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ORIGINAL PAPER

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The production of hemicellulases by *Thermomyces lanuginosus* strain SSBP: influence of agitation and dissolved oxygen tension

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Abstract Shake-flask cultivation of *T. lanuginosus* strain SSBP on coarse corn cobs yielded β-xylanase levels of 56,500 nkat/ml at 50 °C, whereas other hemicellulases (β-xylosidase, β-glucosidase, and α-L-arabinofuranosidase) were produced at levels less than 7 nkat/ml. Cultivation on D-xylose yielded much lower levels of xylanase (350 nkat/ml), although other hemicellulase levels were similar to those produced on corn cobs. The influence of agitation rate and dissolved oxygen tension (DOT) on hemicellulase production was studied further in a bioreactor. On xylose, xylanase activities of 4,330 nkat/ml and 4,900 nkat/ml were obtained at stirrer speeds up to 1,400 rpm to control DOT. At a constant stirrer speed of 400 rpm, xylanase activities of 10,930 nkat/ml and 15,630 nkat/ml were obtained when cultivated on xylose and beechwood xylan respectively, despite DOT levels below 5% for the duration of fermentation. The results indicate that there is an interaction between agitation rate and DOT, impacting on xylanase and accessory enzyme production. Higher agitation rates favoured the production of xylosidase, arabinofuranosidase and glucosidase by *T. lanuginosus* strain SSBP, whereas the lower agitation rates favoured xylanase production. Rheological difficulties precluded cultivation on corn cobs in the bioreactor. Volumetric xylanase productivities of 1,060,000 nkat/l · h and 589,000 nkat/l · h obtained on beechwood xylan and xylose indicate that *T. lanuginosus* strain SSBP is a hyper-xylanase producer with considerable industrial potential.

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Introduction

Thermomyces lanuginosus is a widely distributed thermophilic fungus commonly isolated from self-heating masses of organic debris (Emerson 1968). Some strains of this fungus have been found to produce high levels of thermostable and cellulase-free xylanase with broad pH and temperature optima (Gomes et al. 1993a, b). The production of xylanase by *T. lanuginosus* strains has mainly been studied in shake-flask cultures (Gomes et al. 1993a, b; Purkarthofer et al. 1993a). Under these cultivation conditions, the highest xylanase levels were found on substrates such as corn cobs, with lower levels found on other xylan containing substrates and on xylose. *T. lanuginosus* strain SSBP produces the highest levels of xylanase yet reported when optimally cultivated at 50 °C and pH 6.5 in shake-flasks (Singh et al. 2000). However, the ability of this strain to produce these high xylanase titres in a bioreactor has not been reported. Others have reported much lower production of xylanase by *T. lanuginosus* in bioreactor experiments when compared to studies conducted in shake-flasks. The agitation rate, DOT and aeration rate were thought to influence enzyme productivity in the bioreactor (Gomes et al. 1993a, b, 1994; Hoq and Deckwer 1995; Hoq et al. 1994; Purkarthofer et al. 1993a). However, the optimal conditions are apparently unique for each microbial strain and process.

The objective of this study was to assess the influence of agitation and aeration on the production of xylanase and other hemicellulases by *T. lanuginosus* strain SSBP in a bioreactor, using xylose and beechwood xylan as carbon sources and to compare these activities to those found in shake-flask studies.

Materials and methods

Strain and maintenance

T. lanuginosus strain SSBP was isolated from soil, identified by the Council for Scientific and Industrial Research, Pretoria and deposited in the Industrial Biotechnology MIRCEN Culture Collection, Bloemfontein (accession number PRI 0226). The strain was maintained on potato dextrose agar (Oxoid) by cultivation at pH 6.5 and 50 °C, and stored at 4 °C.

Shake-flask cultures

Triplicate shake-flasks (300 ml), each containing 100 ml medium (medium A; Purkarthofer et al. 1993a) with corn cobs or D-xylose were inoculated with an agar block (1–2 cm^2) from a 5-day-old plate culture and incubated at 150 rpm for 7 days at 50 °C. Dried corn cobs (maize) without kernels were ground in a ball mill grinder (Retsch, Germany) and 2- to 7-mm particles were obtained by sieving. Samples were withdrawn every 24 h to determine enzyme activity in the culture supernatant.

