[a]	Medium serial No.								
	Med 1	Med 2	Med 3	Med 4	Med 5	Med 6	Med 7	Med 8	Med 9
Xylan ($g l^{-1}$)	15	20	25	15	20	25	15	20	25
Yeast extract ($g l^{-1}$)	2.5	2.5	2.5	5.0	5.0	5.0	7.5	7.5	7.5
Results[b]									
Xylanase (nkat ml^{-1})	220	270	285	300	377	389	310	385	400
Final pH	7.30	7.20	7.50	8.20	8.15	8.07	8.34	8.31	8.25

[a] In addition, each medium contained 2.2 $g l^{-1}$ NH_4NO_3 and other ingredients as stated in Table 2; the yeast was cultured at 4°C for 9 days
[b] Values reported are averages from duplicate shake cultures and assays with SD within 10% of the mean values

respective media indicated that birchwood xylan (media 3, 6, and 9), yeast extract (media 1, 2, and 3), NH_4NO_3 (media 3, 5, and 7), and 10 mM $Na_2HPO_4 \cdot 12H_2O/KH_2PO_4$ (media 3, 4, and 8) buffer were the best for maximal xylanase production. Table 2 shows the second Latin square design and the xylanase activities (125–345 nkat ml^{-1}) obtained using three different concentrations each of xylan, yeast extract, and NH_4NO_3. The summarized xylanase activity showed that xylan at 20 $g l^{-1}$ (media 3, 6, and 9), yeast extract at 7.5 $g l^{-1}$ (media 7, 8, and 9), and NH_4NO_3 at 2.2 $g l^{-1}$ (media 2, 4, and 9) were the best concentrations for maximum xylanase production. The highest xylanase yield (345 nkat ml^{-1}) was obtained in medium no. 9.

The concentrations of carbon (xylan) and organic nitrogen (yeast extract) sources were further optimized by performing experiments, arranged in 2^3 factorial designs, as they played the most important roles in xylanase production by *C. adeliae*. The design of the experiment and xylanase activities (220–400 nkat ml^{-1}) obtained in nine different media are shown in Table 3. The results were evaluated by the response surface statistical method as described by Retzlaff et al. (1975). The coefficients of the second-order model were as follow: $b_0 = 326.2$; $b_1 = 40.7$; $b_2 = 53.3$; $b_{11} = -26.7$; $b_{12} = 6.25$; and $b_{22} = -43.7$. The optimum response was obtained at $X_1 = 0.841$ and $X_2 = 1.062$, which corresponded to 24.2 $g l^{-1}$ xylan and 10.2 $g l^{-1}$ yeast extract.

Repeated shake cultures using the optimized medium always yielded about 400 nkat ml^{-1} xylanase activity after 8–9 days of cultivation at 4°C. In addition, very low levels of endoglucanase (25 nkat ml^{-1}), β-mannanase (24 nkat ml^{-1}), β-xylosidase (2 nkat ml^{-1}), β-glucosidase (1 nkat ml^{-1}), and α-L-arabinofuranosidase (0.7 nkat ml^{-1}) and no filter paper cellulase activities were detected in the culture filtrate.

Time course of xylanase production in shake cultures

Cryptococcus adeliae was grown on the optimized medium at 4° and 24°C to compare the growth as well as xylanase production of the yeast; time courses of the enzyme production and yeast growth are shown in Fig. 2. Generally, at 4°C xylanase production and yeast growth increased slowly but steadily, yielding maximum biomass (OD at 600_{nm}, 22) and xylanase activity (400 nkat ml^{-1}) at 168 h (i.e., at the end of logarithmic growth phase), after which time

