Journal of Industrial Microbiology, 13 (1994) 220–224
© 1994 Society for Industrial Microbiology 0169-4146/94/$09.00
Published by The Macmillan Press Ltd

Optimization of cellulase-free xylanase production by a novel yeast strain

K. B. Bastawde, U. S. Puntambekar and D. V. Gokhale

NCIM, Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

(Received 13 December 1993; accepted 10 March 1994)

Key words: New isolate of yeast strain; β-xylanase; α-L-arabinofuranosidase; Xylan fermentation

SUMMARY

A novel yeast strain, NCIM 3574, isolated from a decaying wood produced up to 570 IU ml^{-1} of xylanolytic enzymes when grown on medium containing 4% xylan. The yeast strain also produced xylanase activity (40–50 IU ml^{-1}) in the presence of soluble carbon sources like xylose or arabinose. No xylanase activity was detected when the organism was grown on glucose. The crude xylanase preparation showed no activity towards cellulolytic substrates but low levels of β-xylosidase (0.1 IU ml^{-1}) and α-L-arabinofuranosidase (0.05 IU ml^{-1}) were detected. The temperature and pH optima for the crude xylanase preparation were 55 °C and 4.5 respectively. The crude xylanase produced mainly xylose from xylan within 5 min. Prolonged hydrolysis of xylan produced xylobiose and arabinose, in addition to xylose, as the end products. The presence of arabinose as one of the end products in xylan hydrolysate could be due to the low levels of arabinofuranosidase enzyme present in the crude fermentation broth.

INTRODUCTION

Xylan consists of β-1,4-linked xylose with substituents such as acetyl, arabinosyl and glucuronyl residues [2,19]. Xylan is hydrolyzed by β-xylanases attacking internal xylosidic linkages on the xylan backbone while β-xylosidases release xylose residues by endwise attack on xylooligosaccharides [21]. Since hemicelluloses in many plants also contain substituents such as arabinosyl and/or acetyl groups, complete degradation of xylan generally requires the presence of other enzymes like arabinofuranosidases, in addition to β-xylanases, that remove side chain substituents [9]. Several xylan-degrading enzymes (principally endoxylanases) have been isolated and characterized from filamentous fungi and bacteria [2,21]. Among the yeasts *Cryptococcus albidus* [5], *Aureobasidium pullulans* [13] and *Pichia stipitis* [14] are known to produce xylan-degrading enzymes. Certain naturally occurring isolates of *Aureobasidium pullulans*, previously described as color variants, overproduce extracellular xylanase with exceptionally high specific activity [10]. Very limited information is available on the production of hemicellulolytic enzymes from bacteria, yeast and fungal cultures liberating both xylose and arabinose [7]. Yeasts, being unicellular eukaryotic microorganisms, have good potential as model systems for studying the mechanisms of control of gene expression. Due to the great industrial importance and interest shown in hemicellulose degradation [15], we initiated studies with the aim to isolate yeast strains producing high levels of hemicellulose-degrading enzymes. The present work describes the isolation of a yeast strain from decaying wood producing xylanase in high yields. The production and properties of cellulase-free xylanase produced by this newly isolated yeast strain are reported.

Correspondence to: K.B. Bastawde, NCIM, Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India.

MATERIALS AND METHODS

Organism and culture media

The yeast strain was isolated from decaying sandal wood and maintained on MGYP or PDA slopes. It is deposited with the National Collection of Industrial Microorganisms (NCIM), Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India, with accession No. 3574 [3]. MGYP medium contained (per liter of distilled water) malt extract, 3 g; glucose, 10 g; yeast extract, 3 g; Bactopeptone, 5 g and agar, 20 g. Potato dextrose agar (PDA) contained (per liter of distilled water) extract from 200 g of potatoes, glucose, 20 g; yeast extract, 1 g and agar, 20 g. Aspergillus minimal medium (AMM) [17], supplemented with yeast extract (0.1%, w/v) and Bactopeptone (0.5%, w/v), was used for enzyme production. Soluble or insoluble carbon sources were added as indicated in the text. The pH of the medium was adjusted to 5.5 before the medium was autoclaved.