Bioreactor experiments

Batch cultivations were carried out in a 15-l Biostat C bioreactor equipped with Mettler-Toledo pH and dissolved-oxygen probes (B. Braun Biotech, Melsungen, Germany) using a working volume of 9 l at pH 6.5 and 50 °C. The vessel had four baffles with one propeller at the top and two disk turbine impellers below fitted equidistantly on the stirrer shaft. The medium (per litre) comprised 0.25 g citric acid, 6 g yeast extract, 1.2 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, trace elements solution (Du Preez and van der Walt 1983) and 0.5 ml silicone antifoam (Dow Corning 1520, Seneffe, Belgium). Beechwood xylan (30 g; Sigma) was added to the medium and sterilised in situ for 20 min at 121 °C, whereas xylose (30 g; Merck) was sterilised separately. The bioreactor was aerated at 1 vol/vol · min and stirred at a fixed speed; or the dissolved oxygen tension (DOT) was controlled by automatic adjustment of the impeller speed.

The inoculum was prepared by transferring a culture as described above into 100 ml medium (medium A in a 300-ml Erlenmeyer flask) containing 3% xylose or beechwood xylan. Following incubation at 50 °C for 24 h at 150 rpm, 50-ml aliquots were transferred to two 1-l Erlenmeyer flasks containing 400 ml of medium A and were incubated as above before inoculation into the bioreactor. Samples were taken at regular intervals, centrifuged and the supernatants analysed as described below.

Enzyme and other assays

Xylanase activity was assayed according to Bailey et al. (1992). Xylosidase, arabinofuranosidase and glucosidase activities were measured as described (Herr et al. 1978). An enzyme unit (nkat) was defined as the release of 1 nmol product/s. Soluble protein was analysed according to Lowry et al. (1951). Biomass was determined by vacuum filtration of 5-ml samples using dried and pre-weighed fibre glass filters (Whatman, Maidstone, UK) washed with distilled water and dried for 10 min to constant mass using an infrared drying balance (Mettler LP 16, Greifensee, Switzerland). All assays were conducted in triplicate and the coefficient of variation of the mean never exceeded 10%.

Results

Shake-flask cultivation

Cultivation on corn cobs yielded high xylanase levels, whereas xylosidase, glucosidase and arabinofuranosidase activities were low (Table 1). Xylanase levels were much lower when *T. lanuginosus* was grown on xylose, whereas the activity of the other hemicellulases was only slightly reduced when compared to the corn cob-grown culture. The levels of hemicellulases on xylose were less than expected, since xylose was previously reported to be an inducer of xylanase in *T. lanuginosus* DSM 5826 (Purkarthofer and Steiner 1995). Xylanase levels produced by *Aureobasidium pullulans* during growth on xylose were 60% of those formed on xylan substrates (Myburgh et al. 1991).

Table 1 Hemicellulase activity (nkat/ml) produced by *T. lanuginosus* strain SSBP on corn cobs or xylose media (Purkarthofer et al. 1993a) after growth at 50 °C and pH 6.5 for 7 days in shake-flasks. Values given are means of three determinations with standard deviation

Enzyme	Substrate	
	Coarse corn cobs	D-Xylose
β-Xylanase	56,500 ± 390	350 ± 85
β-Xylosidase	6.6 ± 0.3	1.17 ± 0.1
β-Glucosidase	0.79 ± 0.09	0.77 ± 0.06
α-L-Arabinosidase	0.93 ± 0.08	0.77 ± 0.05

Bioreactor cultivation

When *T. lanuginosus* was grown on xylose, an initial lag of approximately 6 h in xylanase production and xylose utilisation was observed. Thereafter, xylose was rapidly depleted. Xylanase activity attained a maximum of 4,330 nkat/ml after 18 h (Fig. 1, Table 2). A maximum volumetric productivity of 620,000 nkat/l · h and a maximum specific productivity of 11,300 nkat/g · h for xylanase were reached at 12–15 h, respectively (Fig. 1, Table 2). Growth was rapid and a maximum biomass was reached at 18 h, indicating that xylanase production was concomitant with growth. Increasing the stirrer speed from 1,000 rpm to 1,300 rpm at 21 h failed to enhance further xylanase production. Production of the other hemicellulases commenced after 9 h; and continued despite the low DOT values. Arabinofuranosidase and xylosidase were produced at maximum levels of less than 0.5 nkat/ml, whereas glucosidase showed a rapid onset in production after 24 h to reach a maximum of 1.85 nkat/ml. This increase appeared to coincide with the increase in the DOT.

