0273-2289/05/126/0061/$30.00

Production of Poplar Xyloglucan Endotransglycosylase Using the Methylotrophic Yeast *Pichia pastoris*

Monika Bollok, Hongbin Henriksson, Åsa Kallas, Mehmedalija Jahic, Tuula T. Teeri, and Sven-Olof Enfors*

Department of Biotechnology, Royal Institute of Technology, KTH, Alba Nova University Center, Roslagstullsbacken 21, S-106 91, Stockholm, Sweden, E-mail: enfors@biotech.kth.se

Received July 9, 2004; Accepted March 22, 2005

Abstract

The gene *XET16A* encoding the enzyme xyloglucan endotransglycosylase (XET) from hybrid aspen (*Populus tremula x tremuloides* Mich) was transformed into *Pichia pastoris* GS115 and the enzyme was secreted to the medium. The influence of process conditions on the XET production, activity, and proteolytic degradation were examined. Inactivation of XET occurred in the foam, but could be decreased significantly by using an efficient antifoam. Rich medium (yeast extract plus peptone) was needed for product accumulation, but not for growth. The proteolytic degradation of the enzyme in the medium was substantially decreased by also adding yeast extract and peptone to the glycerol medium before induction with methanol. Decreasing the fermentation pH from 5.0 to 4.0 further reduced the proteolysis. The specific activity was further improved by production at 15°C instead of 22°C. In this way a XET production of 54 mg/L active enzyme could be achieved in the process with a specific activity of 18 Unit/mg protein after a downstream process including centrifugation, micro- and ultrafiltration, and ion exchange chromatography.

Index Entries: *Pichia pastoris*; xyloglucan endotransglycosylase; foaming; proteolysis.

Introduction

Xyloglucan is the principal hemicellulose in the primary cell wall matrix in dicotyledons, where it is tightly associated with cellulose microfibrils. In current models of the plant cell wall, these interactions are

*Author to whom all correspondence and reprint requests should be addressed.

thought to give rise to the strength and rigidity of this composite structure. The enzyme xyloglucan endotransglycosylase (XET, EC 2.4.1.207) performs endolytic cleavages of xyloglucan chains with subsequent transfer of the newly created chain ends to the nonreducing ends of a different xyloglucan molecules. By this transglycosylation mechanism, the enzyme has an important role in the reconstruction of the cell wall, which is connected with many functions in the plants, like fruit-ripening, seed germination, cell expansion, response to mechanical stress, and xylem and phloem development *(1–4)*. In addition to the biological importance, the XET reaction has also significant industrial potential. We have recently developed a chemo-enzymatic approach for cellulosic fiber modification based on the tranglycosylating activity of XET and the tight binding of xyloglucan on cellulose *(4a)*. According to the method developed, chemically modified xylogluco-oligosaccharides are attached to polymeric xyloglucan by the XET reaction, followed by adsorption of the chemically modified xyloglucan onto the cellulosic surface of choice. As efficient surface modification of cellulose is achieved without compromising the fibre integrity and strength, the technology can be exploited for activation of cellulosic materials for industrial purposes. For further commercial development of the technology, an efficient production system is needed for XET.

XET enzymes from different sources have been isolated and purified for biochemical, molecular, and enzymatic characterization, and also for functional studies during plant growth *(2,3,5–13)*. The corresponding genes have been cloned and expressed in *Escherichia coli* where the heterologous protein generally appears as inclusion bodies *(6,14–16)*. One XET isoenzyme from *Arabidopsis* has been successfully expressed in insect cells using the baculoviral vectors *(17)* and an isoenzyme of XET from tomato has been expressed in the methylotrophic yeast, *Pichia pastoris (18)*. However, the enzyme yields in all published cases have been relatively low and no attempts have been made to optimize the enzyme production for enhanced yields.

The methylotrophic yeast, *P. pastoris*, is commonly used for heterologous production of proteins with commercial interest *(19–26)*. After insertion of the foreign gene into the *his4* locus of *P. pastoris*, the product is expressed under the control of the strong methanol-inducible *AOX1* promoter. Advantages of this system are the genetic stability of the expression system (decreased deletion mutants) and efficient secretion of correctly folded heterologous products to the culture medium, which simplifies downstream processing. Furthermore, the organism can be grown in methanol fed-batch cultures up to high cell densities in excess of 100 g/L dry cell weight *(26,27)*.

The common approach to fermentation with *P. pastoris* is to use a four-stage fed-batch technique based on (i) initial growth on glycerol to produce repressed cells, followed by (ii) a short exponential glycerol phase, which permits derepression of the AOX promoter thereby allowing a smoother

transition to the (iii) exponential methanol feed phase for induction, and finally (iv) a constant methanol feed phase for produce the protein *(27,28)*.

The productivity of *P. pastoris* is increased by this high-cell-density cultivation technique *(19,27)*. However, the high cell density may also contribute to increased proteolysis problems, because it is likely that the high cell density also results in higher intracellular protease activity from the lysed cells. Modification of fermentation parameters like medium pH and composition, growth temperature, and the use of a protease deficient strain *(19–21,23,24)* are strategies that have been used to overcome proteolysis.

In the present work, the gene *XET16A* encoding the enzyme XET from hybrid aspen (*Populus tremula x tremuloides* Mich) was transformed into *P. pastoris* GS115 strain *(4,28a)* and the enzyme was produced in shake flasks and in methanol limited fed-batch fermentations. The fermentation technique was improved concerning the medium, the antifoam, pH, and temperature to decrease the proteolysis and increase the accumulation of the active enzyme.

Materials and Methods

Strain

P. pastoris GS115 (*his4*) (obtained from Invitrogen, Carlsbad, CA) was used as host. The vector pPIC9 (Invitrogen, San Diego) containing the *xet 16A* gene from *Populus tremula x tremuloides* was linearized with Sal I to transform into the host strain by insertion at the *his4* locus.

