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ScienceDirect
Journal of Biotechnology 130 (2007) 161–170
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Expression of xyloglucan endotransglycosylases of *Gerbera hybrida* and *Betula pendula* in *Pichia pastoris*

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Received 20 October 2006; received in revised form 27 February 2007; accepted 7 March 2007

Abstract

The plant enzyme xyloglucan endotransglycosylase (XET; EC 2.4.1.207, xyloglucan:xyloglucosyl transferase) participates in selective modification of plant cell walls during cell growth. XETs are potential catalysts in various applications. Here, sequences encoding two XETs from *Gerbera hybrida* and *Betula pendula* are reported. The encoded proteins, which are 51% identical at the amino acid level, were expressed in the yeast *Pichia pastoris* in secreted form with the aid of mating factor alpha signal sequence. XET production in shake flask cultivations was better at 22 °C than at 30 °C. Both the yield of protein of expected molecular mass and the XET activity improved at the lower temperature. Under all cultivation conditions studied, higher amounts of XET from *B. pendula* (BXET) were expressed than XET from *G. hybrida* (GXET). Both XET enzymes were produced in 16 l fed-batch bioreactor cultures. GXET was produced in methanol-limited fed-batch cultivation in minimal medium, and BXET in temperature-limited fed-batch (TLFB) in minimal or complex medium. Production was highest in TLFB in complex medium. BXET was purified from the culture filtrate and characterized. Based on the specific activity of the purified protein, 60–70 mg l^{-1} BXET was produced in the TLFB in complex medium.

Keywords: Xyloglucan endotransglycosylase (XET); *Pichia pastoris*; Heterologous expression; Bioreactor cultivation; *Gerbera hybrida*; *Betula pendula*

1. Introduction

Xyloglucan endotransglycosylase (EC 2.4.1.207, xyloglucan:xyloglucosyl transferase, XET) participates in various transformation reactions during plant development. XET cleaves a $\beta(1 \rightarrow 4)$ glucosidic bond in the backbone of xyloglucan, which is non-covalently but tightly bound via hydrogen bonds to cellulose microfibrils and may tether adjacent microfibrils in the cell wall (Fry, 1989; Vissenberg et al., 2005a). The enzyme transfers the xyloglucanyl segment to O-4 of the non-reducing terminal glucose residue of a xyloglucan or a xyloglucan oligosaccharide acceptor. The reactions carried out by XET may be applied *in vitro* on plant-derived cellulosic fibre substrates, *e.g.* pulp and paper raw materials, to produce new characteristics in the fibres. Catalysis by XET may also be applied in order to produce various intermediates for use in the chemical industry (Gustavsson et al., 2005). Furthermore, the importance of renewable resources as a source of raw materials for the pulp and paper, textile, and chemical industries increases interest in biocatalysts such as XET (Kofod and Lund, 1997; Teeri and Brumer, 2003; Wilkins, 2003).

Several XET enzymes from higher plants have been characterized. They are typically encoded by genes which belong to large families having tissue-specific and growth stage-dependent expression (Rose et al., 2002; Yokoyama et al., 2004; Vissenberg et al., 2005b). XETs have also been identified from microbes (Nielsen, 2002). XET enzymes have been heterologously expressed in *Escherichia coli* (Arrowsmith and de Silva, 1995; Purugganan et al., 1997; Schröder et al., 1998; Bourquin et al., 2002), baculovirus/insect cell system (Campbell and Braam,

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doi:10.1016/j.jbiotec.2007.03.004

1999) and in *Pichia pastoris* (Henriksson et al., 2003; Johansson et al., 2003, 2004). Recombinant XET proteins obtained from *E. coli* required solubilization and refolding in denaturating conditions in order to be active. Expression in baculovirus/insect cell system resulted in secretion of active XET into the culture medium (Campbell and Braam, 1999). Expressed protein levels have been very low and XET production has mainly been used for confirmation of identity and activity or for antibody production.

P. pastoris is known to produce foreign proteins at high levels, either intra- or extracellularly, but the production level obtained depends on the protein being expressed, and varies for plant proteins in the range of micrograms to grams per liter (Cereghino and Cregg, 2000). However, *P. pastoris* appears to be a more suitable host for production of heterologous glycoproteins than baker's yeast, *Saccharomyces cerevisiae* (Grinna and Tschopp, 1989). As a methylotrophic yeast, *P. pastoris* is often cultivated in methanol-limited fed-batch cultures, but more recently a temperature-limited fed-batch (TLFB) method was developed (Jahic et al., 2003). The reported benefits of TLFB include decreased cell lysis, reduced release of intracellular proteases to the cultivation medium, and decreased activity of proteases at the low temperature.

Our aim was to test expression of *Gerbera hybrida* and *Betula pendula* XET proteins, GXET and BXET, respectively, in *P. pastoris* and to characterize their properties. Here we report isolation of the two new genes encoding these XETs and their heterologous expression in *P. pastoris*. The TLFB method was tested with two cultivation media, and BXET from one TLFB cultivation was purified and characterized.

2. Materials and methods

2.1. Construction of cDNA libraries

Construction and sequencing of *G. hybrida* cDNA libraries have been described by Laitinen et al. (2005). The *B. pendula* (silver birch) cDNA libraries were constructed from stem tissue isolated from actively growing 10-year-old trees, clone ENSO V5834. Material for the "phloem-enriched cambium library" was prepared by peeling the bark and scraping the phloem side with a scalpel. The "developing xylem-enriched cambium library" was prepared by scraping the exposed surface xylem side from the peeled stem. The "maturing xylem-enriched library" was prepared by scraping the deeper, chip-like xylem region from the stem. The birch cDNA libraries were constructed and sequenced using the same methods as for the *G. hybrida* cDNA libraries, but the sequence cut off length was 250 bp.

