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Ramchuran, Santosh

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PO Box 117
221 00 Lund
+46 46-222 00 00

The modular xylanase Xyn10A from *Rhodothermus marinus* is cell-attached, and its C-terminal domain has several putative homologues among cell-attached proteins within the phylum Bacteroidetes

Eva Nordberg Karlsson^{a,*}, Maher Abou Hachem^{a,1}, Santosh Ramchuran^a, Hugo Costa^a, Olle Holst^a, Åsa Fex Svenningsen^b, Gudmundur O. Hreggvidsson^c

^a Department Biotechnology, Center for Chemistry and Chemical engineering, Lund University, P.O.Box 124, SE-221 00 Lund, Sweden

^b Department Physiological Sciences, Division of Neuroendocrine Cell Biology, BMC F10, Lund University, SE-221 84 Lund, Sweden

^c Prokaria Ltd, Gylfajföt 5, IS-112, Iceland

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Abstract

Until recently, the function of the fifth domain of the thermostable modular xylanase Xyn10A from *Rhodothermus marinus* was unresolved. A putative homologue to this domain was however identified in a mannanase (Man26A) from the same microorganism which raised questions regarding a common function. An extensive search of all accessible data-bases as well as the partially sequenced genomes of *R. marinus* and *Cytophaga hutchinsonii* showed that homologues of this domain were encoded by multiple genes in microorganisms in the phylum Bacteroidetes. Moreover, the domain occurred invariably at the C-termini of proteins that were predominantly extra-cellular/cell attached. A primary structure motif of three conserved regions including structurally important glycines and a proline was also identified suggesting a conserved 3D fold. This bioinformatic evidence suggested a possible role of this domain in mediating cell attachment. To confirm this theory, *R. marinus* was grown, and activity assays showed that the major part of the xylanase activity was connected to whole cells. Moreover, immunocytochemical detection using a Xyn10A-specific antibody proved presence of Xyn10A on the *R. marinus* cell surface. In the light of this, a revision of experimental data present on both Xyn10A and Man26A was performed, and the results all indicate a cell-anchoring role of the domain, suggesting that this domain represents a novel type of module that mediates cell attachment in proteins originating from members of the phylum Bacteroidetes. © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Cell attachment; Xylanase; Bacteroidetes

1. Introduction

Glycoside hydrolase (GH) multiplicity is a common theme among microorganisms capable of degrading the complex and recalcitrant polysaccharide composites found in plant and algal cell walls. These enzymes typically have a modular organisation consisting of catalytic modules (CMs) usually, but not always, joined to

* Corresponding author. Tel.: +46 46 222 46 26; fax: +46 46 222 4713.

E-mail address: eva.nordberg_karlsson@biotek.lu.se (E.N. Karlsson).

¹ Present address: Biochemistry and Nutrition group, Biocentrum DTU, DK-2800 Kgs Lyngby, Denmark

non-catalytic modules (NCMs) by flexible linker sequences [1,2]. The most common types of NCMs are carbohydrate-binding modules (CBMs), but a number of other domains or modules, some of yet unknown function, have been reported and include NCMs involved in cell adhesion, or protein anchoring [3].

Two different types of domains have earlier been suggested to play a role in cell adhesion/anchoring of glycoside hydrolases, these are the so-called fibronectinIII-like domains (Fn3-like domains), and the S-layer homology domains (SLH-domains). The Fn3-like domain, which has a length of approximately 100 residues, is phylogenetically spread and presented in a superfamily of sequences representing receptor proteins or proteins involved in cell-surface binding mainly in eukaryotes. It is also found in some extra-cellular bacterial glycoside hydrolases [4]. These domains are commonly distributed in multiple copies in modular glycoside hydrolases, and are often found between catalytic modules and CBMs. Recently it has however been demonstrated that the Fn3-like domains have a role in hydrolysis of insoluble substrates [5]. The SLH-domain is a domain of about 50–60 residues, found at the N- or C-termini of mature proteins [6] and is believed to be anchored to the peptidoglycan [7], or some other structure in the bacterial cell wall [8]. This domain is almost exclusively bacterial with 63 of the 64 sequences reported to pfam-database (<http://pfam.wustl.edu>) in the bacterial branch, and most commonly occurring within the *Bacillus/Clostridium* group and in related Gram-positive bacteria [6].

The thermophilic marine aerobic bacterium *Rhodothermus marinus* stains Gram-negative and is phylogenetically affiliated to the Bacteroidetes (also known as the Cytophaga/Flexibacter/Bacteroides-group) [9], a phylum with many known degraders of organic matter. This group of bacteria is known to produce a number of cellulose degrading enzymes. Moreover, members of the Cytophaga, one of the better studied genera within this phylum, do not produce soluble extra-cellular cellulose hydrolases, but instead keep their enzymes attached to the cell-envelope [10]. Despite established cell-attachment, only one gene within the phylum Bacteroidetes has been reported to encode a homologue to the SLH-domain (an S-layer protein precursor from *Cytophaga* sp. Jeang 1995).