Shake-Flask Cultures

Media

1. Minimal methanol agar (MM agar): 134 g/L yeast nitrogen base without amino acids (Sigma-Aldrich Chemie, Germany), 400 µg/L biotin, 10 g/L methanol, and 15 g/L agar.
2. Buffered minimum glycerol medium (BMG): 134 g/L yeast nitrogen base without amino acids, 100 mL/L phosphate buffer (132 mL of 1 *M* K_2HPO_4 and 868 mL of 1 *M* KH_2PO_4), 400 µg/L biotin, 10 g/L glycerol.
3. Buffered-glycerol-complex medium (BMGY): 10 g/L BMG plus yeast extract (Bacto Yeast Extract, Becton Dickinson), and 20 g/L peptone (Bacto Tryptone, BD).
4. Buffered minimum methanol medium (BMM): Same composition as BMG, but with 4 g/L methanol instead of glycerol.
5. Buffered methanol complex medium (BMMY): Same composition as BMGY, but with 4 g/L methanol instead of glycerol.

Cultivation Conditions

Shake-flasks with BMG medium were inoculated from MM agar plates. Cells were grown in baffled shake flasks at 30°C on a shaker with 260 rpm. When the OD_{600} reached the value between 2 and 6, the cells were centrifuged at 3000 rpm for 5 min. The supernatant was decanted and the

cell pellet was resuspended to OD_{600} = 1 in a methanol containing medium (BMM or BMMY) to induce the protein production. Four grams per liter of 100% methanol were added every 24 h to maintain the induction (Invitrogen, Pichia expression Kit).

To check the effect of concentrated yeast extract and peptone solution on the growth and protein production shake-flasks were run with BMM medium supplemented with concentrated solution of yeast extract (166 g/L) and peptone (333 g/L) autoclaved separately The shake-flasks were incubated at 22°C on a shaker with agitation of 140 rpm.

The effect of foaming on the XET activity was investigated in BMMY medium inoculated from a BMGY culture. After 68 h induction, a part of the culture was centrifuged at 4200 rpm for 10 min and the supernatant was used for the further experiment. The XET activity was examined by shaking the supernatant or the whole culture at 300 rpm and at 110 rpm with and without MAZU antifoam (0.25 µL/mL). The XET activity was analyzed both in the liquid and in the foam phases after 7 and 23 h.

Fermenter Cultivations

MM agar plates were used for maintenance of the strain. One loop was used to inoculate a 1-L shake-flask with 100 mL of glycerol medium. The initial pH was 6.0. Cells were grown for 20 h at 30°C on a shaker with 160 rpm, which resulted in an OD_{600} between 2 and 5.

The shake-flask culture (BMGY) was then transferred to the fermentor with 3 L fermentor medium: 26.7 mL/L H_3PO_4 85%, 0.93 g/L $CaSO_4 \cdot 2H_2O$, 18.2 g/L K_2SO_4, 14.9 g/L $MgSO_4 \cdot 7H_2O$, 4.13 g/L KOH, 40 g/L glycerol, 10 g/L yeast extract, 20 g/L peptone, and 4.3 mL/L trace element solution. The trace element stock solution contained: 6 g/L $CuSO_4 \cdot 5H_2O$, 0.8 g/L KI, 3 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.2 g/L H_3BO_3, 0.5 g/L $CaSO_4 \cdot 2H_2O$, 20 g/L $ZnCl_2$, 65 g/L $FeSO_4 \cdot H_2O$, 0.2 g/L biotin, and 5 mL conc. H_2SO_4.

To avoid methanol intoxication during the transition from the glycerol to the methanol phase a four stage process was used *(27)*. When the glycerol was consumed after about 19 h (cell concentration about 28 g/L dry weight), a feed containing 555 g/L glycerol, 27 g/L yeast extract, 54 g/L peptone, and 12 mL/L trace element solution was started. The initial feed rate was 40 mL/h and it was increased at a rate of 0.18/h. After 3 to 4 h, when cell concentration was approx 42 g/L dry weight (about OD_{600} = 74), the glycerol feed was replaced with a feed containing 780.6 g/L methanol and 12 mL/L trace element solution. The initial feed rate was 10 mL/h and it was increased up to 20 mL/h, and then kept constant until the end of the process.

The fermentation was carried out in a 10 L standard stirred tank fermentor (Belach Biotek AB, Stockholm). The stirrer speed, pH, temperature, dissolved oxygen tension (DOT), pressure, air flow rate, pump speed, and antifoam addition were automatically controlled. All these parameters and the signals for fermentor weight, feed reservoir weight, NH_4OH reservoir

weight, accumulated antifoam pump run time, and outlet gas composition (CO_2, O_2, and methanol), were monitored and logged. The outlet gas analyses were performed with Industrial Emission Monitor (Bruel Kjaer type 1311, Innova Airtech Instruments A/S, Denmark).

The fermentation conditions were the following under the glycerol phase: temperature, 30°C; pH 5.0 controlled by the addition of 28% NH_4OH. The agitation rate was 800 rpm and the aeration rate was 3 L/min. With these settings the DOT signal did not decline below the level corresponding 30% air saturation. Under the methanol feed phase, 108.6 g/L a solution of yeast extract and 217.4 g/L peptone were added at a feed rate of 6–7 mL/h. During this phase the temperature was kept at 22°C and the pH at 5.0, unless otherwise stated in the text. The air flow rate and/or rpm were gradually increased up to 7 L/min and 1400 rpm, respectively, to maintain the dissolved oxygen level at above 20% saturation. Foaming was automatically controlled by means of a level electrode and addition of antifoam A (A5758, Sigma-Aldrich, Stockholm). Alternatively, 1 mL/L of MAZU antifoam (DF 6000 K, OUVRIE PMC, France) was added at the beginning of the fermentation.