2.2. Annotation of XET-encoding sequences and identification of full-length cDNA sequences

Putative XET-encoding *G. hybrida* and *B. pendula* ESTs (GXETs and BXETs, respectively) were identified through a BLASTX-search (Altschul et al., 1990) with several known plant XET sequences (O80431 (TOBAC), Q39099 (XTH4_ARATH), Q40144 (XTH1_LYCES) Q41638 (XTHA_PHAAN), Q41542 (XTH_WHEAT), Q94IP3 (Q94IP3_SOLSG)) against the local EST libraries. A full-length XET encoding EST (G0000100032F11) was identified in the petal library of *G. hybrida* (Laitinen et al., 2005), and completely sequenced in both directions. For *B. pendula*, an EST clone with the longest 5′ end (Bsp10400017C10) was selected among the highest scoring search hits. To identify a signal sequence region from the selected EST its translated sequence was aligned at the amino acid level with several known XET sequences using the ClustalW program (Thompson et al., 1994). A PCR-amplified sequence of this EST clone was cloned into pGEM-T Easy vector (Promega), and completely sequenced in both directions. The sequences have been deposited in the NCBI genebank with the accession numbers DQ235255 (GXET) and DQ235254 (BXET). The putative signal sequences were analyzed with TargetP v1.01 program (Nielsen et al., 1997; Emanuelsson et al., 2000) to predict the cleavage sites.

2.3. Construction of XET expression strains

P. pastoris strain GS115 (Invitrogen) was used as a host for XET expression. To simplify the subsequent cloning steps, the pPICZαA vector (Invitrogen) was first converted Gateway compatible. It was opened with *Eco*RI and *Asp*718, blunt-ended by fill-in and the Gateway conversion cassette B (Invitrogen) was inserted to obtain vector pJT9. Standard DNA methods were used for cloning (Sambrook et al., 1989). The sequence encoding XET of *G. hybrida*, without its predicted native signal sequence, was amplified by PCR with primers that contained the sequences required for Gateway-specific recombination according to the instructions in the Gateway technology manual (Invitrogen). The PCR product was cloned by *in vitro* recombination to the donor vector pDONR201 (Invitrogen) to create the entry clone pER21. The correct insert sequence was verified by sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems), after which it was further recombined into pJT9. The resulting plasmid was designated pER31 and directs production of GXET from the inducible alcohol oxidase 1 (*AOX1*) promoter. The GXET protein produced also contains mating factor α prepro sequence and a c-myc tag followed by six histidine residues at the carboxy terminus. The XET sequence of *B. pendula* was cloned in a similar manner as GXET, and the resulting entry clone was designated pJT19 and the expression plasmid pJT20. The only difference in the two procedures was the use of donor vector pDONR221 (Invitrogen) in the latter instead of pDONR201. For amplification of the plasmids, *E. coli* strains DH5α and DB3.1 (Invitrogen) were transformed by electroporation. pER31 and pJT20 were targeted into the *AOX1* locus of strain GS115 by linearizing them prior to transformation with a restriction enzyme *Sac*I that recognizes a unique site in the *AOX1* promoter sequence. Transformation of *P. pastoris* cells and selection of correct integrant clones were performed according to instructions of the EasySelect™ *Pichia* Expression Kit (Invitrogen). The control strain for XET expression clones was a pJT9 integrant clone of GS115.

2.4. Media and culture conditions

The expression clones were cultivated according to instructions in the EasySelect™ *Pichia* Expression Kit (Invitrogen), except that buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY), pH 6.0, were supplemented with 1% casamino acids (Bacto™) and the shake flask cultivations were performed at both 30 °C and 22 °C. The GXET and BXET production strains were deposited in the VTT culture collection with the accession numbers VTT C-05622 and VTT C-05623, respectively. *E. coli* was cultivated in LB medium supplemented with either 50 μg ml^{-1} kanamycin or 100 μg ml^{-1} ampicillin, or in low salt LB with 50 μg ml^{-1} zeocin. Inocula for the bioreactor runs were grown on 4× 250 ml 10 g l^{-1} buffered glycerol medium (Invitrogen) for 2 days at 28 °C and 250 rpm. The 1 l inoculum was bulked and transferred aseptically to the bioreactor. The growth medium in the first bioreactor cultivations was minimal medium (MM) containing 40 g l^{-1} glycerol and salts as described in the *Pichia* Fermentation Process Guidelines (Invitrogen), fortified with histidine as described later in the text. In one run a rich medium (RM) was used containing 2× buffered glycerol medium components (Invitrogen) with 4× glycerol (=40 g l^{-1}) and fortified with a feed of 0.1 ml min^{-1} 40 g l^{-1} histidine.

2.5. Bioreactor cultivations

The bioreactor was a Braun Biostat C30 with an initial working volume of 16 l. Process control was with New Brunswick AFS BioCommand, Version 2.62. Cultivation temperature was altered as described below. Minimum pH was maintained at 6.0 throughout the runs with NH_4OH solution. The minimum level of dissolved oxygen (DO) during the growth phase on glycerol was maintained at 25% by agitation (400–800 rpm) and oxygen enrichment of the incoming air at a constant total gas flow of 16 l min^{-1}. XET expression was induced by feeding of pure methanol (MeOH) containing PTM_1 salts (Invitrogen) after the initial growth on glycerol. The method of control of cultivation temperature, DO and methanol feeding rate during the induction phase varied between the three runs as described in the following. After the runs the cells were separated by centrifugation (Sorval RC12BP, 4000 rpm = 3900 × *g*, 8 °C, 20 min).

Run 1 (*GXET/MM*). Production of GXET was carried out using MM with 40 mg l^{-1} histidine at a constant temperature of 22 °C. MeOH was fed in a stepwise increasing manner from 3.6 ml l^{-1} h^{-1} to 10.9 ml l^{-1} h^{-1} as described in the Invitrogen instructions, and DO was maintained at >25% by O_2 enrichment of the incoming air at 800 rpm agitation. MeOH in the culture was analyzed off-line by HPLC of centrifuged samples (storage at −20 °C).