Isolation of yeast strains

The piece of decaying wood was macerated in sterile distilled water. The suspension was plated on MGYP agar containing penicillin (50 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}). Cells from the pink-colored colonies which appeared after 48 h incubation at 30 °C exhibited yeast-like morphology under the microscope. All these colonies were transferred to agar plates containing Aspergillus minimal

medium (AMM) [17], supplemented with yeast extract (0.1%, w/v), Bactopeptone (0.5%, w/v) and xylan (0.2%, w/v). After 48 h incubation at 30 °C, the clearance zone of xylan hydrolysis was visualized using congo red [16]. About 55 colonies showing the clearance zone were further screened for xylanase production in liquid culture.

Xylanase production in liquid culture

Culture was grown in MGYP liquid medium in tube (5 ml) for 48 h at 30 °C on a reciprocal shaker at 200 r.p.m. The seed culture (5 ml) was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of AMM with 1% (w/v) xylan or different carbon sources (see Table 1). The flasks were incubated at 30 °C on a reciprocal shaker and 2-ml samples were removed at various time intervals. The samples were centrifuged at 10 000 r.p.m. for 20 min and the supernatant fluid was used to determine extracellular enzyme activity.

Effect of pH and temperature on enzyme production

The organism was grown at different initial pHs (3.0–7.0) at 30 °C and enzyme production was monitored up to 24 h of incubation. To study the effect of temperature on xylanase production, the culture was grown at six different temperatures (12, 20, 25, 28, 30 and 35 °C). Samples were removed up to 24 h of incubation and analyzed for xylanase activity.

The effect of xylan concentration on xylanase production

The organism was grown in a medium containing different concentrations of xylan (0.5–6.0%, w/v). Samples were removed at 12, 24, 48 and 72 h for determination of xylanase activity.

Enzyme assays

Xylanase (EC 3.2.1.8) activity was assayed as described earlier [1]. A 0.5 ml sample of suitably diluted culture filtrate was mixed with 0.5 ml of 1% (w/v) oat spelts xylan (Sigma, St Louis, MO, USA) solution in citrate buffer (50 mM, pH 4.5) and incubated at 55 °C for 30 min. The reaction was terminated by the addition of 1 ml of dinitrosalicylic acid (DNS). Endoglucanase (EC 3.2.1.4) activity was assayed under conditions similar to those described above except that 1% (w/v) carboxymethylcellulose (CMC, 0.7 DS, Sigma) was used as substrate instead of xylan. β-xylosidase (EC 3.2.1.37) activity was determined by the method of Berghem and Pettersson [4] using *p*-nitrophenyl-β-D-xyloside (Sigma) as substrate. The reaction mixture consisted of 0.9 ml of the substrate (1 mg ml^{-1}) in citrate buffer (50 mM, pH 4.5) and 0.1 ml of suitably diluted culture filtrate. The reaction was carried out at 55 °C for 30 min and then terminated by the addition of 2 ml of 2% (w/v) sodium carbonate solution. The *p*-nitrophenol liberated was measured at 410 nm. α-L-arabinofuranosidase (EC 3.2.1.55) was estimated under conditions similar to those described for β-xylosidase activity using *p*-nitrophenyl-α-L-arabinofuranoside (1 mM) as substrate.

Reducing sugars were determined as xylose or glucose equivalents by the DNS method of Fischer and Stein [8]. One unit of enzyme activity was defined as the amount of enzyme which produced one μmole of xylose, glucose or *p*-nitrophenol per min per ml of crude filtrate from the appropriate substrate.