Proteolysis Studies

To investigate the effect of pH and temperature on the proteolytic degradation of the XET enzyme, samples were withdrawn from the fermenter after 140–145 h of fermentation. These samples were centrifuged to remove the cells and the supernatants were incubated at different pH or temperature on a shaker. One series of samples with pH 5.0 was incubated in the presence of 1 m*M* the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Samples were removed from the incubator at different times and subjected to activity measurement and SDS-PAGE. Band volumes on the gel were evaluated with the Image Master 1D Software (Pharmacia Biotech).

Analyses

Cell Concentration

Cell concentration was monitored by measuring the optical density (OD_{600}) at 600 nm. Dry weight (X) of the cell suspensions was determined by centrifugation of 5 mL cell broth in a preweighted centrifuge tube, followed by drying to constant weight at 80°C in an oven.

Preparation of Xyloglucan Oligomers

Xyloglucan oligomers (XGO) were prepared by dissolving xyloglucan (XG) from tamarind seed (Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland) in water at a concentration of 5 mg/mL (w/v). The solution was heated to 50°C under turbo mixing. Cellulase (5 U/mg) from *Trichoderma reesei*, (Fluka Biochemika, distributed by Sigma-Aldrich) was added to the XG solution (1 mg cellulase to 100 mg XG) for hydrolysis at 37°C overnight. The cellulase in the XGO solution was

removed by using active charcoals followed by cation-exchange resins, and finally freeze-dried before use.

XET Activity Assay

The colorimetric assay was performed similarly to what was reported by Farkas and co-workers *(29)*. 50 µL xyloglucan (XG, 1.75 mg/mL) and 50 µL xyloglucan oligomers (XGO, 3.5 mg/mL) were mixed with 50 µL citrate-phosphate buffer; pH 5.5. 50 µL of sample were added and the mixture was incubated at 30°C or 25°C. A control was set by means of replacing the XGO to water. The reaction was terminated by adding 100 µL hydrochloric acid (1 *M*), 800 µL Na_2SO_4 (20%) and 200 µL iodine reagent (1% KI and 0.5% I_2). The reaction product was stored in darkness for 20 min to let the color develop. The absorbance was measured at 620 nm. The XET activity was expressed as the rate of developing an absorbance difference (ΔA620/min) between the pair of tubes with and without XGO, which is corresponding to unit (U).

The analysis was made in the cell free supernatant and the volumetric activities are expressed as U/mL supernatant.

Protein Assay

Protein concentration was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used as the standard.

SDS-PAGE and Western Blot

Samples were collected by separating the culture medium from the cells by centrifugation at 4200 rpm for 10 min. The samples, containing 65 µL of the supernatant, 25 µL of sample buffer (NuPAGE LDS Sample buffer [4X]), and 10 µL of reducing agent (0.5 *M* DDT [dithiothreitol]) were incubated for 10 min at 70°C. From the reduced samples, 25 µL were added to each well on the 12% *Bis-tris* gel and run with MOPS (20 m*M*) running buffer for 1 h (NuPAGE, Bis-Tris Gel, Invitrogen) The gel was stained with Coomassie Blue R-250 for half an hour and washed with destaining solution for 2–3 h. Western blot analysis were performed and developed with polyclonal anti XET 16A antibodies *(4)*.

Purification

After fermentation, the yeast cells were removed by a two-step (3000 rpm, 10 min and 5000 rpm, 20 min) centrifugation with intermittent discharged the supernatant. The supernatant was then filtered through a 0.45 µm filter (Mini Capsule, Versapor Membrane, Pall Corporation, NY) and the culture filtrate was concentrated about eight times by ultra-filtration (ALPHA MWCO 10 k, Pall Corporation, NY) and buffer changed to 0.1 *M* sodium acetate, pH 5.5 (Buffer A) until the conductivity of the culture filtrate was the same as that of Buffer A. The sample was then applied to a SP-Sepharose Fast Flow column (XK50/30, gel bed = 20 cm × 5 cm i.d., Amersham Biosciences, Uppsala, Sweden) equilibrated by Buffer A. The protein was

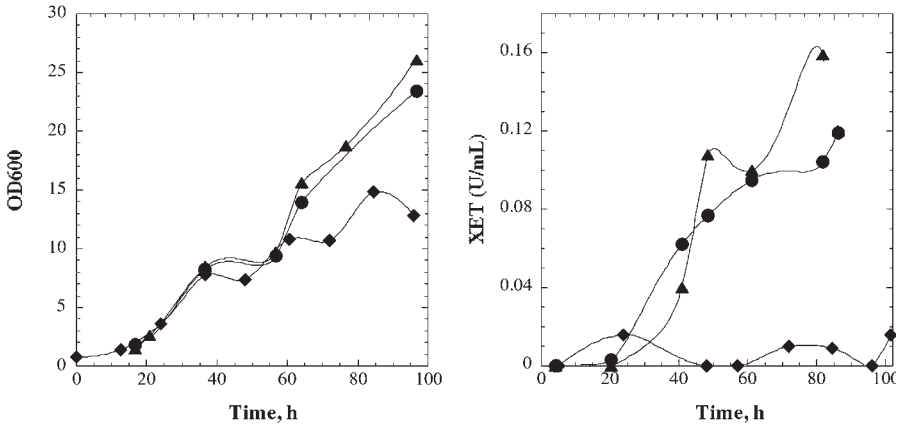

Fig. 1. Influence of yeast extract and peptone on the growth (left panel) and XET activity at 25°C (right panel) in shake flask cultures of *P. pastoris*. Medium: BMM (◆), BMMY (▲), BMMY with the concentrated solution of yeast extract and peptone (●).

eluted by linear gradients of Buffer B (1 *M* sodium chloride in Buffer A) 0–50% Buffer B (2 column volume). The fractions were assayed for XET activity and the active fractions were pooled and analyzed for purity by SDS-PAGE. For further enzyme characterization a second chromatography can be included using S-source 15S (Amersham Biosciences, Uppsala, Sweden) in a high resolution column.