Rhodothermus marinus resembles other microorganisms within this phylum, in its ability to produce a number of glycoside hydrolase activities [11–13], as well as in displaying enzyme activities suggested to be cell-attached. Primary structures of some of the *R. marinus* glycoside hydrolases are known, including one family 10 xylanase [14], and one family 26 mannanase [15]. The xylanase (Xyn10A) is a modular enzyme that consists of two N-terminal family 4 CBMs followed by a domain of unknown function, a catalytic

module classified as GH10 and a finally a 5th domain (D5) at its C-terminus [14,16]. Alignments of the *Rm* Xyn10A-catalytic module with family 10 xylanases, unmasked D5 as an extended C-terminal sequence [14], preceded by a short stretch of repeated glutamic acid and proline residues, typical for the linker sequences often found joining modules in glycoside hydrolases. To cast more light on the possible function of this domain a search for similar sequences was accomplished using accessible databases as well as available partial genome sequences from *R. marinus* and from the related organism *Cytophaga hutchinsonii*. Based on the findings in this search and combined with experimental evidence the possible role in cell-adhesion of the Xyn10A C-terminal domain and its homologues is discussed.

2. Materials and methods

2.1. Sequence analysis and similarity searches

Bioinformatic tools were used to explore the primary structure of D5 in Xyn10A from *R. marinus*. Similarity searches by BLAST, using D5 of *R. marinus* as template, were performed on the NCBI server (<http://www.ncbi.nlm.nih.gov>) or locally using BioEdit v. 5.0.6. on available genome sequences of *C. hutchinsonii* (from the KEGG database), or partial genome sequences of *R. marinus* (available via Prokaria Ltd, Reykjavik, Iceland). Location of a putative signal peptide was predicted by SignalP v.1.1. (<http://www.cbs.dtu.dk/services/SignalP>). Matches with open reading frames of unknown function were subjected to an additional search by BLAST after deletion of the part showing high similarity to D5, to predict the putative function of the remaining part of the ORFs.

The ClustalW tool on the EBI server (<http://www.ebi.ac.uk/clustalw>) was used to create multiple sequence alignments and phylogenetic trees, displayed using Gene doc 2.6.02 [17], and TreeView 1.5 [18], respectively. Theoretical isoelectric points, and amino acid composition of deduced amino acid sequences were analysed by ProtParam (<http://www.expasy.org/tools/protparam.html>), and Microsoft Excel.

2.2. Cultivation of *R. marinus*

Rhodothermus marinus was grown with and without xylan (5 g/L, Birch 7500.1 from Carl Roth, Karlsruhe, Germany) at 65 °C, pH 7.1, with aeration on 5 L/min, in 2.5 L modified M162 medium [11,19] in a 3 L bioreactor inoculated with a 100 ml shake-flask culture ($OD_{620\text{nm}} = 0.7$). Optical density measurements ($OD_{620\text{nm}}$) monitored cell growth. Mannanase producing *R. marinus* were grown in 100 ml shake-flask cultures

using the M162 medium including Locust Bean Gum 6 g/L (Sigma–Aldrich, St. Louis, Mo).

Samples for activity analysis and electrophoresis were withdrawn during the early log, mid-log, late log and in the stationary phases and kept at 4 °C until analysis. The culture supernatant and cell-fraction were separated by centrifugation at 25,000g for 30 min at 4 °C. The whole cell-fraction was washed with 20 mM sodium phosphate buffer at pH 7.0, recentrifuged and resuspended to the original sample volume in the above buffer.

2.3. Activity analysis and electrophoresis

Xylanase activity was measured in the culture supernatant, and on whole cells using the DNS method as described elsewhere [20] with birch xylan (Carl Roth) as substrate and using individual enzyme blanks. Xylanase production in *R. marinus* was also analysed using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) with 10% separation gels [21], activity stained with Congo-red as previously described [22], except for the following modifications: the over-layer agarose gel contained 0.05% oat spelt xylan (Sigma), the buffer used was 50 mM Tris–HCl, pH 7.5, and incubation time at 65 °C was 60 min.

Mannanase activity was determined by a halo plate assay containing 3.5% (w/v) agar and 0.1% (w/v) Azocarob Galactomannan (Megazyme, Bray, Ireland). Samples (80 µL) were loaded into wells and plates were incubated at 65 °C overnight.

2.4. Immunocytochemistry

Drops of cell suspension were dried on SuperFrost microscope slides (Menzel–Gläser, Germany). When completely dry, the cells were fixed for 20 min in Stefani fixative (2% paraformaldehyde and 15% saturated aqueous picric acid solution in 0.1 M phosphate buffer, pH 7.2), followed by repeated rinsing in sucrose-enriched 10% Tyrodes' solution and finally in phosphate-buffered saline (PBS). The cells were then permeabilized and blocked (in the same step) using a blocking solution containing; 0.25% Triton X-100 and 0.25% BSA (both from Sigma) in PBS. This was followed by incubation in a moist chamber with a rabbit anti-CBM4-2 primary antibody [immunoglobulin fraction (10 mg/ml) from serum drawn from a rabbit immunised with recombinant produced purified carbohydrate binding module (CBM4-2) of Xyn10A] in a 1:100 dilution in blocking solution, over night. The next morning excess antibody was rinsed off and the cells were further incubated for 2 h, with fluorescent Rhodamine Red-X-conjugated donkey anti-rabbit secondary antibody (Jackson Laboratories, PA, USA) diluted 1:400 in blocking solution.

The slides were then rinsed 2 × 10 min in blocking solution and once in PBS.

After this procedure fluorescent Sytox green (Molecular Probes, WA, USA) was added in a 1:3000 solution of PBS for 10 min, and then rinsed for another 15 min in PBS before mounting.