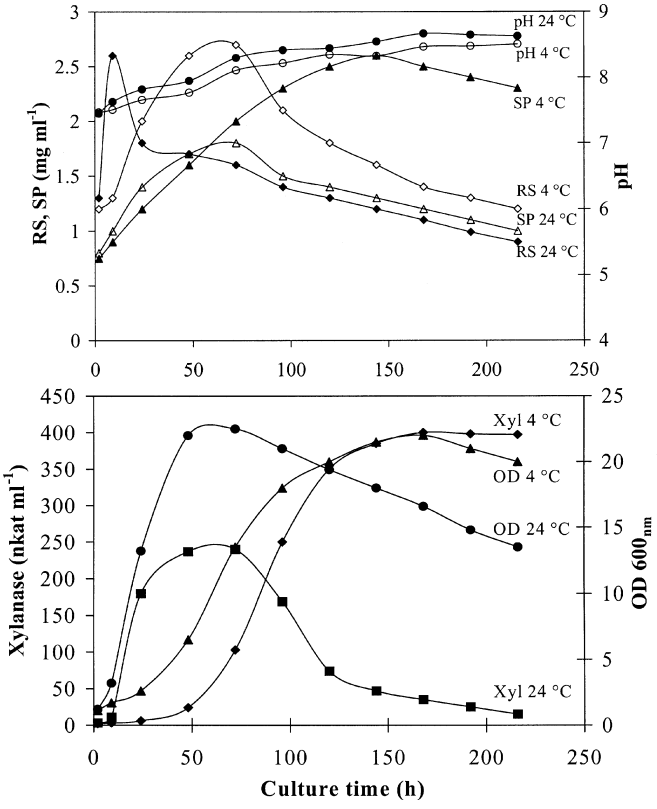

Fig. 2. Growth (OD at 600 nm) and xylanase (Xyl) activity profiles of *C. adeliae*, grown in shake-flasks on the optimized medium at 4° and 24°C. At indicated times, duplicate cultures were used for the determination of pH values, soluble protein (*SP*), reducing sugar (*RS*), and enzyme activity. The results are averages of duplicate cultures and assays with SD within 10% of the mean values

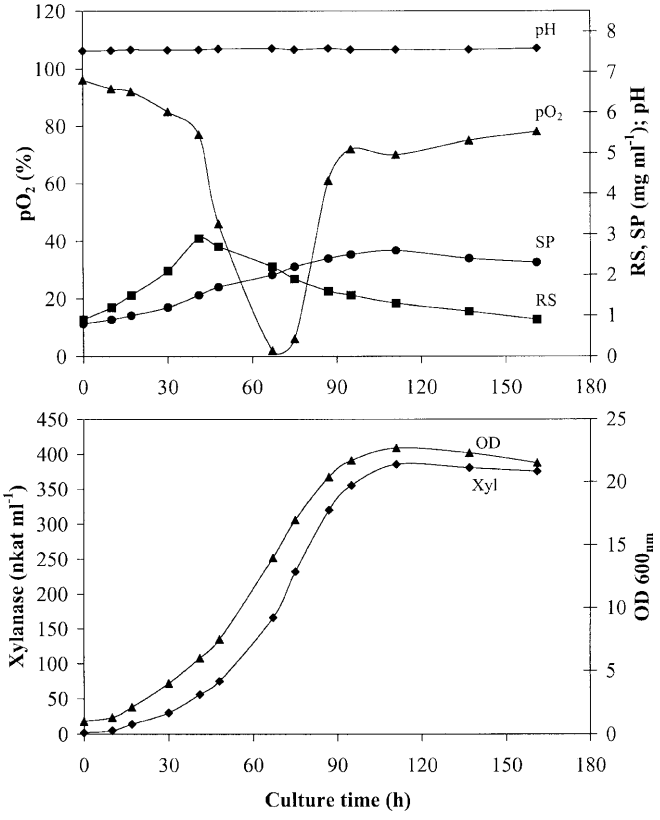

Fig. 3. Time course of a batch cultivation of *C. adeliae* in a 5-l bioreactor using the optimized medium

enzyme activity remained almost at the same level up to 216 h. On the other hand, at 24°C the biomass and xylanase production increased sharply immediately after initiation of shake cultures giving the highest biomass (OD, 22.5) and xylanase titer (240 nkat ml^{-1}) within 72 h, after which the enzyme activity declined very rapidly, reaching only 15 nkat ml^{-1} at 216 h. The level of soluble protein increased with the increase of enzyme activity and then declined with the decline in activity. The pH value, initially adjusted to 7.5, increased slowly with the progress of cultivation.