Chromatographic analysis of xylan hydrolysis products

End product analysis was carried out by hydrolyzing xylan with crude enzyme preparation (100 IU ml^{-1}). The hydrolysis was carried out at 55 °C for 5, 10, 30, 60 min and the hydrolyzed samples were boiled for 10 min to stop the reaction. The end products were identified using ascending paper chromatography with a solvent system of *n*-butanol:pyridine:water (46:36:19, v/v) by the method of Trevelyen et al. [20]. After 20 h the chromatographic paper was air dried and sprayed with 0.1% (w/v) silver nitrate in aqueous acetone. Subsequently the paper was sprayed with 0.5% (w/v) NaOH in aqueous ethanol to intensify the reducing sugar spots. Finally, the chromatograph was washed

TABLE 1

The effect of carbon sources on xylanase production

Carbon source	Xylanase activity IU ml^{-1} (±SD)	pH
Xylan	220 ± 20	6.8
Wheat bran	150 ± 20	6.5
Xylose	50 ± 5	7.2
Arabinose	40 ± 6	6.0
Galactose	62 ± 4	7.6
Maltose	50 ± 7	7.3
Lactose	1.2	6.9
Glucose	0	7.5
Glycerol	0	8.0
Sucrose	0	6.0
Inositol	0	6.4
Inulin	0	6.3
Carboxyl methylcellulose	0	5.6

TABLE 2

The effect of different xylan concentrations on xylanase production

% Xylan	Xylanase activity (IU ml^{-1})							
	12 h		24 h		48 h		72 h	
	IU	pH	IU	pH	IU	pH	IU	pH
0.5	27	6.3	198	7.3	215	7.2	210	7.4
1.0	27	5.4	215	6.4	227	6.7	293	7.2
2.0	17	5.3	184	6.5	213	6.7	249	7.1
3.0	11	4.7	145	6.0	196	6.3	237	6.4
4.0	22	4.2	132	5.5	236	6.0	570	6.4
5.0	21	4.0	100	4.9	191	5.6	517	6.0
6.0	12	3.8	72	4.2	101	4.8	477	5.4