The immuno-labelled cells were visualized using an Olympus BX-60 microscope connected to an Olympus DP-50 digital camera. Photomicrographs were taken with the viewfinder Lite software.

3. Results and discussion

3.1. Similarity between C-terminal parts of *R. marinus* xylanase and mannanase

Initially, the only domain among the publicly accessible sequences found to share primary structure similarity with the Xyn10A D5 domain of *R. marinus*, was from another hemicellulose degrading enzyme originating from the same organism (*Rm* Man26A) [15]. The similarity was restricted to the C-terminal part of the two enzymes (33% identity) (Fig. 1). Evaluation of a multiple sequence alignment including the *R. marinus* mannanase and a number of known catalytic modules (CMs) of GH 26 suggested also the C-terminal part (residues 939–1021) of this enzyme to be a separate domain, as it flanks the CM downstream the consensus region, rather than lying within it even though no linker sequence separating it from the catalytic module is distinguishable in this enzyme (data not shown).

It was also noted that D5 of RmXyn10A (residues 913–997, in the Xyn10A-sequence) has a theoretical *pI* value of 11.05, which is strikingly higher than either the full-length xylanase or any isolated module thereof (all with *pI*s of 4–4.5) but in better agreement with the C-terminal domain of *Rm* Man26A (*pI* 11.65). The high similarity between these two domains and their occurrence at the C-terminus of two modular hemicellulases

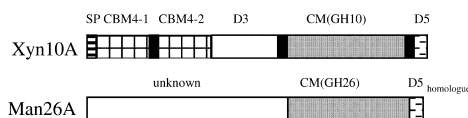


Fig. 1. Domain structure of the xylanase Xyn10A and mannanase Man26A of *Rhodothermus marinus*. The identified domains (D) or unknown regions in the primary sequence are shown as blocks. Regions/domains of unknown function are shown in white, catalytic modules (CM) in grey and carbohydrate binding modules (CBM) in a squared pattern. Identified linker sequences are shown in black, and signal peptides striped. The C-terminal domains of the respective protein are marked by dashes.

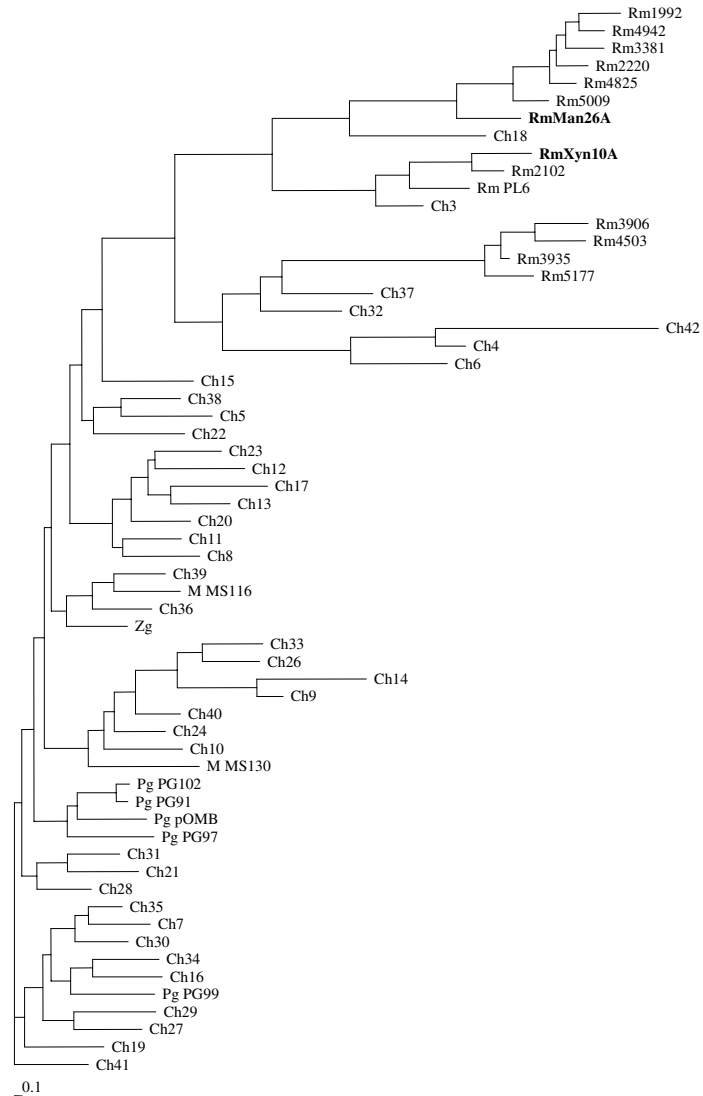


Fig. 2. Phylogram over the putative domains found by BLAST - similarity search using D5 of *Rm Xyn10A* as template. Open reading frames from the respective organism are numbered, and labelled: Ch (*Cythophaga hutchinsonii*), M (*Microscilla* sp.), Pg (*Porphyromonas gingivalis*), Rm (*Rhodothermus marinus*), Zg (*Zobellia galactanivorans*). The phylogram is displayed using TreeView, and created using the EBI-ClustalW-tool with the output in the phylip format, and using default parameters, with correct dist. "on", and ignore gaps "off".

from the same organisms suggested the possibility of a common function. Affinity electrophoresis analysis in a previous work failed to show any mannan, xylan or glu-

can-binding capacity of the xylanase domain, ruling out a carbohydrate binding function to substrates related to either of the catalytic modules [23].