Using similar conditions, except that the DOT was maintained at 20% for most of the cultivation, a second experiment (Fig. 2) was conducted to assess whether the low DOT could have influenced the level of xylanase produced. After an initial lag of 3 h, xylanase production increased sharply after 6 h, reaching a maximum activity level of 4,900 nkat/ml at 24 h. To maintain a DOT of 20% saturation, a sharp increase in agitation occurred between 6 h and 15 h which coincided with an increase in biomass. At 15 h, the DOT dropped below

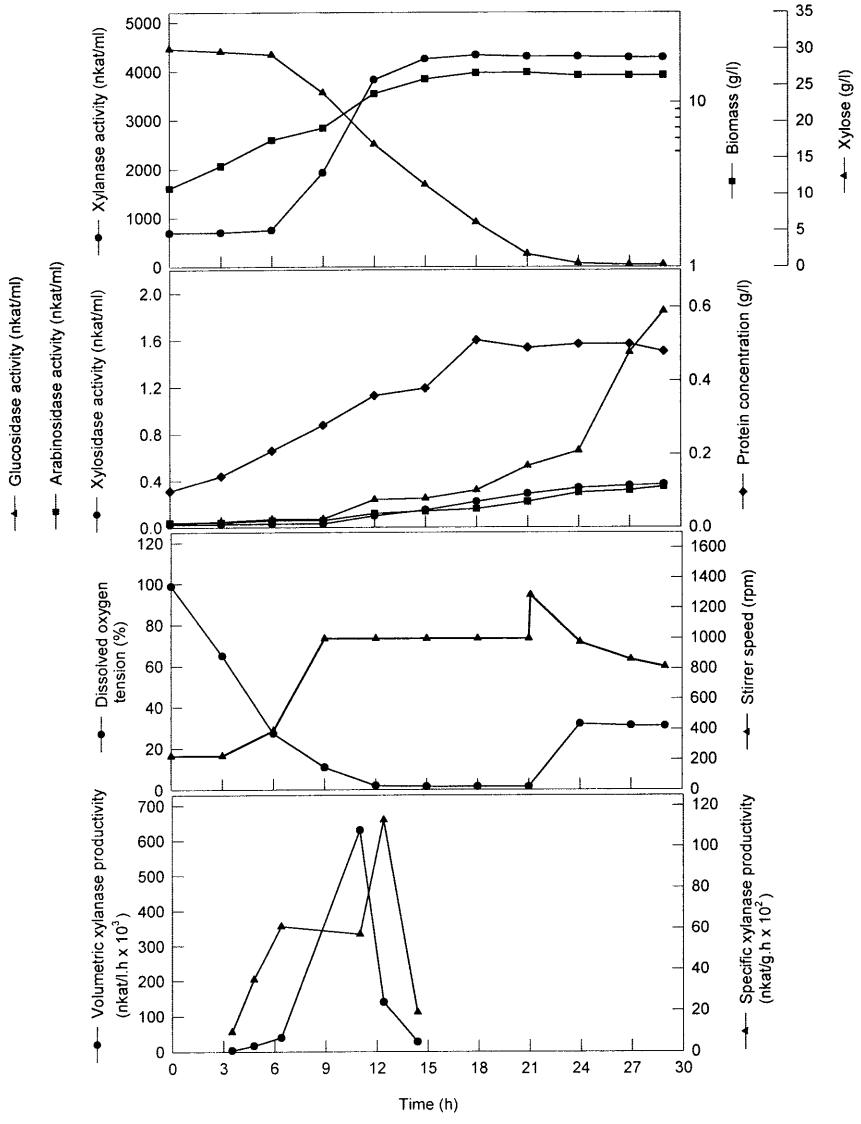

Fig. 1 Time-course of a batch cultivation of *T. lanuginosus* strain SSBP in a 15-l bioreactor (9 l working volume) on 30 g xylose/l. The dissolved oxygen tension setpoint was 30% of saturation controlled by the automatic adjustment of the stirrer speed between the limits of 224–1,000 rpm up to 21 h; and subsequently the maximum stirrer speed limit was increased to 1,300 rpm

Table 2 Effect of agitation and type of carbon substrate on growth and hemicellulase production by *T. lanuginosus* strain SSBP in a bioreactor at 50 °C and pH 6.5. All values represent maximum levels obtained. *TMX* Time required for maximum β-xylanase activity, *X1* β-xylanase, *X2* β-xylosidase, *A* α-L-arabinosidase, *G* β-glucosidase