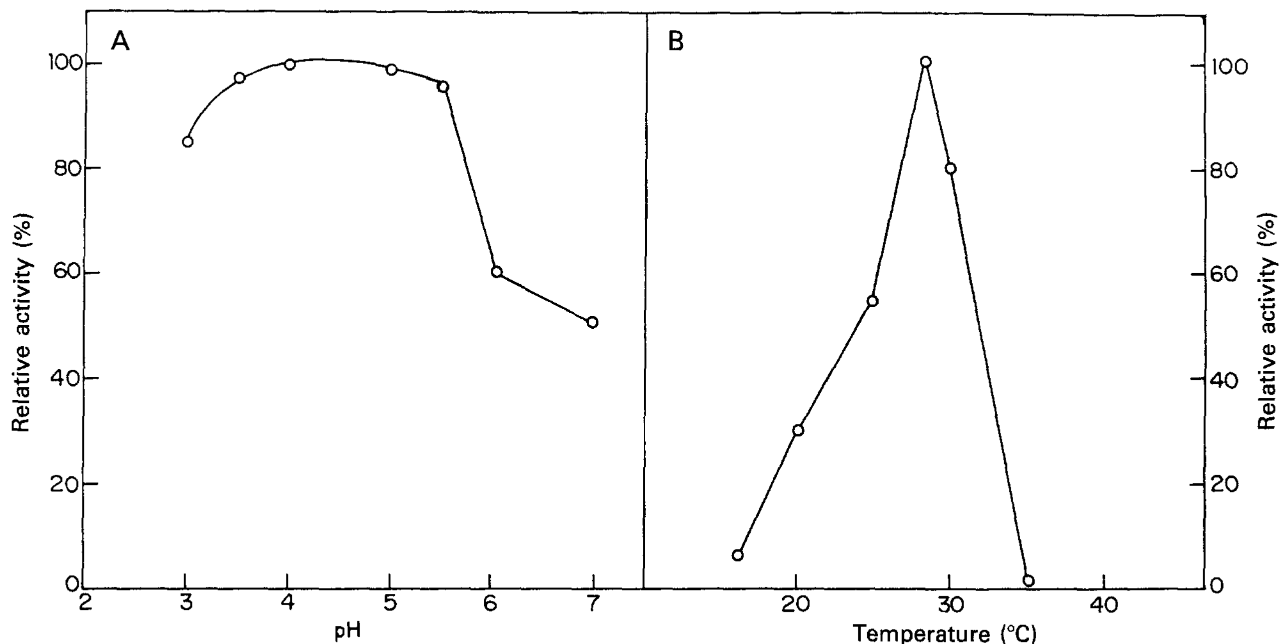

Fig. 1. Xylanase production by yeast at different initial pHs (A) and temperatures (B). Xylanase activity was determined after 24 h of incubation. Enzyme activity is expressed as the relative activity.

with 5% (w/v) sodium thiosulfate followed by thorough washing with distilled water and then air dried.

RESULTS

Isolation of yeast strains

More than 50 pink colonies exhibiting yeast-like morphology were tested for the production of xylanase in liquid culture. On the basis of this screening, one of the yeast isolates, NCIM 3574, producing high levels of xylanase was selected for further studies. When grown at 30 °C in AMM with larchwood xylan, the culture produced a good amount (200 IU ml^{-1}) of xylanase but low levels of β-xylosidase (0.1 IU ml^{-1}) and α-L-arabinofuranosidase (0.05 IU ml^{-1}). The xylanase preparation did not show activity towards carboxymethylcellulose (CMC).

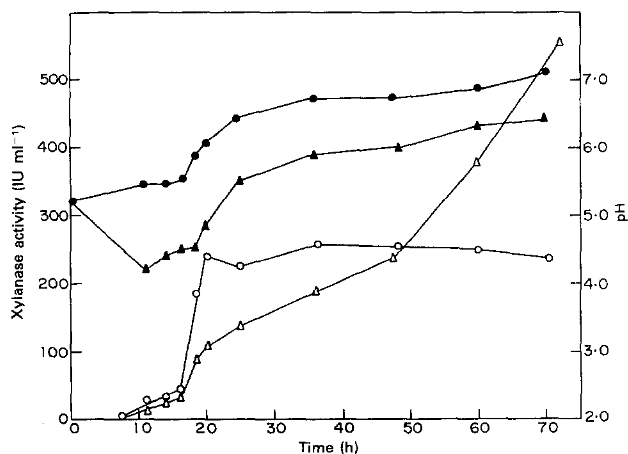

Fig. 2. The time course effect on xylanase production by yeast using 1% xylan: enzyme activity (○), pH (●); and 4% xylan: enzyme activity (△), pH (▲).

Effect of different carbon sources on xylanase production

The effect of different carbon sources on xylanase production are summarized in Table 1. Growth on xylan resulted in maximum production of xylanase activity whereas soluble carbon sources like xylose, arabinose, galactose and maltose induced only ~30% as much activity. Other soluble carbon sources including glucose, lactose, glycerol and sucrose failed to induce xylanase. Different concentrations of xylan were tested and 4% xylan produced the highest yields of enzyme (Table 2). Though low levels of xylanase were detected initially, high yields were obtained after 72 h. Since xylan was the best inducer, further studies were carried out using xylan as the carbon source.

Effect of temperature and initial pH on enzyme production

The effect of initial pH on xylanase production is shown in Fig. 1(A). Growth at pH values above 5.5 decreased xylanase production. Maximum xylanase production was observed at pH values between 3.5 and 5.5. The optimum temperature for xylanase production was 28 °C, whereas no enzyme production was observed at 35 °C (Fig. 1(B)). The organism grew well between temperatures 25–30 °C but no growth was obtained at 35 °C (data not shown).