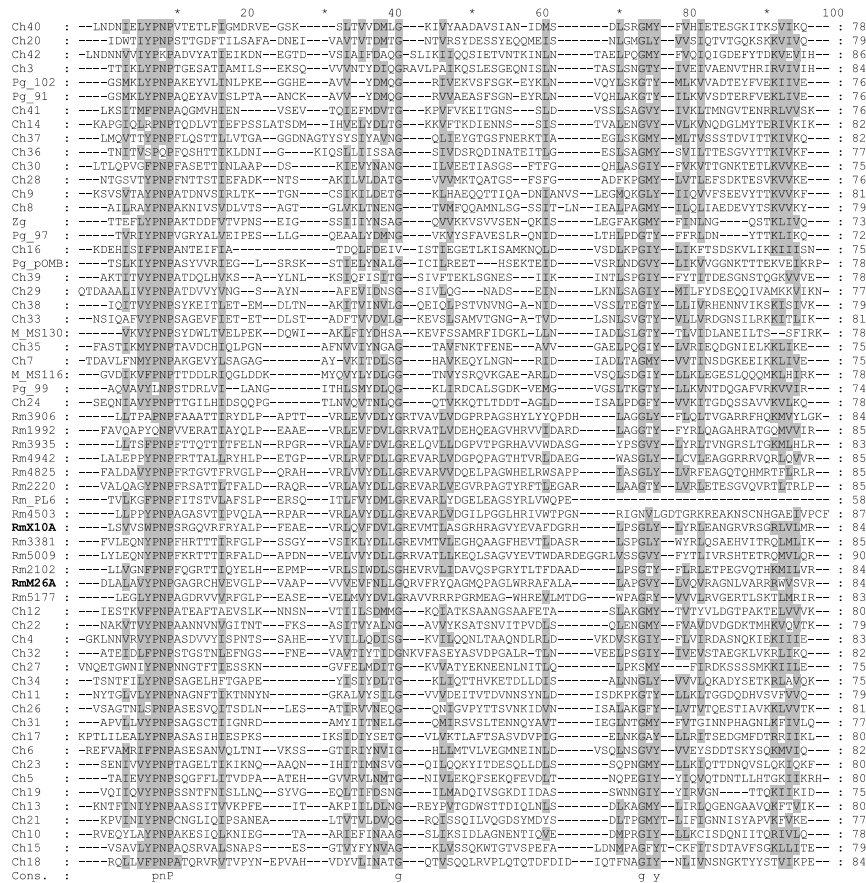


Fig. 3. Multiple sequence alignment of C-terminal domains encoded in the proteins found by BLAST - similarity search using D5 of *Rm Xyn10A* as template. The first consensus region starts at position 7 in the alignment (residue 915, *Xyn10A* numbering) and spans seven residues with the motif [(I/L/M/V), X, (I/L/M/V), (F/W/Y), P, N, P]. The second consensus region spans positions 37–44 [(I/L/V), X, (I/L/M/V), (I/L/M/T/V/F/W/Y), (D, N), (I/L/M/V), X, G], and mostly involves conserved hydrophobic residues. This is also true for the third region, which is located at position 74–85 in the alignment, and has 1–2 inverted residues in a few of the sequences [(I/L/M/V), X, X, G, (I/L/M/T/V), Y, -, -, (F/I/L/M/V), (I/L/M/V), X, (I/L/M/V)]. The domains originate from five species all affiliated to the Bacteroidetes phylum. Open reading frames from the respective organism are numbered, Ch (*Cytophaga hutchinsonii*), M (*Microsilla* sp.), Pg (*Porphyromonas gingivalis*), Rm (*Rhodothermus marinus*), Zg (*Zobellia galactanivorans*). The alignment is created using the EBI-ClustalW-tool, and default parameters. The resulting alignment was analysed in GeneDoc. Conserved residues are identified [The following residues are grouped, and considered conserved within the group: 1.(D,N); 2.(E,Q); 3.(S,T); 4.(K,R); 5.(F,Y,W); 6.(L,I,V,M)] and shaded if present in more than 60% of the sequences.

3.2. Homologous C-terminal domains are encoded in multiple genes in *R. marinus* and related organisms

The observations presented above prompted an extended search for homologues to D5 of *Xyn10A* using partial genomic sequence data from *R. marinus* (available via Prokaria Ltd, Iceland), and publicly available sequence databases attempting to unravel the function

of this domain. Using this approach, a number of hits in mostly putative open reading frames were found, invariably located at the C-termini in the deduced amino acid sequence of the respective gene. The highest score hits originated from five different microorganisms all classified within the phylum Bacteroidetes. Most of the hits originated from genome sequences of two microorganisms (*R. marinus* and *C. hutchinsonii*), but

Table 1

A summary of average properties for the C-terminal domains found in the five microbial species, in which genes encoding the putative homologue to the C-terminal domain of *Rm* Xyn10A were found