Run 2 (*BXET/MM*). Production of BXET was carried out using both MM (Run 2) and RM (Run 3, see below). A histidine feed was applied as described in Section 3. Initial temperature during the growth phase was 24 °C. At the beginning of the MeOH induction phase a control loop was constructed to effect control of DO on the basis of decreasing temperature. The process loop was composed of two logical commands:

1. IF DO < 25% SET [$T_{sp} = T_{csp} - 0.1$ °C], FALSE [$T_{sp} = T_{csp} + 0.1$ °C]; limits 20 °C/12 °C, where T_{sp} = temperature set point, T_{csp} = temperature current set point.
2. IF MeOH < 1.5 g l^{-1} AND IF DO > 22% SET PUMP = 25%, FALSE = 0%.

Thus (line 1) DO was maintained at 25% by incremental 0.1 °C increases or decreases in temperature set point from the current set point value (adjustments at 30 s intervals), with maximum and minimum permitted set point values of 20 °C and 12 °C, respectively. In order to avoid accumulation of MeOH under these non-substrate-limited conditions, the MeOH feed was made conditional so that the addition pump would operate only at low MeOH concentration in the culture (<1.5 g l^{-1}) and at DO levels above 22% (line 2). On-line monitoring of MeOH was performed using a MeOH/EtOH on-line probe (Frings, Germany) and analyzer. The induction phase of this run was operated with constant agitation (500 rpm) and aeration (16 l min^{-1}) without O_2 enrichment.

Run 3 (*BXET/RM*). BXET was also produced in RM with a continuous feed of 0.1 ml min^{-1} 40 g l^{-1} histidine (=15 mg l^{-1} h^{-1} based on the initial volume of 16 l). The temperature during batch growth was 22 °C, and was reduced to a minimum of 12 °C during the MeOH induction phase using the same algorithm as in Run 2 at 500 rpm and without O_2 enrichment. The concentration of histidine in the culture supernatant samples was analyzed using HPLC according to the Waters AccQ*Tag Chemistry Package Instruction Manual (Waters Corp., Milford, MA, WATO52874, rev0.).

2.6. Biomass and protein analyses

Yeast biomass was determined as centrifuged wet weight (ww, g l^{-1}). Samples of 2.0 ml were centrifuged in tared Eppendorf tubes for 4 min at 10,000 rpm, the liquid phase was discarded and the mouth of the inverted tube was blotted on absorbent paper (the pellet remained firmly attached to the bottom of the tube). The tube was then reweighed and the wet weight of the biomass was calculated.

Proteins from the culture supernatants were precipitated with trichloroacetic acid (TCA) and dissolved in Laemmli sample buffer prior to separation by SDS-PAGE in 10% acrylamide gels (Laemmli, 1970). Prestained low range SDS-PAGE standards (Bio-Rad Laboratories) were used to determine the molecular mass of the Coomassie-stained proteins. For Western analysis, the proteins were transferred onto nitrocellulose filters (Bio-Rad Laboratories), detected with antibodies against c-myc (9E10, ATCC CRL-1729), His-tag (Dianova GmbH) or bacterially produced Sso2p (Jäntti et al., 1994) and visualized with alkaline phosphatase conjugated secondary antibodies (Bio-Rad Laboratories) and BCIP/NBT Color Development Substrate (Promega). Protein concentration was determined with the Bio-Rad DC kit. Complete EDTA-free Protease Inhibitor Coctail (Roche) was added to the culture filtrate samples, and 0.1 mM PMSF (Sigma) and 1 μM Pepstatin A (Sigma) to the final bioreactor supernatant.

2.7. Measurement of XET activity

XET activity was measured using the method of Fry (1997) which was modified as follows: Tamarind xyloglucan (XG) (Megazyme) was used as the donor in the activity assay. The acceptor was prepared from Tamarind XG by endoglucanase digestion (Sulova et al., 1995). Undigested XG was separated from the xyloglucan oligomers by ultrafiltration with PM 10 membranes (Millipore). The xyloglucan oligomers in the permeate were hepta-, octa- and nonasaccharides as indicated by HPLC (Dionex, CarboPac PA1 column). The oligomers were labeled with sulphorhodamine (Lissamine rhodamine B sulfonyl chloride, Molecular Probes) as described by Fry (1997). The activity assay contained 0.1 mg XG donor and 0.05 nmol labeled XG-oligosaccharide acceptor in 100 μl of 0.35 M Na-succinate buffer, pH 5.5, and 50 μl of culture filtrate. When necessary, samples were concentrated with Vivaspin concentrators (10,000 MWCO, PES, VivaScience). In the control, the enzyme was replaced by buffer. After 2 h incubation at room temperature (22 °C) the reactions were monitored by fluorescence measurement. Ten microliter aliquots of reaction mixtures were spotted onto Whatman no. 1 paper. Unreacted acceptors were washed away with ethanol/formic acid/H_2O (1:1:1) for 1 h, followed by H_2O for 5 min. The paper was dried at 105 °C, attached to a Millipore MAFLS 40 microtiter plate and fluorescence was detected with excitation at 579 nm/emission at 615 nm (Victor 1420 V^2 equipment, Wallac). The fluorescence value of the control sample was subtracted from the values of the samples and the XET activities were expressed in arbitrary units, fluorescence counts per second per milliliter (cps ml^{-1}).

2.8. Purification and characterization of Betula XET

BXET was purified from 490 ml of clarified culture filtrate from Run 3 using the IMAC-technique on a Ni-substituted Chelating Sepharose FF column (Pharmacia, 10 ml). The enzyme was bound to the column in 50 mM Na phospate buffer, pH 7.2 containing 0.5 M NaCl, and was released with the same buffer containing 0.5 M imidazole. Fractions were analysed for XET activity and active fractions were combined, dialyzed against 0.35 M Na-succinate buffer, pH 5.5, and stored at −80 °C until further analysis. Purification was monitored by SDS-PAGE and Western analysis. Deglycosylation of purified BXET was carried out with endoglycosidase H (EC 3.2.1.96) according to the manufacturer's (Roche) instructions. The effect of pH on BXET activity was determined at 22 °C in McIlvaine universal buffers over the pH range 2.2–8.2 in 2 h incubation assays. The pH-stability was monitored by analyzing the residual activity in the normal activity assay after 1 h, 4 h and 24 h preincubation in McIlvaine buffers. The temperature dependence of BXET activity was studied at 2 °C, 10 °C, 22 °C, 30 °C, 40 °C, 50 °C and 60 °C. The temperature stability was estimated by determining the residual activity in the normal 2 h incubation assay at 22 °C after 15–180 min preincubation at 30 °C, 40 °C, 50 °C and 60 °C. Potential inhibitor or activator effects of 10 mM, 0.1 M and 1.0 M $CaCl_2$ and NaCl as well as 0.1 mM, 1.0 mM and 10 mM dithiothreitol (DTT) in succinate buffer at 22 °C were also tested.