Results and Discussion

Influence of Rich Medium

The production of XET was first investigated in shake-flask cultures (Fig. 1). It is evident that while absence of yeast extract and peptone had only a minor effect on the cell growth, it completely prevented accumulation of active XET. Western blot analyses confirmed that no measurable XET protein was present in the cultures with minimal medium, whereas a band accumulated at the 32-kDa level in analysis of the rich medium cultures (data not shown). The enzyme activity increased at about a constant rate throughout the cultivation and reached about 0.12 U/mL (at 25°C), after 100 h, when the cell density was about 16 g/L.

Based on these shake flask experiments, a fermentation was carried out using buffered minimum glycerol medium during the glycerol phase. If the constant production shown in Fig. 1 could be reached also in a fed-batch fermentation a higher productivity was expected as a result of the higher cell density. However, in spite of reaching 150 g/L cell dry weight after 170 h, only 0.15 U/mL (at 25°C) enzyme activity was obtained (Fig. 2).

Thus, compared to the shake flask cultures, a 10 times increased cell mass produced only 22% more active XET. This low XET productivity could be a result of proteolytic degredation.

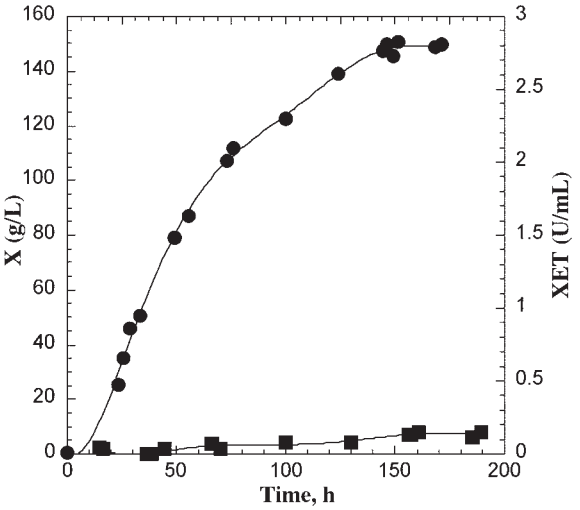

Fig. 2. Fed-batch fermentation with *P. pastoris* using minimal glycerol medium (BMG): cell dry weight (●), and XET activity (■) (at 25°C).

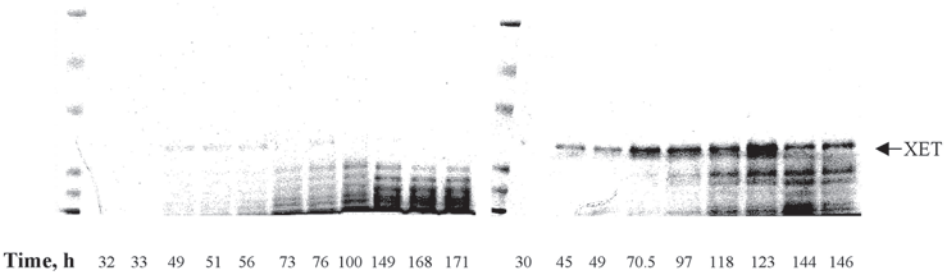

Fig. 3. Coomassie stained SDS-PAGE analyses of samples from the fed-batch fermentation shown in Fig. 2. (left panel). SDS-PAGE of samples from a fed-batch fermentation with the rich glycerol medium during the glycerol growth phase (right panel).

SDS-PAGE analysis of the medium during this process revealed that very little protein accumulated at the 32-kDa level (Fig. 3). Instead, a number of proteins with lower molecular weight, possible products of proteolysis, accumulated and this pattern became more evident in the later stage of the process.

There are many indications in the literature *(19,24,26)* that during cultivation recombinant proteins are more proteolytically degraded using minimal medium than in rich medium. When the rich glycerol medium was used during the glycerol phase, more protein of the XET size accumulated and relatively less of the low molecular weight compounds was observed (Fig. 3). However, in spite of this, the enzyme activity was not increased with the rich medium (data not shown). A possible reason for this is the behavior of the XET under the strongly foaming conditions of these cultures, in which antifoam A was used against foaming.

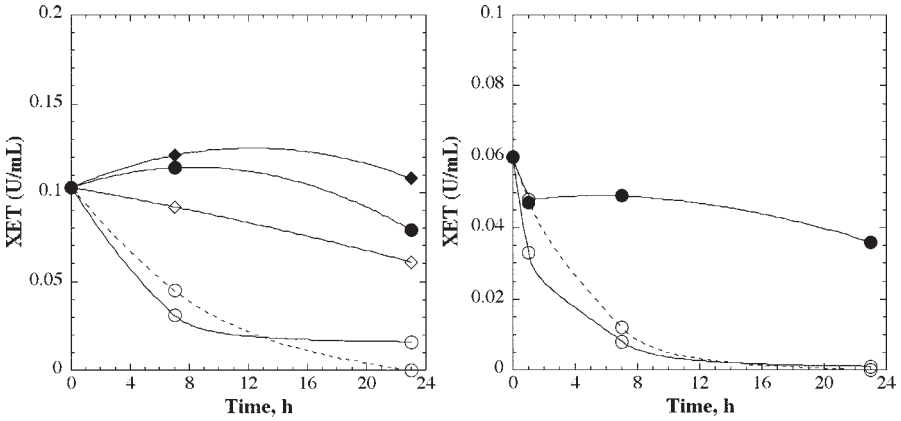

Fig. 4. The effect of foaming on the XET activity in cell-free supernatant (left panel) and in whole culture (right panel). Open symbol: incubation without antifoam. Closed symbol: incubation with antifoam. Dashed line: analysis of the foam phase. Solid line: analysis of the liquid phase. ◆, ◇: incubation at 110 rpm; ●, ○: incubation at 300 rpm.