	Ch	M	Pg	Rm	Zg ^a
Number of genes	39	2	5	13 ^c	1
Average length (residues)	79 ± 0.5	78 ± 0	75 ± 1	85 ± 0.5	73
Theoretical molecular weight (kDa)	8.64 ± 0.06	8.93 ± 0.04	8.43 ± 0.13	9.54 ± 0.09	7.97
PI	5.6 ± 0.2	5.4 ± 0.3	7.5 ± 0.7	9.4 ± 0.5	6.0
Content of selected residues ^b (% of total number of residues)					
Ala	6.1 ± 0.5	4.5 ± 1.9	5.9 ± 1.4	8.7 ± 1.2	4.1
Arg	1.6 ± 0.2	3.8 ± 1.3	4.5 ± 0.9	11.2 ± 0.9	0
Asn	7.4 ± 0.4	3.2 ± 0.6	4.8 ± 0.3	2.4 ± 0.4	8.2
His	0.9 ± 0.1	1.3 ± 0	1.3 ± 0.02	2.4 ± 0.4	0
Ile	9.5 ± 0.6	7.0 ± 2.0	6.1 ± 1.1	2.0 ± 0.6	9.6
Lys	6.2 ± 0.3	7.7 ± 0	7.7 ± 1.3	1.0 ± 0.3	8.2
Pro	4.1 ± 0.2	3.2 ± 0.6	3.7 ± 0.7	6.6 ± 0.6	4.1
Ser	7.9 ± 0.5	7.0 ± 2.0	6.1 ± 0.8	3.2 ± 0.6	11

The microbial species are: *Cytophaga hutchinsonii* (Ch), *Microscilla* sp. (M), and *Porphyromonas gingivalis* (Pg), *Rhodothermus marinus* (Rm), and *Zobellia galactanivorans* (Zg). Values are given as the average ± SEM.

^a Only one gene found, no statistics possible.

^b Values are given for those residues where the domains from enzymes of thermophilic *R. marinus* differ significantly from the domains from the mesophilic microorganisms.

^c The ORF encoding a putative family 6 pectate lyase was excluded, due to a short C-terminal domain (58 residues) indicating a frameshift possibly caused by a sequencing error.

additional hits were also found within three other species, from the same phylum. In addition, three of these five microorganisms (*R. marinus*, *C. hutchinsonii*, and a *Microscilla* sp.), were classified into the same class and order (Sphingobacteria; Sphingobacteriales), and both *R. marinus* and *C. hutchinsonii* to the same family (Chrenotrichaceae) within the bacterial lineage. The remaining two microorganisms (*Porphyromonas gingivalis* and *Zobellia galactanivorans*), belong to separate classes of Bacteroidetes: Bacteroides and Flavobacteria, respectively. In the phylogram analysis, most of the sequences originating from *R. marinus* ($n = 14$) clustered together, and had the shortest distance to some of the sequences originating from its closest relative, *C. hutchinsonii* ($n = 39$). Sequences from the latter were, however, distributed throughout the phylogram (Fig. 2). Presence of only two genes from *Microscilla* sp., and one from *Z. galactanivorans*, rendered discussion of evolutionary relationships premature. In contrast, four of the five sequences from *P. gingivalis* ($n = 5$) clustered together in the phylogram, in a position relatively distant to the *R. marinus* sequences.

A multiple sequence alignment including all the putative domains revealed three consensus regions of which the first was most conserved (Fig. 3). Some physico-chemical properties of respective domain were also analysed, revealing that most of the *R. marinus* sequences, which are on average a few residues longer, have a relatively high pI (Table 1). It was also noted that the content of a number of amino acid residues in the *R. marinus* domains differed significantly from the domains of the other species, probably reflecting

the thermophilicity of this organism. There is for instance a dramatic increase in the Arg/Lys-ratio, as previously reported for many thermophilic proteins [24], a decrease of residues prone to deamidation (in this case seen as a decrease in Asn content), and an increase in Pro (which could increase rigidity in the structure).

Most of the genes (except Xyn10A and Man26A) found during the search were uncharacterised ORFs, which motivated analysis of the deduced polypeptide regions upstream the D5 homologues. Analysis of the full-length genes revealed that all had a potential N-terminal signal peptide. Furthermore, the best matches encoded proteins predominantly implicated in extra-cellular functions including glycoside hydrolases, and various other cell attached proteins (Table 2), while regions encoding polypeptides of clear intra-cellular function were absent. The relatively ubiquitous occurrence of this domain within taxonomic subdivisions of a single phylum, its conserved location at the C-termini, and the identification of a conserved motif within its primary structure (which is likely to reflect a structural common fold or motif), along with the common extra-cellular nature of the proteins or activities the domain is associated to, strongly suggest that this is a novel type of module conserved among members of this particular phylum. This along with a revision of the experimental evidence on the cellular location of the *R. marinus* enzymes (see below) makes it extremely tempting to postulate that the cell-attachment of glycoside hydrolases and of other enzymes could be mediated by this domain within the addressed taxonomic group.