Xylanase production in bioreactor

The production of xylanase by *C. adeliae* was studied in a 5-l bioreactor using the optimized culture medium. A typical batch cultivation time course under controlled pH is shown in Fig. 3. After inoculation with a 72-h-old preculture (6% v/v), the yeast continued to grow progressively and the oxygen concentration (pO_2) dropped from an initial value of 96% to 2% within 67 h. Very little xylanase was secreted up to 48 h, after which the enzyme secretion increased remarkably, giving a final titer of 385 nkat ml^{-1} at 111 h at which point the biomass yield (OD, 22.7) was also maximum.

Influence of some carbon and nitrogen sources on enzyme production by *C. adeliae*

Among the carbon sources tested (Table 4), the highest xylanase activity (395 nkat ml^{-1}) was induced by xylan, followed by pretreated wheat straw (93 nkat ml^{-1}) and bagasse (65 nkat ml^{-1}. Xylanase production on other carbon sources was very low (13–38 nkat ml^{-1}). Among the organic nitrogen sources tested, the highest xylanase activity was obtained with yeast extract (397 nkat ml^{-1}), followed by soymeal (389 nkat ml^{-1}), Pharmamedia (363 nkat ml^{-1}), and Alburex (337 nkat ml^{-1}). Enzyme production on other organic nitrogen sources was 212–287 nkat ml^{-1}.

Properties of crude xylanase

Cryptococcus adeliae xylanase was most active at 45°–50°C, with a 5-min reaction time, showing 3% and 40% of the maximal activity at 4° and 60°C, respectively (Fig. 4a). The enzyme appeared to be very thermolabile as it retained full activity for 120 min at 30°C and 50% activity for 78 min at 35°C, whereas only 5%, 10%, and 29% of the maximum activity was retained after 5-min heating at 50°, 45°, and 40°C, respectively (Fig. 4b). The xylanase hydrolyzed xylan maximally at pH 5.0–5.5, exhibiting only 21% and 11% activity at pH 4 and 8, respectively (Fig. 5a). The xylanase was fairly stable between pH 4 and 9 after 21-h incubation

Table 4. Effects of some carbon and nitrogen sources on xylanase production by *C. adeliae*

	Xylanase (nkat ml^{-1})[c]	Final medium pH
Carbon sources[a]		
Arabinogalactan	27	8.45
Bagasse (alkali-treated, 0.25 mm)	65	8.44
Corn cobs (untreated, 0.25 mm)	35	8.42
Carboxymethyl cellulose	15	8.50
Jute (steamed, 0.25 mm)	23	8.41
Lactose	14	7.98
Locust bean gum	38	8.25
Solka floc	13	8.41
Starch	15	8.35
Wheat straw (steamed, 0.25 mm)	93	8.41
Xylan	395	8.28
Xylose	25	7.14
Nitrogen sources[b]		
Alburex (potato protein)	337	7.50
Bactopeptone	269	8.30
Casein peptone	212	8.04
Fish peptone	287	8.24
Meat peptone	220	8.26
Pharmamedia (cotton seed protein)	363	7.73
Solulys (spray dried corn-steep liquor)	278	8.37
Soymeal	389	7.87
Yeast extract	397	8.35

[a] The optimized basal medium was supplemented with 24.2 g l^{-1} carbon source and 10.2 g l^{-1} yeast extract

[b] Each organic nitrogen source was added at equal concentration (1.1 g N l^{-1}) to the basal medium containing 24.2 g l^{-1} xylan; the yeast was cultivated for 9 days at 4°C

[c] The data shown are means of duplicate shake cultures and assays with SD within 10% of the mean value

at 4°C, showing the highest stability at pH 7 (Fig. 5b). Figure 6 shows that xylan was hydrolyzed rapidly by the crude *C. adeliae* xylanase up to 6 h, releasing 3.8 g l^{-1} (19% hydrolysis) and 4.5 g l^{-1} (22.5% hydrolysis) total reducing sugars at 25° and 30°C, respectively. Further hydrolysis up to 48 h proceeded very slowly, yielding only 5.2 g l^{-1} (26% hydrolysis) and 5.9 g l^{-1} (29.5% hydrolysis) reducing sugars at 25° and 30°C, respectively.