3. Results

3.1. Expression of XET proteins in shake flask cultivations

The genes encoding xyloglucan endotransglycosylase of *G. hybrida* (GXET) and *B. pendula* (BXET) were identified from cDNA libraries. The proteins encoded by the two genes are 51% identical at the amino acid level and contain putative signal sequences (Fig. 1). Their sequences also contain one putative *N*-glycosylation site and four conserved cysteine residues. BXET is more similar to poplar PttXET16A (Bourquin et al., 2002) than GXET. At the amino acid level they are 87.5% and 53.5% identical, respectively, to PttXET16A.

Nine out of ten analyzed GXET transformants showed correct integration of the expression plasmid, as did all the 14 BXET transformants studied. Based on Western analysis, two of the GXET clones produced GXET in secreted form in BBMY medium at 30 °C. Three other clones produced less secreted GXET and four clones did not secrete measurable amounts of GXET. Most of the product detected by c-myc-specific antibody was of higher molecular mass than expected, approximately 54 kDa. Based on calculated molecular mass, this form could contain the prepro region of mating factor α. In addition, a smaller amount of a protein representing the expected molecular mass of 38 kDa was visible in the Western analysis (Fig. 2A).

Since the protein detected in Western analysis had a higher molecular mass than expected, a lower cultivation temperature (22 °C) was used in an attempt to improve the processing efficiency and to increase the amount of active product. The abundance of the 38 kDa and 54 kDa proteins varied depending on the cultivation temperature. At 22 °C the smaller, 38 kDa form dominated and the higher, 54 kDa form was barely visible, whereas at 30 °C the larger protein was the major product (Fig. 2A). Furthermore, only the supernatant from cultures at 22 °C contained detectable GXET activity (Fig. 2B). One of the clones analyzed showed higher expression and activity (2413 cps ml^{-1}) than the other clones, and was chosen as the production strain (VTT C-05622) for bioreactor cultivation.

The pattern of protein expression for BXET was similar to that observed with GXET; most of the protein was of correct size (*ca.* 39 kDa) when the cells were cultivated at 22 °C (Fig. 2A). Five of the clones analyzed showed XET activity at 22 °C, with one clone producing higher activity than the others (Fig. 2A). Its activity corresponded to 92,487 cps ml^{-1}, which was almost 40-fold higher than that of the best GXET-producing clone (Fig. 2B). This BXET-producing clone (VTT C-05623) was chosen for cultivation in the bioreactor.

3.2. XET production in bioreactor cultivations

GXET was produced in Run 1 at a constant temperature of 22 °C. After a long initial lag phase, probably resulting from insufficient histidine in the medium, the biomass reached 167 g l^{-1} and methanol (MeOH) feeding was started at

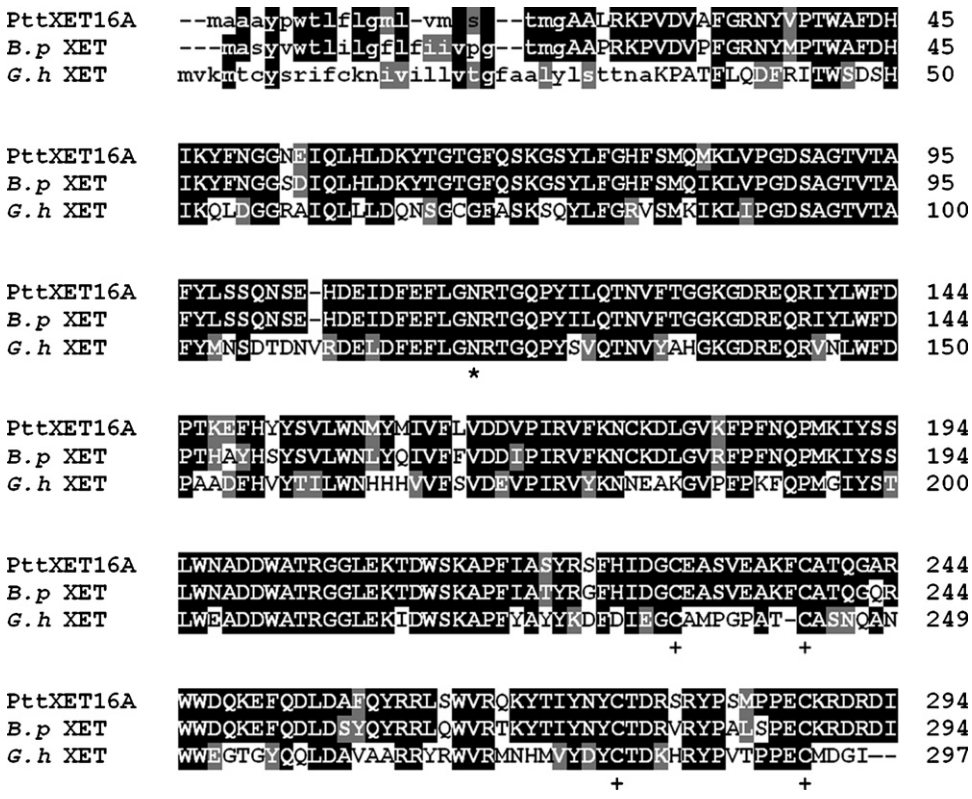

Fig. 1. Amino acid sequences of xyloglucan endotransglycosylases from poplar (PttXET16A), *Betula pendula* and *Gerbera hybrida*. The identical amino acids are marked in black boxes and the similar ones in grey boxes. The predicted signal sequences are denoted with lower case letters, the predicted site for *N*-glycosylation with * and the conserved cysteine residues with +.