Table 2
Putative function of ORF's (in the cases where similarity could be identified) encoding modular proteins that contain a putative homologue to the C-terminal domain of Xyn10A from *Rhodothermus marinus*

Bacterial lineage	Gene or ORF	N-terminal function	Identification
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Crenotrichaceae; <i>Cytophaga hutchinsonii</i>	ORF 4, 10, 19, 27, 42	Cell wall surface anchor protein/cell surface protein	By sequence similarity (Q97P71); (Q8TJE3); (Q8TJE5); (Q8TJS8); (Q97P71)
	ORF5, 23, 31	RCC1 repeats protein	By sequence similarity (Q97FL4); (Q97FL4); (Q97FL4)
	ORF6	Surface antigen	By sequence similarity (Q8TTC5)
	ORF7	Putative signal peptide protein	By sequence similarity (Q8XQF7)
	ORF 8, 9, 15	Hemagglutinin/hemolysin related protein	By sequence similarity (Q8XQP2); (Q8Y366); (Q8XPU1)
	ORF11	Putative β -agarase	By sequence similarity (Q9347)
	ORF13, 14	GH 9	By sequence similarity (Q9RBJ4); (P71140)
	ORF16	Subtilase fam. protein	By sequence similarity (Q8YWJ8)
	ORF17	Secreted metal-binding prot. (plastocyanin/azurin fam.)	By sequence similarity (Q8TR26)
	ORF18, 33, 38	GH 10	By sequence similarity (AAM21605); (Q9FIV3); (BAC16332)
	ORF20	Putative autotransporter protein	By sequence similarity (Q8PKM0)
	ORF22	Putative RTX-family exoprotein	By sequence similarity (Q8X4H5)
	ORF24	Keleh-like protein	By sequence similarity (Q8P3F9)
	ORF26	Secreted metalloprotease	By sequence similarity (Q97TN3)
	ORF28, 41	GH 18	By sequence similarity (Q30678); (Q9C105)
	ORF29	Integrin like repeats	By sequence similarity (Q97F11)
	ORF30	predicted secreted metalloprotease and	By sequence similarity (Q97TN3 and Q9KQP6)
	ORF32	GH 18	
	ORF35	GH 8 (and 18)	By sequence similarity (P37701 and Q9C105)
	ORF36, 40	GH 26	By sequence similarity (Q30654)
		GH 5	By sequence similarity (Q9AQH0); (Q95YN1)
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Flexibacteraceae; <i>Microscilla</i> sp.	ORF1, 2	MS130 and MS116, putative β -agarase	Annotated (Q93PA1); (Q93416)
Bacteroidetes; Sphingobacteria; Rhodothermus marinus	ORF4825	Extracellular serine protease	By sequence similarity (Q8YDM7)
	ORF3935	GGDEF family protein	By sequence similarity (Q8P9F2)
	ORF PL6	Polysaccharide lyase family 6	By sequence similarity (Q9RKE2)
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; <i>Zobellia galactanitiorans</i>	ORF1	β -Agarase A precursor	Annotated (Q9R GX9)

3.3. Experimental data support cell-attachment

A series of batch-cultivations of *R. marinus* were performed in order to induce expression of the native xylanases and monitor their cellular location as to provide experimental evidence for the hypothesis presented above. The gene encoding *Rm* Xyn10A encodes an N-terminal putative signal peptide [14], ruling out an intracellular location. This finds support in a previous investigation in which it was demonstrated that xylanase activity was not located in the intra-cellular protein fraction [11].

In the presence of xylan, *R. marinus* had a specific growth rate of 0.35 h^{-1} , and grew to a final $\text{OD}_{620 \text{ nm}}$ of approximately 5. Without xylan, the final $\text{OD}_{620 \text{ nm}}$ was lower, and xylanase activity not detectable. Xylanase activity was hence measured in both *R. marinus* cell slurries (cell attached activity), and in the cultivation medium, of the culture grown in presence of xylan. In both fractions, the activity was increasing throughout the cultivation and the cell associated activity was always higher than that of the cultivation medium (Fig. 4(a)), while intracellularly, it was below the detection limit of the assay as determined by protein fractionation [11]. Evidence that the cell-bound activity originated from Xyn10A emerged from two techniques: activity staining and immunocytochemistry. Activity staining after electrophoresis of samples (identical to those analysed for activity) showed a single band of xylanohydrolytic activity of the expected molecular mass ($M_r \approx 110 \text{ kDa}$) in the cell fraction (showing intracellular and cell-attached proteins, Fig. 4(b)), excluding the possibility that the cell-bound activity arose from additional cell-attached xylanases produced by the organism. Definite proof of the attachment of Xyn10A to *R. marinus* cells was collected after immunocytochemical analysis, in which primary antibodies against the carbohydrate binding module of Xyn10A (produced as a recombinant protein in *Escherichia coli*) were effectively bound to the cells (Fig. 5).

Two activity bands were observed in samples from the cultivation medium, one with high ($\approx 100 \text{ kDa}$) and one with low ($< 25 \text{ kDa}$) apparent molecular mass (Fig. 4(b)). The low molecular mass activity band proved presence of an additional secreted xylanase in *R. marinus*. Interestingly the M_r differences between the higher molecular mass (100 kDa) activity-band and that of Xyn10A conformed well to the M_r of D5, suggesting the possibility of release after (proteolytic) cleavage at the linker preceding D5. This would explain the previously observed slow release of xylanase to the growth medium during the stationary phase [11]. An alternative explanation for the release would be cell lysis, but the apparent decrease in molecular mass in our current results supports the former. Further support for proteolytic cleavage was col-