The Effect of Foaming on the XET Activity

The loss of enzyme activity could be associated with the appearance of foaming in which the protein can be enriched or inactivated by denaturation *(30)*.

When XET was incubated in a cell free medium without antifoam (Fig. 4), the enzyme activity decreased rapidly at the high agitation rate when foaming was intensive. At the lower agitation rate, the foaming was less intensive and the enzyme showed much more stability. Correspondent experiment with the MAZU antifoam efficiently ceased the foaming and resulted in a considerable stabilization of the enzyme activity. Presence of cells in these incubation did not influence the effect of foaming.

Comparative analysis of the XET protein on the Coomassie-stained SDS-PAGE in the liquid and the foam fraction showed that the disappearance of the XET activity in foaming medium was not due to enrichment of the enzyme in the foam. Thus, the foam induced denaturation is the possible explanation.

When fermentation was carried out using antifoam A to control the foaming, low values of XET activities were measured either in the medium and the foam. When MAZU antifoam was added after 79 h of fermentation corresponding to 55 h in the induction phase, the foam disappeared and three times higher XET activity was achieved in the medium after 94 h fermentation. However, still with a considerable proteolytic degradation (data not shown).

The Effect of pH on the Proteolytic Degradation of XET

The influence of pH on the product yield has been demonstrated in several fermentation processes using *P. pastoris (21,24,31–33)*. This has been

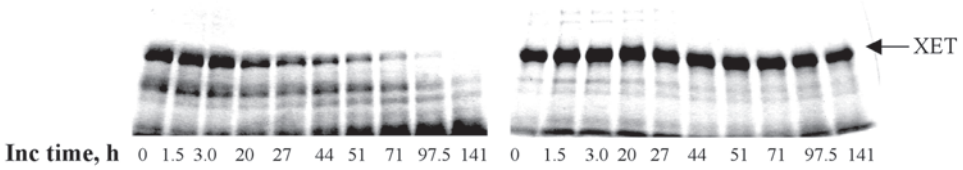

Fig. 5. Coomassie-Blue stained SDS-PAGE analysis of XET protein degradation in culture supernatant incubated at pH d 5.0 (left panel) and pH d 4.0 (right panel).

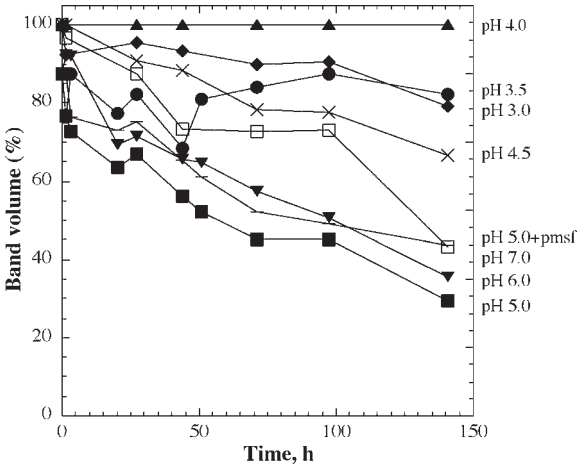

Fig. 6. Effect of pH on XET protein degradation in culture broth of 140 h of fermentation. The data show the remaining band volume of Coomassie Blue-stained SDS-PAGE after different incubation time at pH 3.0 (◆), pH 3.5 (●), pH 4.0 (▲), pH 4.5 (X), pH 5.0 (■), pH 5.0 + PMSF (□), pH 6.0 (▼), and pH 7.0 (–).

ascribed to the activity of pH-dependent extracellular proteases. In the present work, the effect of pH on the degradation of the XET protein was investigated, in the supernatant of a 142 h fermentation. Samples were withdrawn from the fermenter, centrifuged to remove the cells and the pH of the supernatant was adjusted in the interval 3.0–7.0. These samples were further incubated on a shaker at the cultivation temperature (22°C).

Figure 5 shows examples how the XET band disappeared at pH 5.0 while it remained relatively constant at pH 4.0. Also the bands below the XET gradually became weaker with incubation time. It is likely that these bands represent degradation products of XET, since in the pH 4.0 incubation, when the XET band was quite stable, less of the bands below the 32-kDa level appeared. These bands could also represent native *P. pastoris* proteins. However Fig. 8 shows that no such bands are visible in the samples in the fermentation medium directly after induction.

Scanning of the remaining band volume of the full length XET of these analyses revealed the kinetics of degradation in more detail (Fig. 6). The results showed that in the pH range 5.0–7.0 the degradation rate was approximately the same. After about 100 h, 50% of the protein had disap-

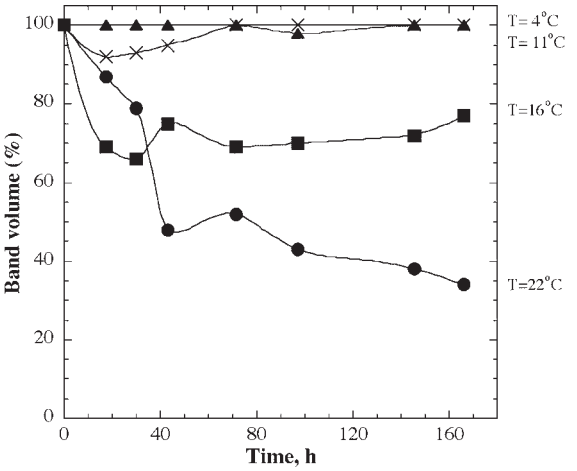

Fig. 7. Effect of the incubation temperature on protease activity in culture broth of 146 h fermentation. The data show the remaining band volume of Coomassie Blue-stained SDS-PAGE after different incubation time at 22°C (●), 16°C (■), 11°C (▲), and 4°C (X).

peared. At lower pH the degradation rate was lower and at pH 3.5–4.0 no degradation at all was observed. PMSF had a little effect (at pH 5.0), indicating that the responsible proteases were not of the alkaline serine type. This is contrary to the result obtained from production of a fusion protein Cellulose binding module and lipase, where almost all proteolytic activity was found to be due to PMSF sensitive protease *(33)*. In that case the host strain was *P. pastoris* SMD 1168 (*pep4*, *his4*) that is a protease A deficient strain.