143 h (see Fig. 3A). Methanol was limiting (MeOH < 0.1 g l^{-1} in all samples, data not shown) throughout the feeding phase. The maximum GXET activity in the culture supernatant (20,606 cps ml^{-1}) was obtained approximately 70 h post-induction (Fig. 4A). Analysis of the protein expression showed that, despite the relatively low cultivation temperature, the majority of the product was in the higher molecular mass form (Fig. 4B). Degradation products were also detected (Fig. 4B). The very long lag phase of 100 h (on glycerol) before exponential growth began may have resulted in some cell lysis and release of proteinases to the culture medium. Culture supernatant samples were analyzed with antibodies raised against a *Saccharomyces cerevisiae* plasma membrane protein, Sso2p (Aalto et al., 1992; Jäntti et al., 1994), which can also recognize a homologous protein in cell lysates of *P. pastoris* (Toikkanen et al., 2004). The analysis indicated that cell lysis had occurred during the cultivation (data not shown). However, the GXET activity obtained in the cultivation was more than eightfold higher than that obtained in shake flask cultivations at 22 °C (Figs. 2B and 4A).

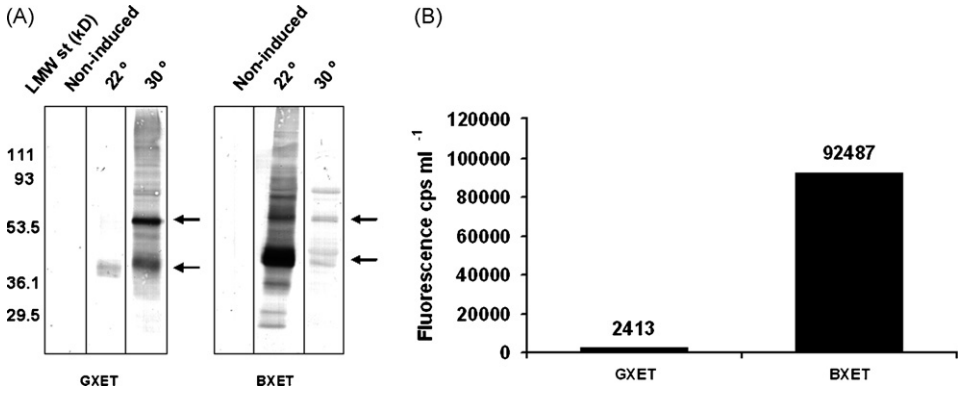

Fig. 2. Production of XET proteins by the strains VTT C-05622 (GXET) and VTT C-05623 (BXET) in shake flask cultivations at 30 °C and 22 °C. (A) Western analysis of culture supernatant (400 μl) 72 h post-induction with c-myc-specific antibodies. The molecular mass marker protein sizes are indicated on the left in kilodaltons. The proteins of expected size (lower arrows) and the higher molecular mass forms are indicated (higher arrows). (B) XET activity in culture supernatant (1 ml) taken 72 h post-induction at 22 °C. The arbitrary XET activities were measured after 2 h incubation at room temperature. Mean values from three cultivations are presented.

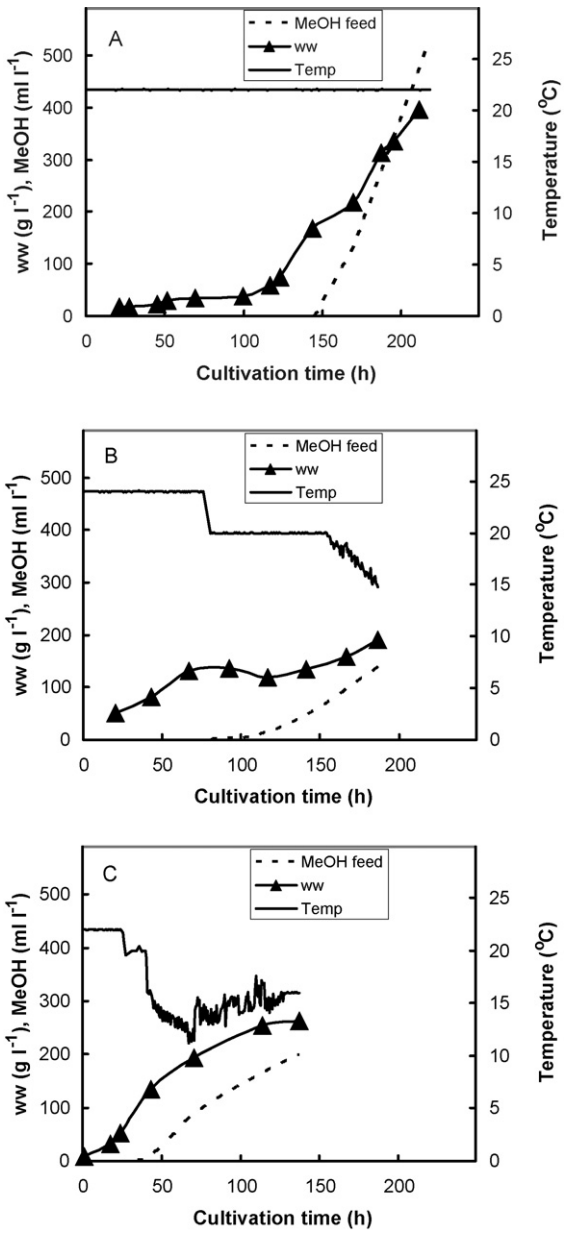

Fig. 3. Temperature, centrifuged wet weight (ww) and accumulated methanol feed (MeOH) in three bioreactor cultivations of *Pichia pastoris* GS115 for production of xyloglucan endotransglycosylase (XET) proteins. (A) GXET, minimal medium, MeOH substrate-limited fed-batch; (B) BXET, minimal medium, temperature-limited fed-batch (TLFB); (C) BXET, rich medium, TLFB.