Xylanase production as a function of time

Xylanase production by yeast using 1% and 4% xylan is shown in Fig. 2. With 1% xylan, xylanase activity appeared only after 12 h and reached its maximum after 20 h of incubation. No significant increase in xylanase activity was

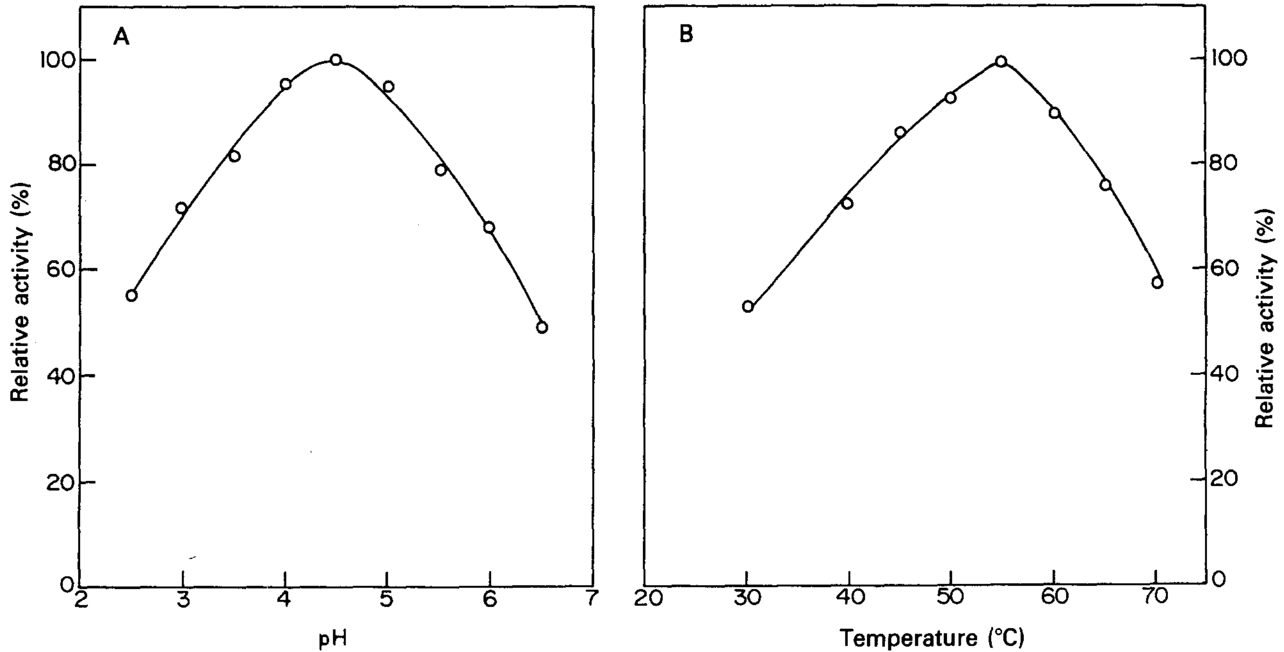

Fig. 3. Effect of pH (A) and temperature (B) on xylanase activity. Xylanase activity was determined using 0.05 M sodium citrate buffer.

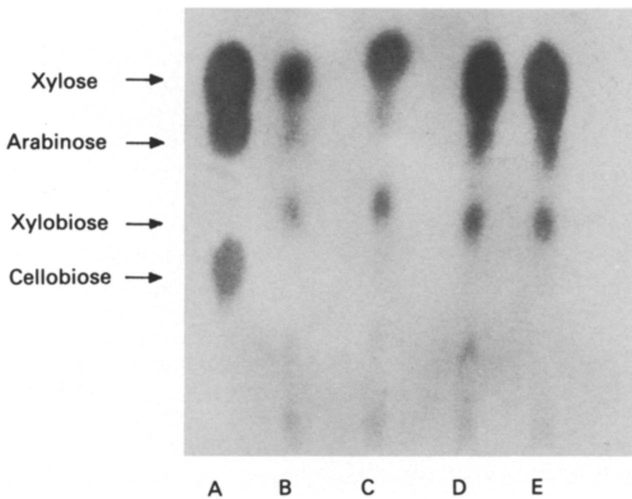

Fig. 4. Paper chromatography of the products of xylan hydrolysis by a crude xylanase preparation after different time intervals: (A) standards (B) 5 min (C) 10 min (D) 30 min (E) 60 min.