Based on the result of the incubation experiments, several runs of fed-batch fermentation were carried out using pH 4.0 under the induction (methanol feed) phase. The activity of XET reached about 0.65 U/L after 71 h fermentation. After purification the concentration of enzymatically active XET was about 29 mg/L with a specific activity of 11 U/mg. However, after 71 h the protease activity increased considerable (data not shown).

The Effect of Temperature on the Proteolysis and the Activity Stability

P. pastoris fermentations are usually performed at the optimal temperature for growth, i.e., 30°C *(26)*. Temperatures below 30°C have been reported to increase the yield of the recombinant proteins *(21,23)*, which was assumed to be because of the enhanced protein folding, and increase in the cell viability with less protease release from the dead cells *(23)*. Because the data in Fig. 6 show a clearly pH-dependent extracellular proteolytic activity, it is plausible to assume that this activity also should be strongly temperature dependent. The proteolysis rate and the activity stability were therefore determined in the medium supernatant incubated at different temperatures. This revealed a considerably lower degradation rate and higher activity stability of XET at the reduced temperature (Fig. 7).

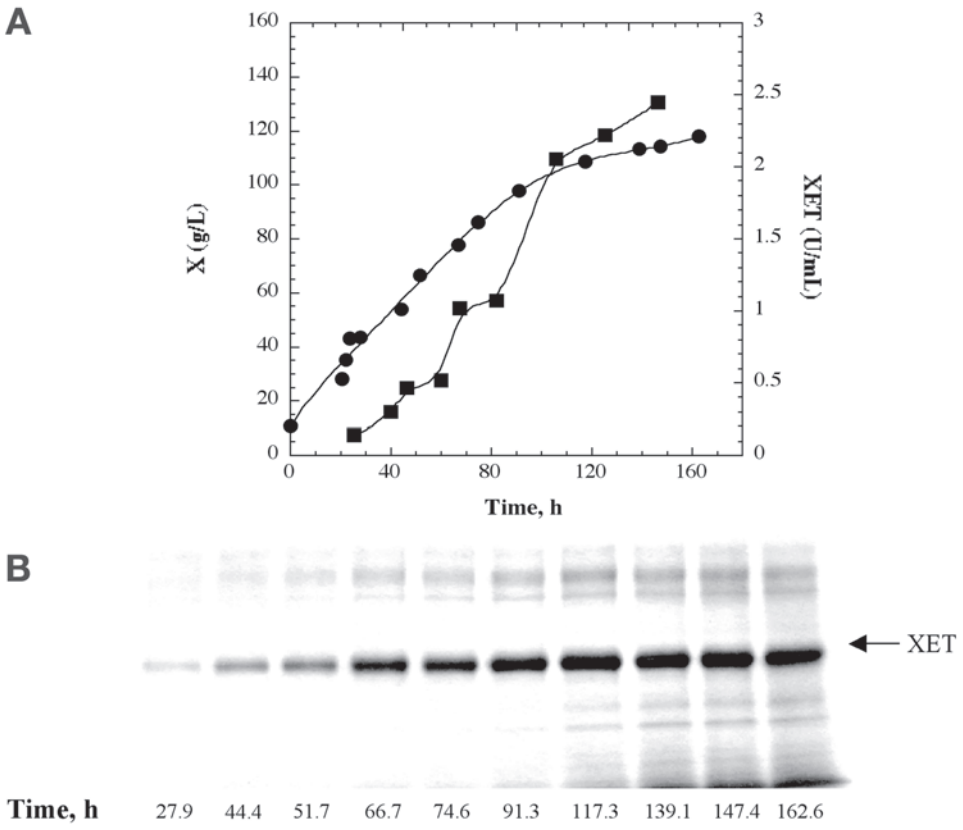

Fig. 8. The fermentation profile of a fed-batch fermentation with *P. pastoris*, at pH 4.0 and a temperature of 15°C in the induction phase **(A)**: cell dry weight (●), and XET activity (■) (at 30°C). Analysis of samples from fermentation culture with Commassie-stained SDS-PAGE **(B)**.

A fermentation was carried out to improve the product yield using lower temperature during the methanol feed phase. Using tap water for cooling, the temperature could be decreased to minimum 18°C under the given process conditions. After about 70 h of fermentation, XET activity reached 1.31 U/L. After purification the concentration of 53 mg/L active XET was reached with a specific enzyme activity of 9 U/mg of protein. SDS-PAGE analysis of the medium during the process revealed that a few hours after induction only a product band appeared. No other proteins visible at induction. Later on large amount of bands with lower molecular weight were appeared probably due to product degradation. Final activity after 163 h of fermentation was 2.55 U/L. After purification the concentration of 36.5 mg/L active XET was achieved with a specific enzyme activity of 14.5 U/mg protein.