Discussion

To our knowledge, this is the first detailed report on the production and properties of a thermolabile xylanase produced by an Antarctic yeast. Among the various factors influencing the microbial performance, pH is of major importance in microbial growth and enzyme production. *C. adeliae* grew best and secreted the highest level of xylanase in the medium having initial pH 7.5. The differences in the ability of the yeast to utilize different xylans for its growth and xylanase production are most likely due to the presence of different side groups on the xylans originating from birchwood, oat spelt and beech wood. Statistically designed shake-culture experiments were found to be effective in optimizing culture medium composition for the increased production of xylanase by the yeast. The first Graeco-Latin square experiments helped to select the best carbon and nitrogen sources as well as the strength of phosphate buffer for increased xylanase production. Xylan was found to be a potent inducer of xylanase, whereas only a constitutive level of enzyme was produced in the presence of locust bean gum and xylose. The greater production of xylanase in the presence of yeast extract is most probably the result of its better amino acid composition and mineral and vitamin content. As summation of xylanase activity (second Graeco-Latin experiment) for different concentrations of xylan and yeast extract showed greater variation, it was clear that carbon and organic nitrogen sources had more influence on xylanase production. Final optimization of the concentrations of xylan and yeast extract using central composite designs yielded 400 nkat ml^{-1} xylanase, which was 4.3 times higher than the value obtained in the preliminary experiment. This yield of xylanase by a psychrophilic yeast is appreciable. There are no data available in the literature on psychrophilic xylanase yield. Further, detection of very low levels of endoglucanase, β-mannanase, β-xylosidase, β-glucosidase, and α-L-arabinofuranosidase and absence of filter paper cellulase activity indicate this yeast *C. adeliae* to be a predominant producer of xylanase.

Secretion of extracellular xylanase commenced already with the early growth phase of *C. adeliae*, when cultured at both 4° and 24°C. The much more rapid yeast growth at 24°C and secretion of the highest xylanase activity (240 nkat ml^{-1}) within 72 h followed by rapid and continuous loss of activity led us to assume that combined effects of temperature (24°C) and shear force (due to shaking at

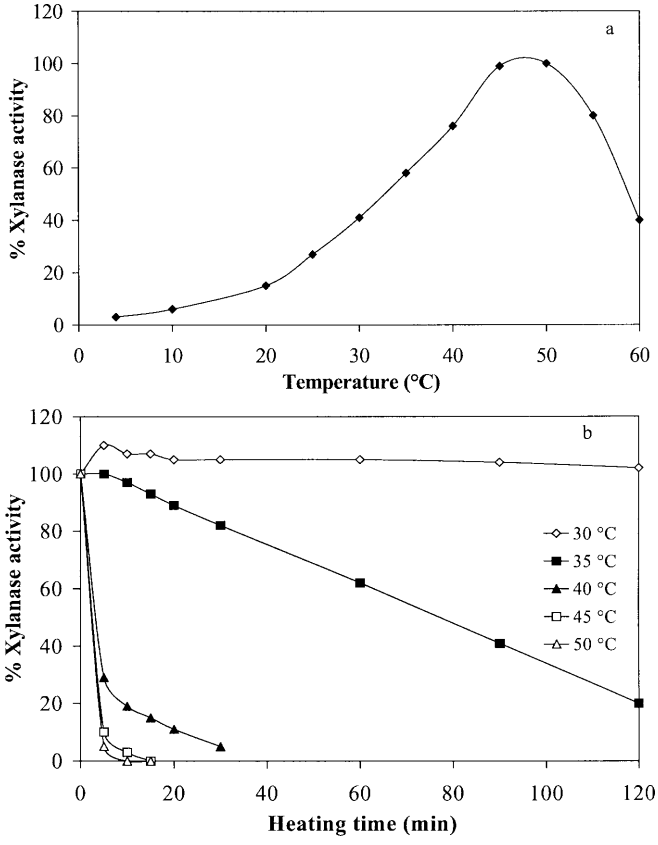

Fig. 4a,b. Effect of temperature on (**a**) activity and (**b**) stability of *C. adeliae* xylanase. Data shown are averages of triplicate assays with SD within 10% of the mean values