Substrate	Stirrer speed (rpm)	TMX (h)	Biomass (g/l)	Protein (mg/ml)	β-Xylanase productivity		Hemicellulase produced (nkat/ml)			
					Volumetric (nkat/l · h)	Specific (nkat/g · h)	X1	X2	A	G
Xylose	224–1,000[a] 1,000–1,300[b]	18	15.1	0.51	620,000	11,300	4,330	0.37	0.35	1.85
Xylose	224–1,400[c]	24	16.5	0.48	370,000	47,000	4,900	7.00	1.52	4.10
Xylose	400	68	13.1	0.58	589,000	101,600	10,930	3.80	0.95	0.45
Beechwood xylan	400	72	11.7	0.65	1,060,000	70,800	15,630	13.10	1.20	2.10

[a] Up to 21 h with automatic setting
[b] From 21 h with automatic setting
[c] Automatic setting of stirrer speed

20% of saturation when the stirrer reached its maximum setpoint of 1,400 rpm. The maximum volumetric and specific xylanase productivity values were 370,000 nkat/l · h and 47,000 nkat/g · h, respectively. The production of xylosidase and glucosidase commenced after 6 h and 9 h respectively, reaching maximum levels of 7.0 nkat/ml and 4.1 nkat/ml. The extracellular production of both enzymes occurred during the rapid increase in stirrer