By using cooling water system, the temperature could be further reduced to 15°C during the methanol feed phase. Under these conditions, an almost constant accumulation up to 2.48 U/L XET activity was obtained (Fig. 8). After purification the concentration of 54 mg/L active XET was

Table 1
Summary of Fermentation Data

Ferm conditions	Batch time (h)	Cell dry weight (g/L)	Total extracellular protein (g/L)	XET activity (U/L)	XET conc (mg/L)[a]	Mean productivity (mg/L·h)	Filtrate yield (L/L)
T = 22°C	71.4	54	0.247	465	46.5	0.65	0.72
T = 18°C	72	74	0.392	917	102	1.41	0.7
T = 18°C	163.4	103.5	0.437	1582	109	0.67	0.62
T = 15°C	162.6	118	0.413	1541	87	0.53	0.63

Data are based on liter of fermentation broth. [a]XET concentration (mg/L) based U/mg purified protein.

reached with a specific enzyme activity of 18 U/mg of protein. The corresponding SDS-PAGE analyses revealed that most of the product was of full length, even if some accumulation of low molecular bands were observed.

Summary of Fermentation Yields

Table 1 summarizes the yield of biomass, total produced protein and XET activity, and productivity obtained at different temperature during the methanol feed phase.

A significant proteolytic activity was observed after 70 h of fermentation at 22°C which contributed to loss in enzyme activity, so the optimal harvesting time was around 70 h. By decrease in temperature the production of active enzyme increased and then by extending the fermentation after 70 h, further active enzyme could be produced due to decreased proteolytic degradation.

Purification

For recovery of the XET from the fermentation broth, the cells were removed by centrifugation and the supernatant was filtered by microfiltration. The filtrate volumes were about 62 to 72% of the total broth volume due to the high cell density. The filtrate was concentrated by ultrafiltration and applied to a cation exchange chromatography column and eluted as described in Materials and Methods.

The target protein, XET, was captured by the cation-exchange gel and eluted mainly as three peaks, A1+A2 and B (Fig. 9). The B peak is the dominant part of XET. SDS-PAGE analysis (Fig. 10) of the B peak showed that this procedure results in one dominant XET band with high purity. Purity and recovery data from the purification are summarized in Table 2. From the 15°C culture 40% of the XET was recovered with a specific activity of 18 U/mg protein.

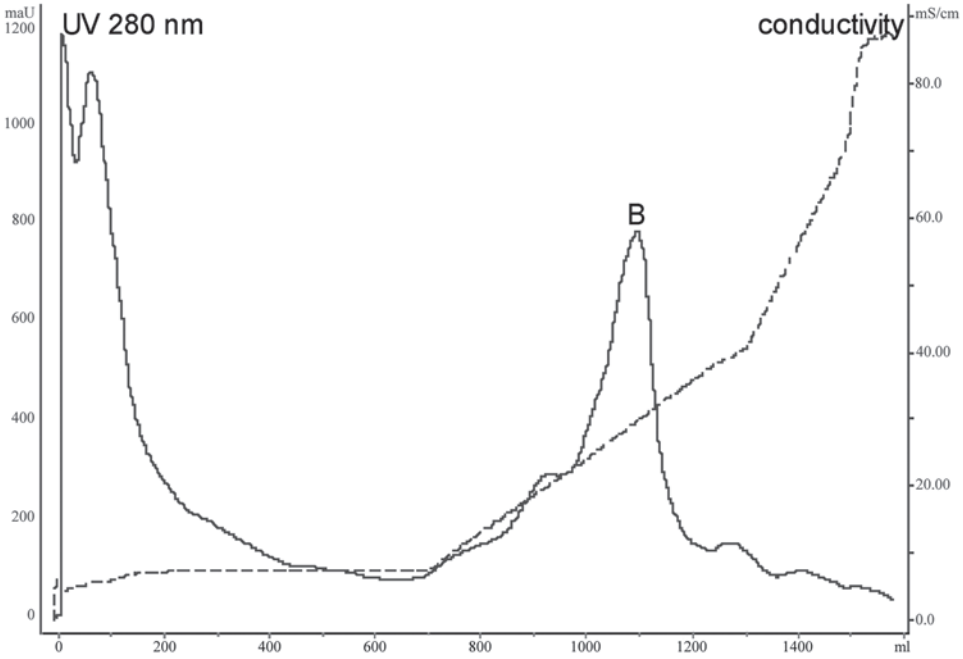

Fig. 9. Chromatogram of XET from fermentation culture filtrate by cation-exchange chromatography. Refer to the methods for details.

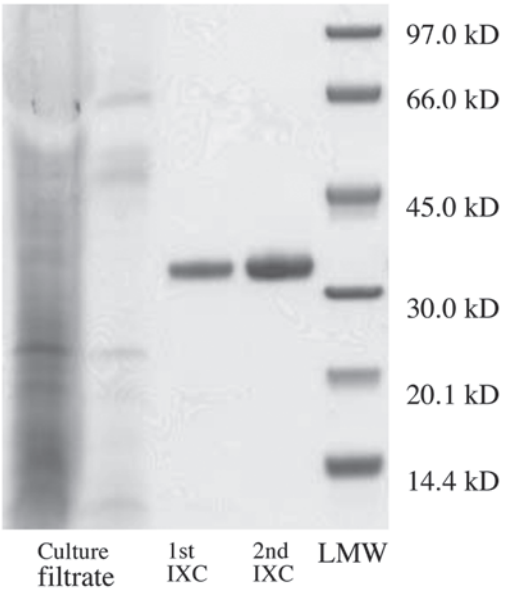

Fig. 10. SDS-PAGE of XET in the culture filtrate and after the 1st and 2nd cation exchange chromatographies, respectively.