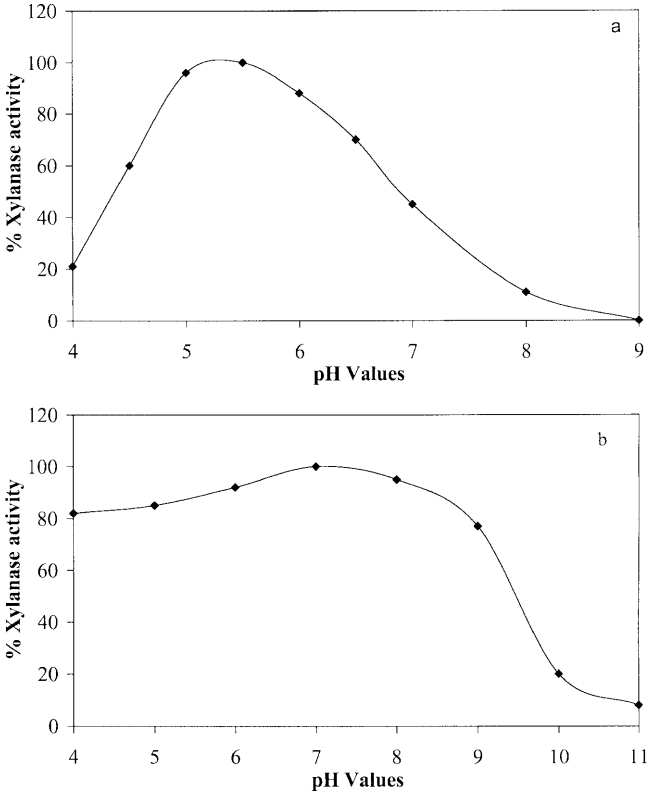

Fig. 5a,b. Effect of pH on (**a**) activity and (**b**) stability of *C. adeliae* xylanase; 100% values for **a** and **b** represent 318 nkat ml^{-1} and 378 nkat ml^{-1}, respectively. Data shown are averages of triplicate assays with SD within 10% of the mean values

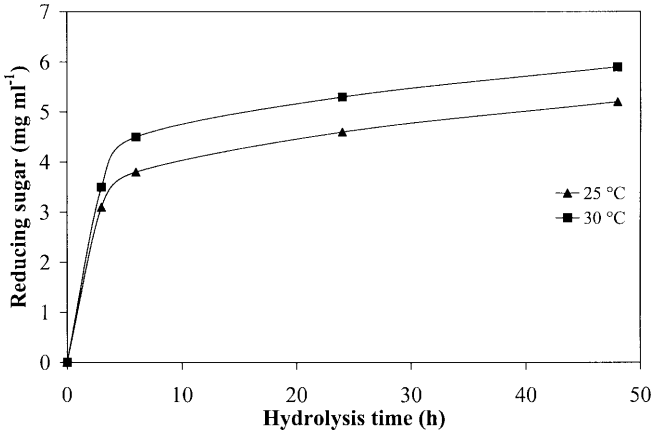

Fig. 6. Time courses of xylan hydrolyses by the crude *C. adeliae* xylanase (at pH 5.5, 25°, and 30°C). Data shown are averages of duplicate hydrolyses and assays with SD with 10% of the mean values

175 rpm) caused irreversible denaturation of the enzyme protein structure. It is also presumed that the rate of enzyme synthesis was much faster than its inactivation rate up to 72 h, when enzyme synthesis ceased but the inactivation process continued. On the other hand, the slower but steady yeast growth and enzyme secretion at 4°C, yielding maximum biomass (OD, 22) and xylanase activity (400 nkat ml^{-1}) at 168 h, followed by no remarkable loss of activity indicate that shear force from shaking at 175 rpm had no effect on enzyme activity at this temperature. The enzyme synthesis occurring at steady state at low temperature is one of the adaptation mechanisms of the organisms to the surrounding environment (Gounot 1991; Margesin and Schinner 1994; Brenchley 1996). Similar observations were made with other psychrophiles that grow more rapidly at higher temperatures, but the secreted enzyme lose activity during cultivation time (Feller et al. 1994; Turkiewicz et al. 1999).