Because of the production of high molecular weight GXET protein at 22 °C, a temperature-limited fed-batch (TLFB) method (Jahic et al., 2003) was used for the BXET production. In Run 2, a continuous feed of 1.75 mg l^{-1} h^{-1} histidine was installed during batch growth on glycerol at 26 h and increased in several stages to 26 mg l^{-1} h^{-1} at 117 h. The lag phase in this BXET culture on MM with extra histidine (Fig. 3B) was shorter than that of the GXET culture (Fig. 3A). The host strain GS115 is auxotrophic for histidine and apparently requires addition of histidine to the cultivation medium throughout the cultivation, not only as a batch addition at the beginning. Methanol induction was commenced at 80 h. To avoid overfeeding of MeOH at reduced temperature, its concentration in the fermentation medium was monitored on-line. MeOH was not growth-limiting during the induction phase with temperature limitation. Some cell lysis occurred during the fermentation as detected with antibodies against Sso2p (data not shown), but BXET degradation products were not detected by Western analysis (Fig. 4B). Furthermore, the majority of the protein produced during the induction phase was of the expected molecular mass, and only a small fraction appeared to be unprocessed. The temperature during the induction phase eventually decreased to a minimum of 15 °C in order to maintain DO > 25%. The maximum BXET activity was 408,920 cps ml^{-1}, obtained 107 h post-induction. This value was *ca.* 20-fold higher than that obtained in GXET culture (Fig. 4A).

BXET was also produced in a rich medium supplemented with a continuous feed of 0.1 ml min^{-1} 40 g l^{-1} (15 mg l^{-1} h^{-1}) additional histidine throughout the run. No lag in initial cell growth was observed (Fig. 3C). The amount of MeOH present in the medium during induction under conditions of temperature limitation was controlled at 1.5 ± 0.5 g l^{-1} (Fig. 5). The temperature decreased to 12 °C during the induction phase beginning at 34 h. Some cell lysis occurred during the cultivation, based on Sso2p detection in Western analysis (data not shown), but active BXET was obtained. Most of the protein appeared to be correctly processed and there was no detectable degradation (Fig. 4B). The maximum activity, obtained 102 h post-induction, was 1,527,200 cps ml^{-1}, which was almost fourfold that observed in the previous fermentation on minimal medium with histidine feed (Fig. 4A). A decrease in the production rate of BXET occurred after 70 h (arrowed in Fig. 5), at which time 7.5 l culture was removed. This partial harvesting resulted in a decrease in DO (Fig. 5), probably because only one of the bioreactor's impellers was in use at the low culture volume. Reduced mean DO in turn decreased the rate of MeOH feeding according to the conditions of the control algorithm (PUMP = 0 IF DO < 22%).

3.3. Purification and characterization of BXET

Ni-IMAC was the best of the various chromatographic techniques tested for purification of BXET. 7.8-fold purification was obtained, and the purified active BXET was visible as a polypeptide band with an average molecular mass of 42 kDa on a Coomassie blue-stained SDS-PAGE (Fig. 6A) and in a Western blot detected with myc-specific antibodies (data not shown). Endo H treatment decreased the molecular mass of purified BXET to *ca.* 39 kDa (Fig. 6A). This size difference (Grinna and Tschopp, 1989) indicated that the *N*-glycosylation site in the BXET sequence had been glycosylated in *Pichia*. Based on the specific activity of purified BXET, expressed in arbitrary units (fluorescence per protein), the amount of active BXET protein in the culture filtrate was approximately 60–70 mg l^{-1}.

BXET activity was optimal at approximately pH 6 and 30 °C (Fig. 6B and C). The enzyme was 100% stable for 24 h in the pH range 5–7 at room temperature, but at pH values below and above this range 50–75% of the activity was lost (Fig. 6D). BXET was sensitive to increased temperature, with a half-life of only

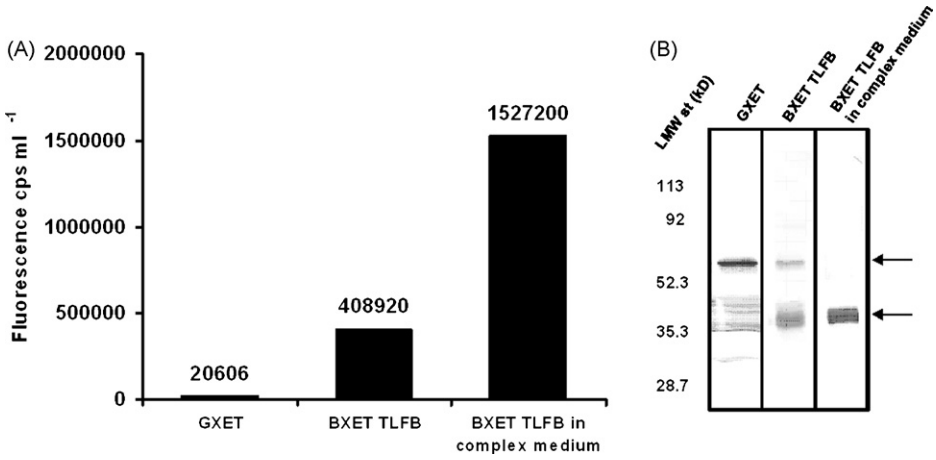

Fig. 4. XET production in three bioreactor cultivations. (A) Arbitrary XET activities present in 1 ml of bioreactor culture supernatants measured after 2 h incubation at room temperature; (B) Western analysis of 50 μl culture supernatant taken at the end of the induction phase detected with c-myc-specific antibody.

30 min at 30 °C (Fig. 6E). BXET activity was reduced 55% in the presence of 1 M $CaCl_2$, whereas NaCl had hardly any effect on its activity. BXET activity was increased by 30% in the presence of 1 mM DTT (results not shown).

4. Discussion

The properties of XET enzymes make them interesting for several applications, but it is still a challenge to produce them heterologously in adequate quantities for these applications. Here we have reported the isolation of two new genes encoding XET from *G. hybrida* and *B. pendula*, and their expression in *P. pastoris* in secreted form using the mating factor α (Mfα) prepro sequence in place of the native signal sequences.