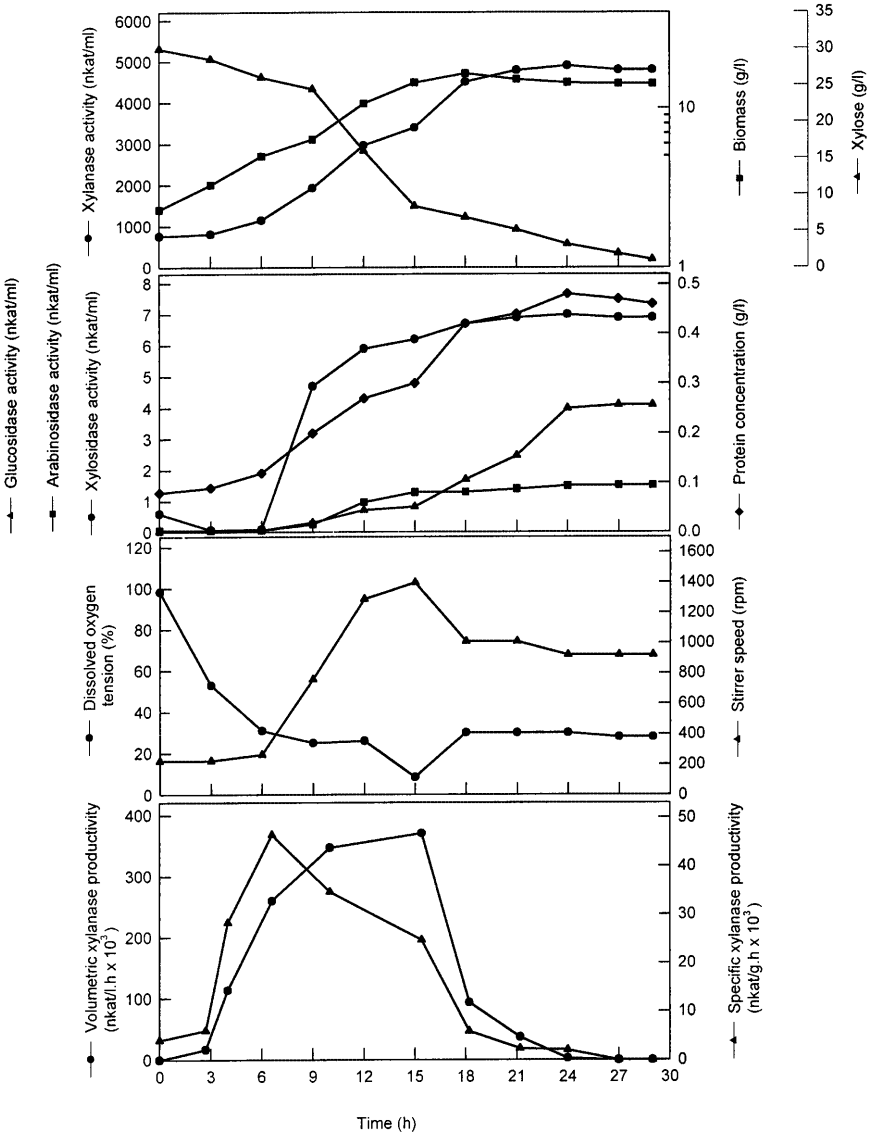

Fig. 2 Time-course of a batch cultivation of *T. lanuginosus* strain SSBP in a 15-l bioreactor (9-l working volume) on 30 g xylose/l at 50 °C and pH 6.5 with aeration at 1 vvm. The dissolved-oxygen tension set-point was 20% of saturation, controlled by the automatic adjustment of the stirrer speed between the limits of 224–1,400 rpm

speed. Arabinofuranosidase was also produced at a low level (less than 2 nkat/ml). This experiment indicated that DOT was not critical for increasing the production of xylanase when *T. lanuginosus* was grown on xylose.

When *T. lanuginosus* was grown on xylose at a constant stirrer speed of 400 rpm, the DOT decreased during active growth and reached less than 1% DOT after 20 h (Fig. 3). There was a marked increase in the levels of xylanase produced after 24 h, compared to previous fermentations, in spite of the final biomass in the reactor being lower (Table 2). Xylanase production displayed a lag of almost 10 h, with a steady increase in production over 8–68 h, reaching a level of 10,930 nkat/ml with maximum volumetric and specific productivities of 589,000 nkat/l · h and 101,600 nkat/g · h, respectively. Even when the DOT fell to below 10% after 12 h and remained below 1% for the rest of the fermentation, xylanase production continued to increase in parallel with biomass. However, the decrease in DOT (below 20%) after 10 h limited fungal growth. In contrast, xylosidase production displayed a 20 h lag, with a rapid increase thereafter, reaching a maximum level of 3.8 nkat/ml.

Rheological difficulties with corn cobs precluded the use of this substrate in bioreactor experiments. Beechwood xylan, however, presented no cultivation difficulties in the bioreactor and was chosen for investigating xylanase production at a constant agitation rate of 400 rpm. A lag in xylanase production, biomass growth and a slow decrease in the DOT were evident during the first 6 h (Fig. 4). Biomass steadily increased after 4 h before reaching a maximum level of 12 g/l after 20 h. The reduction in the DOT to below 10% after 16 h occurred concomitantly with a cessation of growth. Xylanase production coincided with a DOT of less than 30% and continued thereafter, despite a DOT below 5%. When the DOT increased at 56 h, a sharp increase in xylanase production was observed. A xylanase activity of 15,630 nkat/ml was evident after 72 h, with maximum volumetric and specific activities of