Although the final xylanase titer of 385 nkat ml^{-1} obtained in the bioreactor was slightly lower than that obtained in the shake cultures, maximum xylanase productivity (3737 nkat l^{-1} h^{-1}, i.e., 355 nkat ml^{-1} at 95 h) in the bioreactor was higher than that (2917 nkat l^{-1} h^{-1}, i.e., 350 nkat ml^{-1} at 120 h) in shake flasks. The higher volumetric productivity of xylanase in the bioreactor was the result of reduced lag phase and fermentation time. Cultivation of the yeast at a higher temperature, i.e., 10°C, did not show any adverse effect on the production and stability of the xylanase. Accordingly, large-scale production of xylanase by the Antarctic *C. adeliae* should be possible at this temperature.

The pH optimum and pH stability observed for this thermolabile xylanase are comparable to those reported for

many mesophilic and thermophilic microorganisms (Biely 1985; Gomes et al. 1992; Bajpai 1997). The optimal temperature (45°–50°C) found for *C. adeliae* xylanase activity, with 5-min reaction time, is similar to the values reported for the proteases of psychrophilic bacteria (Margesin and Schinner 1991; Hamamoto et al. 1994). On the other hand, psychrophilic α-amylase (Feller et al. 1994), β-lactamase (Feller et al. 1995), lipase (Arpigny et al. 1997), and protease (Turkiewicz et al. 1999) have been reported to exhibit maximum activity at 15°–30°C. High activity at low temperature is another important feature of all psychrophilic enzymes. For example, α-amylase (Feller et al. 1994), β-lactamase (Feller et al. 1995), lipase (Arpigny et al. 1997), and protease (Margesin and Schinner 1991; Hamamoto et al. 1994) from psychrophilic organisms have been reported to exhibit 5%–30% of the maximal activity at 0°–10°C. However, the lower level of activity (3%–6% of the maximal activity) of the *C. adeliae* xylanase at 4°–10°C compared with other psychrophilic enzymes is most likely due to the weak solubility of the assay substrate xylan at these temperatures.

The very low thermal stability of the *C. adeliae* xylanase is most probably the result of increased protein flexibility. Similar reduced thermal stability of many cold-adapted enzymes compared with their temperate counterparts has been observed (Feller et al. 1995; Gerday et al. 1997). Although some microbes are known to produce multiple xylanases (Gilbert et al. 1992; Biely et al. 1993), the isoenzymic composition of the thermolabile crude xylanase of *C. adeliae* could not be determined. As this xylanase was able to act over a very broad range (4°–60°C) of temperature, lost 71%–95% activity after 5-min incubation at 40°–50°C, and retained some activity (5%–29%) for a longer time, it is assumed that this xylanase exists in multiple forms. The characteristics such as low optimal temperature, catalytic activity at low temperature, and very low thermal stability of the *C. adeliae* xylanase can be related to its ability to adapt to permanently cold environments. Hydrolysis of xylan by the crude xylanase was limited (26% at 25°C and 29.5% at 30°C), most probably because of the deficiency of β-xylosidase in the enzyme complex or partial inactivation of the enzyme with the course of hydrolysis at the working temperatures.

The production of thermolabile xylanase by the Antarctic yeast *C. adeliae* was increased remarkably by optimizing the medium composition using statistical designs. At present no biotechnological applications of cold-active xylanase have been established. However, cold-active xylanase may be suitable for applications such as digestion of industrial or sewage wastes and decomposition of agricultural residues at low or ambient temperatures. Moreover, the yeast *C. adeliae*, with its ability to degrade plant polysaccharides and produce hydrolases, may find application as a probiotic inclusion and therapeutic agent in feed or food.

Acknowledgments The financial support of the EC project Extremophiles as Cell Factories (contract ERB BIO4 CT96 0488) and of the Austrian Federal Ministry of Science and Transports, Vienna, is gratefully acknowledged. I. Gomes thanks Austrian Academic Exchange Service for offering a postdoctoral fellowship, and Bangladesh Jute Research Institute is thanked for granting a study leave.

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