observed during further incubation. During growth of yeast on 1% xylan the pH increased from 5.2 to 6.7. Growth on 4% xylan gave maximum enzyme activity after 72 h when the pH had increased to 6.5 after an initial drop to 4.2.

pH and temperature optima of xylanase

pH and temperature optima were determined for the crude xylanase preparation. The optimum pH for enzyme activity was 4.5 (Fig. 3(A)) while the optimum temperature for enzyme activity was 55 °C (Fig. 3(B)).

End-product analysis of xylan hydrolysate

The main breakdown product of xylan hydrolysis was xylose with traces of xylobiose within 5 min of hydrolysis (Fig. 4). During further digestion of xylan up to 60 min, increases in xylose, arabinose and xylobiose were observed.

DISCUSSION

Xylanases are receiving much attention due to their applications in industry as well as in various fields of biochemistry. Xylanases from fungi have been well documented and intensively studied.

Among yeasts, *Trichosporon* [18], *Cryptococcus albidus* [6] and *Cryptococcus flavus* [22] produce low levels of xylanase. Recently a naturally occurring color variant of *Aureobasidium pullulans* was reported to produce very high levels of xylanase [10,12]. The results in this paper reveal that the newly isolated yeast strain from decaying wood, produced xylanase in high quantities (200–250 IU ml^{-1}) when it was grown on xylan. This level of xylanase activity is quite high compared to those of other yeast strains including the color variant of *Aureobasidium pullulans* [10,12]. The extracellular xylanase produced by our yeast strain is a typical inducible enzyme and was induced by xylan more effectively than by xylose or arabinose. The xylanase of *Cryptococcus flavus* is induced by xylose, xylooligosaccharides or xylan [22]. Xylan in combination with β-methylxyloside acted as the best inducer of xylanase in *Aureobasidium pullulans* [12]. Essentially no growth or xylanase production by yeast NCIM 3574 was observed on cellulose 123 or carboxymethylcellulose, indicating the absence of cellulases in this yeast strain. Similar observations were reported for *Aureobasidium pullulans* [11,12].

The xylanase production profile showed that the newly isolated yeast strain produced 240 IU ml^{-1} of xylanase activity within 20 h incubation using 1% xylan as the only carbon source. Growth on 4% xylan resulted in production of xylanase in high amounts (570 IU ml^{-1}) only after prolonged incubation (72 h). This activity appears to be very high compared to that produced by *Aureobasidium pullulans* [10]. Hydrolysis of xylan with crude enzyme produced xylose as a major product within a short time indicating its exo-type action. In addition, arabinose was also detected in the hydrolysate which could be due to the presence of low levels of α-L-arabinofuranosidase in the culture filtrate. The further characterization of these two enzymes is in progress. The potential industrial application of such cellulase-free xylanase, especially from yeast strains having activity at neutral pH, will be attractive in the paper and pulp industries to improve paper pulp quality as well as to minimize environmental pollution which occurs due to the use of hazardous chemicals by these industries.

ACKNOWLEDGEMENTS

We are grateful to Mrs Sandhya Sudage for her technical assistance during this work.

REFERENCES

1 Bastawde, K.B. 1992. Cellulolytic enzymes of a thermotolerant *Aspergillus terreus* strain and their action on cellulosic substrates. World J. Microbiol. Biotechnol. 8: 45–49.

2 Bastawde, K.B. 1992. Xylan structure, microbial xylanases and their mode of action. World J. Microbiol. Biotechnol. 8: 353–368.