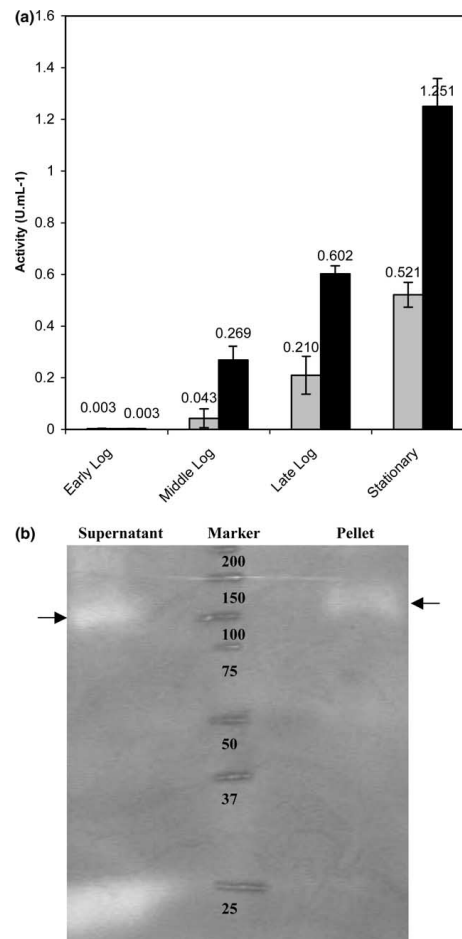


Fig. 4. Xylanase activity in the culture supernatant and on *R. marinus* whole cells. Activity was analysed by the DNS-assay for reducing sugars (a) using birch xylan as the substrate, and by activity staining after electrophoretic separation (by SDS-PAGE) and in-gel refolding (b), using Congo-red staining of a xylan-containing overlay-gel. Samples for activity analysis (a), were taken at times corresponding to different phases of the growth-curve, after 3 h (early log phase), 9 h (mid-log phase), 13 h (late log-phase) and 16 h (stationary phase) and the activity of the supernatant (grey) and the whole cells (black) was analysed. The positions of the standards in the prestained molecular mass ladder (Biorad) after separation is indicated by a scalpel in the overlay gel, and activity bands from the mid-log sample (9 h) of the supernatant (left) and cell pellet (right) of a size corresponding to Xyn10A are indicated by arrows (b).

lected from production patterns of recombinant *Rm* Xyn10A and truncated variants in *E. coli*, which showed two linker positions in the heterologous full-

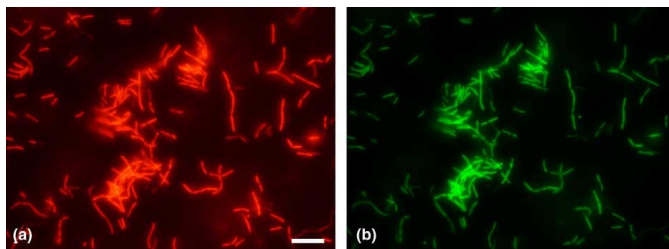


Fig. 5. Fluorescence microscopy image after immunocytochemical staining of *R. marinus* cells from the late growth phase grown in the presence of xylan. Binding of the primary xylanase-antibody to the cells is visualised after incubation with secondary antibody conjugated to the fluorophore Rhodamine Red-X (a). Subsequent staining of all cells with fluorescent Sytox Green nucleic acid stain (b) demonstrated that the xylanase was expressed on the surface of all cells. Bar represents 10 μm .

length enzyme to be susceptible to proteolytic cleavage, one of them being the linker preceding D5 [23]. Since the functions of the other modules in this enzyme are known except for the third domain (D3), cell attachment could theoretically be mediated by either domain. The third domain, however, has no homologues in this microorganism or in related ones (an does not so far show significant sequence similarities to any deposited sequence), which practically discounts it from any common function such as cell attachment, and leaves D5 as the most credible site of cell anchoring.

Cultivations in presence of galactomannan (locust bean gum) in our laboratory showed also the major part of the mannanase activity associated with the cell slurry (data not shown). This finding together with the facts that the only shared similarity between the two *R. marinus* enzymes is in the C-terminal domain, and the data from Politz and coworkers [15] promote the cell-attachment hypothesis. Politz and co-workers found cell-bound (and no intracellular) mannanase activity after protein fractionation of *R. marinus* cells. No N-terminal signal peptide was recognised in Man26A by Politz and coworkers, but as the collected data on the location of the enzyme proved export of the enzyme at least to the periplasmic space [15] we have (using the program SignalP) recognised a likely location of a signal peptide on the N-terminal side of the catalytic module (comprising the sequence MTLLLVWLIFTGVA). In accordance with the xylanase experiments and with the activity graphs published by Gomes and Steiner [13], some mannanase activity was also released to the culture medium during the later growth phase and the stationary phase.

3.4. How are the domains attached to the cells?

Currently we can only speculate on how Xyn10A is attached to the *R. marinus* cell. The domains found in this search did not share significant sequence similarities

with SLH-domains (proposed to anchor GH to many G-positive microorganisms by noncovalent interactions with secondary cell wall polymers, in turn covalently linked to peptidoglycan [7,8]) so no conclusion can be made based on their mechanism. Another difference is that *R. marinus* is a G-negative bacterium [25]. Based on the observation of the three conserved regions (with a number of hydrophobic residues) in the domain, attachment could be mediated by hydrophobic stretches (specific or unspecific), but a protein-carbohydrate interaction (e.g., via lipopolysaccharides (LPS), found to often be mediated by Ca^{2+}) [6] is also possible. At this point further discussion will have to await isolation and/or characterisation of *R. marinus* cell wall polymers, in order to study possible alternatives.