In the shake flask cultivations the yield and quality of the produced enzymes GXET and BXET were improved by decreasing the cultivation temperature from 30 °C to 22 °C. The efficiency of post-translational protein processing, such as folding in the endoplasmic reticulum, may improve at lower temperatures and thus result in increased secretion of properly matured protein (Hurtley and Helenius, 1989; Ellgaard and Helenius, 2003). In the case of GXET and BXET the reduced cultivation temperature probably also affected the efficiency of removal of the Mfα prepro region. In the supernatant samples from 30 °C cultivations a higher molecular mass form dominated, which probably contained unprocessed Mfα prepro region. This form was absent or clearly less pronounced in the samples obtained from the shake flask cultivations at 22 °C (see Fig. 2). Previously, the processing of Mfα prepro sequence has also appeared to be a rate-limiting step in heterologous protein expression from *Pichia*, and heterologous proteins with variable amino termini have been observed (Cereghino and Cregg, 2000). Furthermore, the cleavage sites of the native signal sequences of these XET proteins were predicted by computer analysis and if they were not accurate, the yield of native proteins may have been affected.

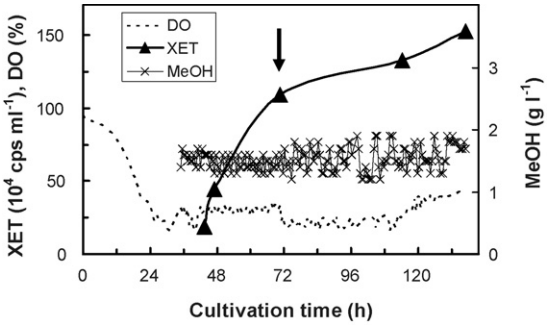

Fig. 5. Dissolved oxygen (DO), medium methanol concentration (MeOH) and xyloglucan endotransglycosylase activity (XET) in the temperature-limited fed-batch laboratory bioreactor cultivation for BXET production on rich medium. Partial harvesting (7.5 l of culture) was performed at 70 h (arrow).

Surprisingly, when GXET was produced in the bioreactor at 22 °C most of the product had a higher molecular mass than expected. This may have been due to the higher overall growth rate in the bioreactor than in shake flasks, with a corresponding reduction in the capacity of the cells to process the heterologous protein. Therefore, we used the TLFB method (Jahic et al., 2003) to further limit cell growth during MeOH induction in the BXET cultures.

Application of the TLFB method resulted in a high yield of active BXET, with little BXET of higher molecular mass product and reduced cell lysis. The biomass concentration produced in the BXET TLFB processes was lower than in the MeOH-limited GXET process (Fig. 3). Use of the TLFB method also had advantages for downstream processing. The very high yeast biomass produced in MeOH-limited cultivations of *P. pastoris* limits the amount of heterologous protein recovery in downstream processing because of loss of liquid associated with the packed biomass fraction, which is typically *ca*. 50% of the volume of the culture after centrifugation (Porro and Mattanovich, 2004). Attempts to reduce this loss by rinsing and filtration or re-centrifugation would increase process costs and might also result in increased levels of intracellular impurities. The TLFB method required less MeOH during the induction phase than was required in the MeOH-limited feed but still resulted in higher product yield, which is an obvious economic advantage. Furthermore, oxygen enrichment of the incoming air was not necessary in TLFB, which improves both economic and safety aspects of the process.

Cell growth in the TLFB process in MM appeared to be at least partly substrate-limited (histidine or other nutrient), because the temperature did not decrease to 12 °C during the protein production phase as it did in the RM culture. No histidine was detected in the culture supernatant during the induction phase (data not shown). In RM supplemented with histidine, more cell biomass was produced than in MM and the temperature did decrease to 12 °C. Almost fourfold higher BXET activity was produced in RM compared to MM (Fig. 4A). These observations clearly suggest that GS115-derived strains should either be converted to His^+ or should be grown in rich medium for efficient production of heterologous proteins.

Based on the specific BXET activity of the purified protein, the amount of BXET produced in RM was 60–70 mg l^{-1}. On the basis of the production curve in Fig. 5, final BXET activity would probably have been even higher without the partial harvesting of culture at 70 h, which was performed in order to be certain of obtaining material for purification and characterization of the BXET. Concentrations of XET protein in culture medium of recombinant *P. pastoris* have been reported to be 10 mg l^{-1} for tomato XET (Chanliaud et al., 2004) and 54 mg l^{-1} for poplar XET (Bollok et al., 2005). The heterologus XET expression and the purification of recombinant XET for estimation of its specific activity have been challenging in *E. coli* and the baculovirus/insect cell system (Arrowsmith