3 Bastawde, K.B. and D.V. Gokhale. 1991. A process for the preparation of extracellular arabinose liberating exoxylanase from a pure yeast strain. Indian Patent Application no. 1119/DEL/91 dated 18.11.91.

4 Berghem, L.E.R. and L.G. Pettersson. 1973. The mechanism of enzymic cellulose degradation: purification of a cellulolytic enzyme from *Trichoderma viride*, active on highly ordered cellulose. Eur. J. Biochem. 37: 21–30.

5 Biely, P., Z. Kratky, A. Kochova-Kratochilova and S. Bauer. 1978. Xylan degrading activity in yeast: growth on xylose, xylan and hemicelluloses. Folia Microbiol. 27: 366–371.

6 Biely, P., M. Vrsanka and Z. Kratky. 1980. Xylan degrading enzymes of the yeast *Cryptococcus albidus*. Identification and cellular localization. Eur. J. Biochem. 108: 313–321.

7 Dekker, R.F.H. 1985. Degradation of the hemicellulose. In: Biosynthesis of Wood Components (Higuchi, T., ed.), pp. 505–533, Academic Press, Tokyo.

8 Fischer, E.H. and E.A. Stein. 1961. Amylase from human saliva. Biochem Preparations 8: 27–33.

9 Johnson, K.G., M.C. Silva, C.R. Mackenzie, H. Schneider and J.D. Fontana. 1989. Microbial degradation of hemicellulosic materials. Appl. Biochem. Biotechnol. 21: 245–257.

10 Leathers, T.D. 1986. Color variants of *Aureobasidium pullulans* overproducing xylanases with extremely high specific activity. Appl. Environ. Microbiol. 52: 1026–1030.

11 Leathers, T.D. 1989. Purification and properties of xylanase from *Aureobasidium*. J. Ind. Microbiol. 4: 341–348.

12 Leathers, T.D., R.M. Detroy and R.J. Bothast. 1986. Induction and glucose repression of xylanase from a color variant strain of *Aureobasidium pullulans*. Biotechnol. Lett. 8: 867–872.

13 Leathers, T.D., C.P. Kurtzman and R.M. Detroy. 1984. Overproduction and regulation of xylanase in *Aureobasidium pullulans* and *Cryptococcus albidus*. Biotechnol. Bioeng. Symp. 14: 225–240.

14 Lee, H., R.J.B. To, R.K. Latta, P. Biely and H. Schneider. 1987. Some properties of extracellular acetylxylan esterase produced by the yeast *Rhodotorula muciloginosa*. Appl. Environ. Microbiol. 53: 2831–2834.

15 Linko, M. 1989. Enzymes in the forefront of food and feed industries. Food Biotechnol. 3: 1–9.

16 Mackenzie, C.R. and R.E. Williams. 1984. Detection of cellulase and xylanase activity in isoelectric focussed gels using agar substrate gels supported on plastic film. Can. J. Microbiol. 30: 1522–1525.

17 Pontecorvo, G., J.A. Roper, L.M. Hemmons, K.D. McDonald and A.W.J. Bufton. 1953. Genetics of *Aspergillus nidulans*. Adv. Genet. 5: 142–238.

18 Stevens, B.J. and J. Payne. 1977. Cellulase and xylanase production by yeasts of the genus *Trichosporon*. J. Gen. Microbiol. 100: 381–393.

19 Thompson, N.S.C. 1983. Hemicellulose as a biomass resource. In: Wood and Agricultural Residues. Research on Use for Feed, Fuel and Chemicals (Soltes, E.J., ed.), pp. 101–119, Academic Press, New York.

20 Trevelyen, W.E., D.P. Procter and J.S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature 166: 444–445.

21 Wong, K.K.Y., L.U.L. Tan and J.N. Saddler. 1988. Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol. Rev. 52: 305–317.

22 Yasui, T., B.T. Nguyen and K. Nakanishi. 1984. Inducers for xylanase production by *Cryptococcus flavus*. J. Ferment. Technol. 62: 353–359.