Table 2
Summary of XET Purification From 1-L Culture Filtrate
After Purification With a One-Step Cation Exchange Chromatography (*see* Fig. 10)

Ferm conditions/ batch time	Filtrate total protein (mg)	(1 L) XET activity (U)	Vol (mL)	Total XET protein (mg/L)	Peak B XET activity (U/L)	Spec activity (U/mg protein)	XET recovery	Purification
T = 22°C/71.4 h	345	646	110	29	317.5	11	0.49	5.8
T = 18°C/72 h	561	1310	180	53	480	9	0.37	3.87
T = 18°C/163.4 h	704	2553	360	36.5	528	14.5	0.21	3.99
T = 15°C/162.6 h	660	2447	200	54	970	18	0.40	4.83

Conclusions

Active xyloglucan endotransglycosylase enzyme could be produced in methanol limited fed-batch fermentations with the methylotrophic yeast *P. pastoris*. However, severe inactivation resulting from proteolysis and denaturation in the foam was observed. The production of active XET was significantly improved by avoiding the foaming. Off-line determination of the influence of pH on the proteolysis was used to optimize the process pH, which further improved the product yield. Further reduction of the proteolysis was obtained by reducing the process temperature to 15°C.

XET enzyme was obtained through a one-step chromatography of the culture filtrate with a yield of about 54 mg XET protein per liter filtrate, with a specific activity of 18 U/mg protein.

Acknowledgments

We would like to thank to European Commission (QLK3-CT-2000-51211) for financial support.

References

1. Pauly, M., Albersheim, P., Darvill, A., and York, W. S. (1999), *The Plant J.* **20,** 629–639.
2. Fry, S. C., Smith, R. C., Renwick, K. F., Martin, D. J., Hodge, S. K., and Matthews, K. J. (1992), *Biochem. J.* **282,** 821–828.
3. Smith, R. C. and Fry, S. C. (1991), *Biochem. J.* **279,** 529–535.
4. Bourguin, V., Mellerowicz, E., Christiernin, M., et al. (2002), *Plant Cell.* **14,** 3073–3088.
4a. Brumer, H., Zhou, A., Bauman, M., Karlsson, K., and Teeri, T. T. (2004), *J. Am. Chem. Soc.* **126,** 5715–5721.
5. Steele, N. M. and Fry, S. C. (2000), *Phytochemistry* **54,** 667–680.
6. Schröder, R., Atkinson, R. G., Langenkämper, G., and Redgwell, R. J. (1998), *Planta* **204,** 241–251.
7. Nishitani, K. and Tominaga, R. (1992), *J. Biol. Chem.* **267,** 21058–21064.
8. Sulová, Z., Takácová, M., Steele, N. M., Fry, S. C., and Farkas, V. (1998), *Biochem. J.* **330,** 1475–1480.
9. Sulová, Z. and Farkas, V. (1999), *Protein Expr. Purif.* **16,** 231–235.
10. Thompson, J. E., Smith, R. C., and Fry, S. C. (1997), *Biochem. J.* **327,** 699–708.
11. Steele, N. M., and Fry, S. C. (1999), *Biochem. J.* **340,** 207–211.
12. Redgwell, R. J. and Fry, S. C. (1993), *Plant Physiol.* **103,** 1399–1406.
13. Percy, A. E., O'Brien, I. E. W., Jameson, P., Melton, L. D., Macrae, E. A., and Redgwell, R. (1996), *Physiol. Plant.* **96,** 43–50.
14. Purugganan, M. M., Braam, J., and Fry, S. (1997), *Plant Physiol.* **115,** 181–190.
15. Xu, W., Purugganan, M., Polisensky, D. H., Antosiewicz, D. M., Fry, S. C., and Braam, J. (1995), *Plant Cell* **7,** 1555–1567.
16. Arrowsmith, D. A. and de Silva, J. (1995), *Mol. Biol.* **28,** 391–403.
17. Campbell, P. and Braam, J. (1999), *Plant J.* **18,** 371–382.
18. Catala, C., Rose, J. K. C., York, W. S., Albersheim, P., Darvill, A. G., and Bennett, A. B. (2001), *Plant Physiol.* **127,** 1180–1192.
19. Clare, J. J., Romanos, M. A., Rayment, F. B., et al. (1991), *Gene* **105,** 205–212.
20. Weib, H. M., Haase, W., Michel, H., and Reiländer, H. (1995), *FEBS Lett.* **377,** 451–456.
21. Inan, M., Chiruvolu, V., Eskridge, K. M., et al. (1999), *Enzyme Microb. Technol.* **24,** 438–445.
22. Files, D., Ogawa, M., Scaman, C. H., and Baldwin, S. A. (2001), *Enzyme Microb. Technol.* **29,** 335–340.

23. Li, Z., Xiong, F., Lin, Q., et al. (2001), *Protein Expr. Purif.* **21,** 438–445.
24. Kobayashi, K., Kuwae, S., Ohya, T., et al. (2000), *J. Biosci. Bioeng.* **89,** 55–61.
25. Austin, A., Jones, C. E., and Van Heeke, G. (1998), *Protein Expr. Purif.* **13,** 136–142.
26. Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993), *Biotechnology* **11,** 905–910.
27. Jahic, M., Mulder, J. C., Martinelle, M., Hult, K., and Enfors, S.-O. (2002), *Bioproc. Biosyst. Eng.* **24,** 385–393.
28. Higgins, D. R. and Cregg, J. M. (1998), *Pichia Protocols Methods in Molecular Biology 103*, Humana, Totowa, NJ.

28a. Kallas, Å., Piens, K., Denman, S., et al. (2005), *Biochem. J.*, in press.

29. Sulová, Z., Lednická, M., and Farkas, V. (1995), *Anal. Biochem.* **229**, 80–85.
30. Vardar-Sukan, F. (1998), *Biotechnology Adv.* **16,** 913–948.
31. Sreekrishna, K., Brankamp, R. G., Kropp, K. E., et al. (1997), *Gene* **190,** 55–62.
32. Brierley, R. A., Davis, G. R., and Holtz, G. C. (1994), United State Patent No. 5,324,639 US.
33. Jahic, M., Gustavsson, M., Martinelle, M., Jansen, A.-K., and Enfors, S.-O. (2003), *J. Biotechnol.* **102,** 45–53.