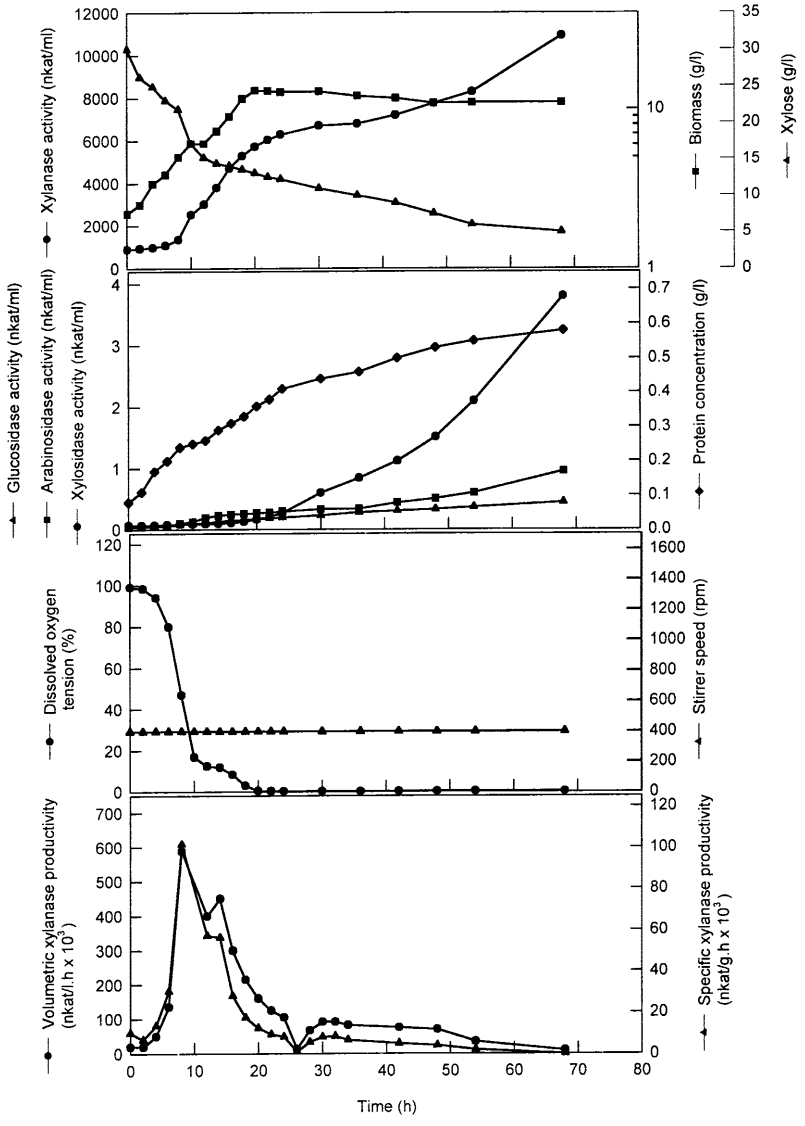

Fig. 3 Time-course of a batch cultivation of *T. lanuginosus* strain SSBP in a 15-l bioreactor (9 l working volume) on 30 g xylose/l at a constant stirrer speed of 400 rpm

1,060,000 nkat/l · h and 70,800 nkat/g · h, respectively during the fermentation. The xylosidase level of 13.1 nkat/ml was considerably higher than found in experiments with xylose as substrate; and most accumulation occurred during a phase that was probably oxygen-limited. Arabinofuranosidase and glucosidase were also produced at levels higher than when *T. lanuginosus* was cultivated on xylose (Table 2).

Discussion

These results show that xylanase production by *T. lanuginosus* strain SSBP grown on xylose can be considerably improved by cultivation in a bioreactor. This significant increase in xylanase production in bioreactors compared to shake-flasks was surprising. This apparent contradiction may be related to the particular carbon substrate used. Other researchers have found the opposite trend, namely that xylanase activity levels were lower in bioreactors than shake-flasks when *T. lanuginosus* was cultivated on xylan substrates (Gomes et al. 1993a, b; Hoq et al. 1994; Purkarthofer et al. 1993a).

Shear stress is known to cause morphological and physiological changes to some filamentous fungi (Mitard and Riba 1988; Tanaka et al. 1975). Purkarthofer et al. (1993b) grew *T. lanuginosus* strain DSM 5826 on corn cobs at shaking speeds of 100–250 rpm and reported that 120 rpm was optimal for xylanase production. They ascribed the lower xylanase activity at the slower shaking speed to poor oxygen transfer within the medium, whereas at higher shaking speeds the xylanase production was lower, concomitant with an increase in hyphal branching. Similarly, the bioreactor studies of Hoq et al. (1994) found that *T. lanuginosus* strain RT9 had an optimum agitation rate of 200 rpm and an increase in the oxygen transfer rate appeared to increase xylanase production. Our results also point to agitation affecting xylanase production when grown on xylose. Sustained and maximal xylanase production on xylose was found