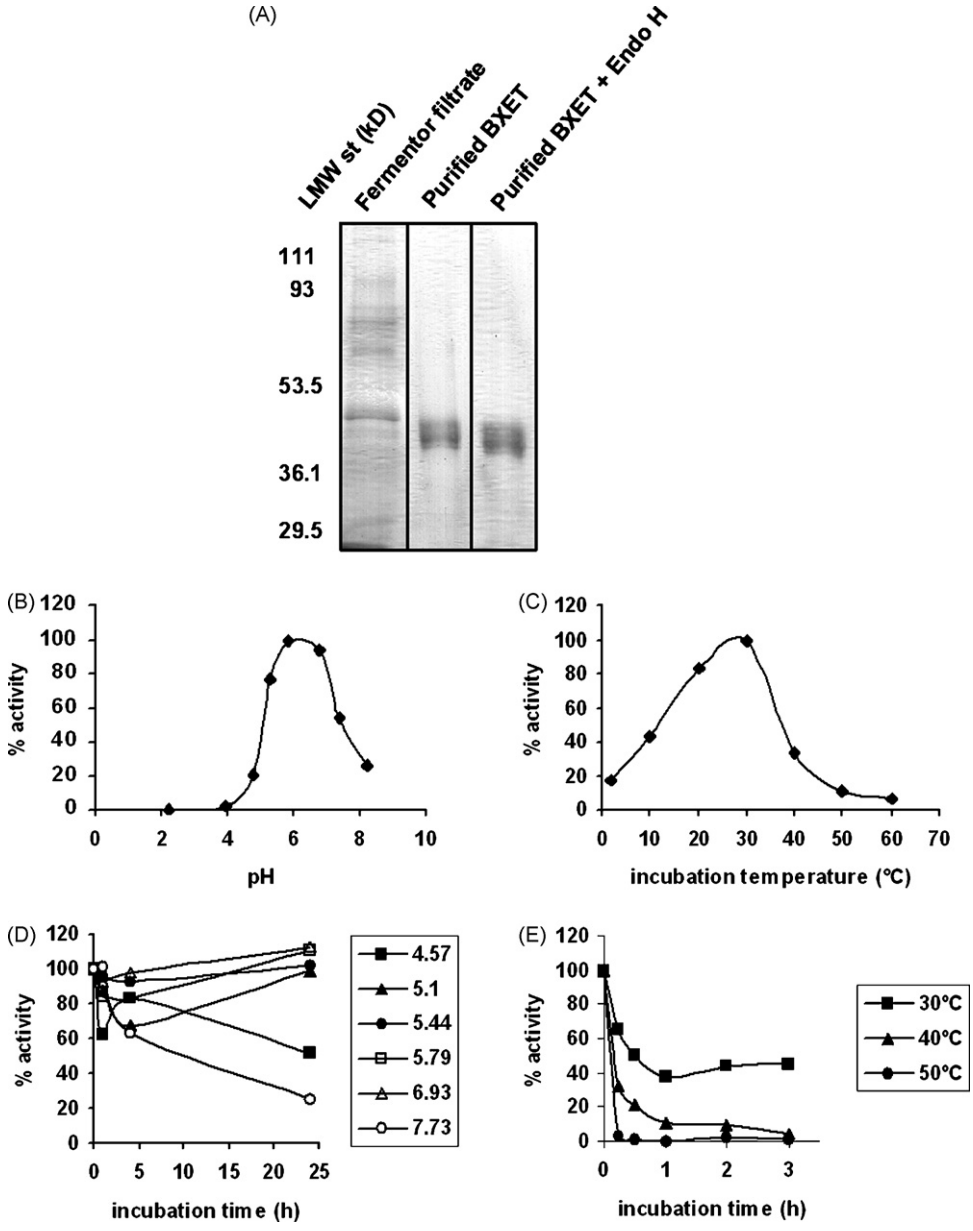

Fig. 6. Properties of purified BXET. (A) Purification and Endo H treatment of BXET. 3.4 μg total protein from bioreactor culture filtrate and 0.9 μg of purified BXET, with or without Endo H treatment, were separated in 10% SDS-PAGE and stained with Coomassie blue. The sizes of the molecular mass marker proteins are indicated on the left in kilodaltons. (B) Optimal pH and (C) temperature, and (D) the pH and (E) temperature stability of BXET. Activity was measured as described in Section 2.

and de Silva, 1995; Purugganan et al., 1997; Schröder et al., 1998; Campbell and Braam, 1999; Bourquin et al., 2002). Comparison of expression levels is also complicated by the use of different activity assays. Some assays, *e.g.* using radioactivity or fluorescence in the acceptors (Fry et al., 1992; Fry, 1997; Vissenberg et al., 2000), estimate the end product of the transfer reaction. Others, *e.g.* viscosimetric and spectrophotometric methods (Lorences and Fry, 1993; Sulova et al., 1995), measure the first step of the reaction, cleavage of the donor in the presence of acceptor. The method developed and used here was a simplified assay for fluorescent end product. It was valid for screening of the *P. pastoris* clones expressing plant XETs and for monitoring the purification and characterization of BXET.

BXET was purified in this work by Ni-IMAC chromatography. Previously both this method (Bourquin et al., 2002; Schröder et al., 1998; Arrowsmith and de Silva, 1995) and cation exchange chromatography (Henriksson et al., 2003; Bollok et al., 2005) have been used in purification of recombinant XETs. The only detailed figures for purification of recombinant XET are available from the work of Bollok et al. (2005). They reported 4.83-fold purification for poplar XET expressed in *P. pastoris* using cation exchange, whereas in this work 7.76-fold purification was obtained for BXET with the Ni-IMAC technique.

BXET has similar characteristics to other plant XET enzymes (http://www.brenda.uni-koeln.de), which have pH optima in the range of 5–6.5 and are stable over the pH range 4–8. As with other plant XETs (maximum activity at 12–37 °C), BXET had a relatively low temperature optimum for activity (*ca.* 30 °C). BXET was less stable than some other plant XETs, being rapidly inactivated at 30 °C. Both GXET (*ca.* 38 kDa) and BXET (*ca.* 39 kDa) are similar in size to other XET proteins (http://www.brenda.uni-koeln.de).

In conclusion, the two plant XET enzymes were successfully expressed in the methylotrophic yeast *P. pastoris*. Considerable improvement in production and post-translational processing was obtained by cultivation at reduced temperatures. In the bioreactor cultivations, use of the temperature-limited fed-batch (TLFB) technique led not only to higher production of active protein but also to improved process feasibility. The yields of active XET protein could probably be further increased by optimization of the TLFB technique. Use of a prototrophic yeast strain would also probably lead to improvements in the production process. The properties of the BXET purified and characterized in this work were similar to those reported for other plant XET enzymes in the literature.

Acknowledgements

The expression of XET proteins was carried out by the Expression Service of VTT Biotechnology. Hanna Kuusinen, Riitta Isoniemi and Birgit Hillebrandt-Chellaoui are thanked for skillful technical assistance, Juha Tähtiharju for help with the fermentations, Tapani Suortti for analysis of histidine, and Marilyn Wiebe and Ilkka Virkajärvi for useful discussions. This work (within project 40408/01) was supported by the Finnish National Technology Agency (Tekes) and was part of the research programme "VTT Industrial Biotechnology" (Finnish Centre of Excellence programme, 2002–2005, Project no. 64330).

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