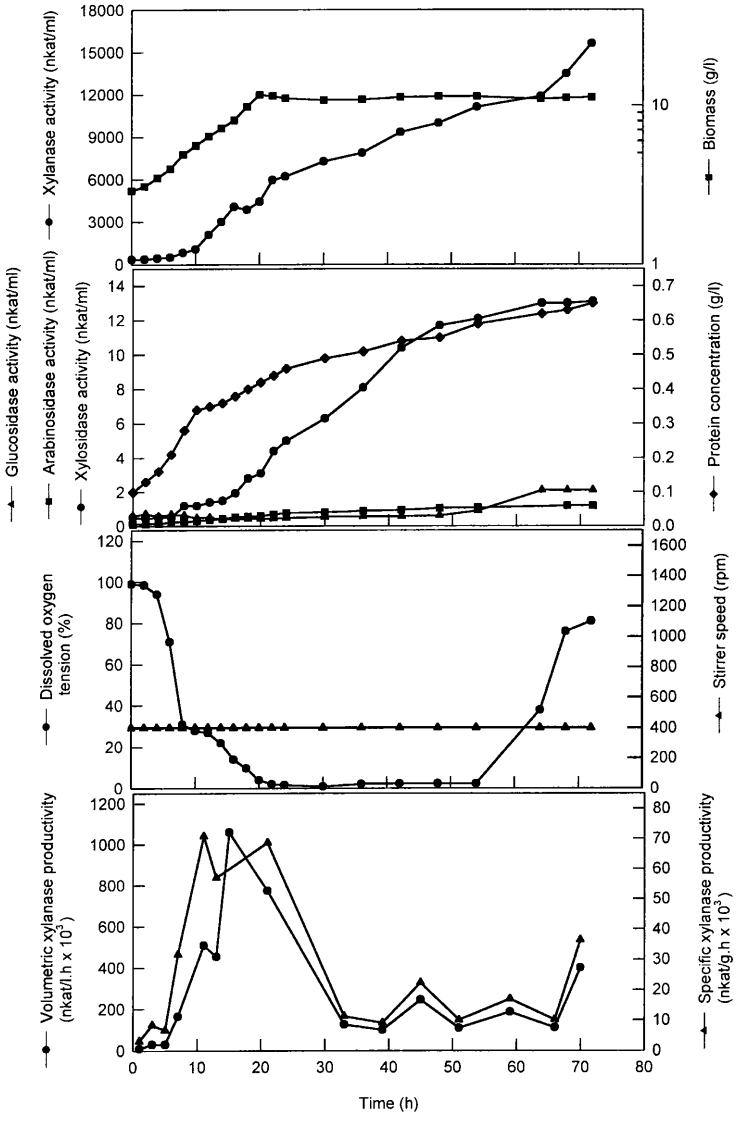

Fig. 4 Time-course of a batch cultivation of *T. lanuginosus* strain SSBP in a 15-l bioreactor (9 l working volume) on 30 g beechwood xylan/l at a constant stirrer speed of 400 rpm

at a low stirrer speed (Fig. 3), even though the culture probably was oxygen-limited after about 20 h, as indicated by the very low DOT values. Apparently an oxygen limitation decreased the rate of xylanase production, so that any increase was probably determined by the rate of oxygen transfer. Furthermore, high stirrer speeds curtailed xylanase production well before the depletion of the xylose (Figs. 1, 2), possibly due to the effects of hydrodynamic stress. Therefore, these data point to an interaction between agitation rate and DOT that impacts on xylanase production. These interactions require further study to optimise growth of and xylanase production by *T. lanuginosus* strain SSBP, by minimising stress while simultaneously ensuring oxygen-sufficient conditions.

The effectiveness of fermentation processes is often determined by the productivity of organisms in producing an enzyme. A remarkable volumetric productivity was obtained with *T. lanuginosus* strain SSBP and the maximum values of 1,060,000 nkat/l · h and 620,000 nkat/l · h on beechwood xylan and xylose, respectively, exceed or are comparable to the values of 178,880 nkat/l · h and 666,080 nkat/l · h reported previously for other *T. lanuginosus* strains grown respectively on beechwood xylan and birchwood xylan (Gomes et al. 1993a, b; Hoq et al. 1994).

The levels of other hemicellulases produced by *T. lanuginosus* strain SSBP were low compared to xylanase production, as in other *T. lanuginosus* strains (Gomes et al. 1993a, b; Hoq et al. 1994; Purkarthofer et al. 1993a). When grown on xylose, much higher xylosidase levels were found in most of our bioreactor cultures than in shake-flask cultures. However, the xylosidase activity produced by our strain was still lower than the 22 nkat/ml produced by *T. lanuginosus* strain RT9 grown on birchwood xylan (Hoq et al. 1994). The levels of arabinofuranosidase and glucosidase were comparable to levels of other strains (Gomes et al. 1993a, b; Hoq et al. 1994; Purkarthofer et al. 1993a). At higher stirring speeds, fungal hyphae may become

disrupted, thereby releasing extracellular or membrane-bound hemicellulases (Myburgh et al. 1991).

The results of these initial bioreactor studies indicate that *T. lanuginosus* strain SSBP is the most efficient xylanase producer yet described. Further optimisation of the bioreactor conditions might enhance xylanase productivity. Significant increases in other hemicellulases through bioreactor optimisation appear to be less promising.

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