4. Conclusion

Bioinformatic and experimental lines of evidence are analysed in this study to elucidate the function of the fifth domain (D5) in Xyn10A. Our results clearly demonstrate that this domain is relatively wide-spread within subdivisions of the Bacteroidetes taxonomic group and that it displays a common primary structure motif and location at the C-termini which reflects its common function and evolutionary history. Moreover, experimental evidence from *R. marinus* cells in combination with evidence gathered from previous studies leads to the suggestion that this domain type mediates cell-attachment in proteins produced by members of the Bacteroidetes. Additional studies are motivated to highlight the mechanism of cell-attachment and the structural basis of this function. Finally, identification of a potential cell receptor for this module (if any) opens the door for use of the receptor/module interaction in biochemical studies, and for biotechnological applications.

Acknowledgement

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PAPER II

S. O. Ramchuran · E. Nordberg Karlsson · S. Velut ·
L. de Maré · P. Hagander · O. Holst

Production of heterologous thermostable glycoside hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of *Escherichia coli* BL21(DE3)

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Abstract Metabolic stress is a phenomenon often discussed in conjunction with recombinant protein production in *Escherichia coli*. This investigation shows how heterologous protein production and the presence of host cell proteases is related to: (1) Isopropyl- β -D-thiogalactopyranoside (IPTG) induction, (2) cell-mass concentration at the time of induction, and (3) the presence of metabolites (glutamic acid or those from tryptone soy broth) during the post-induction phase of high cell density fed-batch cultivations. Two thermostable xylanase variants and one thermostable cellulase, all originating from *Rhodothermus marinus*, were expressed in *E. coli* strain BL21 (DE3). A three-fold difference in the specific activity of both xylanase variants [between 7,000 and 21,000 U/(g cell dry weight)], was observed under the different conditions tested. Upon induction at high cell-mass concentrations employing a nutrient feed devoid of the metabolites above, the specific activity of the xylanase variants, was initially higher but decreased 2–3 h into the post-induction phase and simultaneously protease activity was detected. Furthermore, protease activity was detected in all induced cultivations employing this nutrient feed, but was undetected in uninduced control cultivations (final cell-mass concentration of 40 g/L⁻¹), as well as in induced cultivations employing metabolite-supplemented nutrient feeds. By contrast, maximum specific cellulase activity [between 700 and 900 U/(g cell dry weight)] remained relatively unaffected in all cases. The results demonstrate that detectable host cell proteases was not the primary reason for the decrease in post-induction activity observed under certain conditions, and possible causes for

the differing production levels of heterologous proteins are discussed.

Introduction

Cellulases and xylanases, are glycoside hydrolases that catalyze hydrolysis of the major constituents of plant cell walls (cellulose and hemicellulose). Thermostable variants of these enzymes have received considerable research attention, in order to exploit their potential in environmentally friendly industrial technologies [e.g. food, feed, textile, and pulp and paper sectors (Wong and Saddler 1993; Bergquist et al. 1996)]. These types of applications demand efficient enzyme production methods (even for pilot-scale trials), and consequently also the use of genetically modified microorganisms.

Escherichia coli is commonly used for heterologous protein production, but due to the lack of a natural secretion mechanism (the only translocation of proteins is to the periplasm), enzyme activity is proportional to cell concentration. Hence, successful establishment of high cell density processes is of paramount importance in achieving cost effective and efficient production of the desired protein, and for this purpose fed-batch cultivations are often employed. Certain limitations in substrate feed-rate are, however, crucial in order to prevent overflow metabolism, (i.e. production of acetate under glucose excess conditions) (Yee and Blanch 1992; Riesenberg et al. 1991), which otherwise inhibit cell growth (due to metabolic by-product accumulation) and reduce specific cellular product yields (e.g. yield of heterologous protein). Various nutrient feeding strategies have been proposed to circumvent this phenomenon (Lee 1996). Åkesson et al. (2001) recently proposed a strategy based on superimposed pulses in the substrate feed, followed by evaluation of the responses in the dissolved oxygen signal, which is then used for feedback control of the flow rate of the nutrient feed.

In addition, optimization of the time of induction with respect to cell-mass concentration is important in order to

S.O. Ramchuran (✉) · E. Nordberg Karlsson · O. Holst
Dept. Biotechnology,
Center for Chemistry and Chemical Engineering, Lund University,
P.O.Box 124, SE-22100 Lund, Sweden
e-mail: santosh.ramchuran@biotek.lu.se
Tel.: +46-46-2224948
Fax: +46-46-2224713

S. Velut · L. de Maré · P. Hagander
Automatic Control, Lund University, Lund, Sweden

maximize production levels of the desired protein. Induction of heterologous gene expression in *E. coli* often results in redirection of cellular metabolism (Harcum et al. 1992), due to the strength of the promoters used in controlling expression. A decrease in growth rate (Nordberg Karlsson et al. 1999; Andersson et al. 1996; Yee and Blanche 1992) is therefore often associated with the onset of recombinant protein production. Of importance for the yield of heterologous proteins are also factors such as the composition of the growth medium and cell-associated factors (e.g. in vivo proteolysis). It is well known that the growth rate is increased by the presence of metabolites such as amino acids and nucleosides in the medium due to reduced de novo synthesis of these metabolites. Furthermore, incorporation of certain key metabolites and precursors during the post-induction phase of cultivation has rendered significant improvement in target protein yields (Ramirez and Bentley 1995; Yee and Blanche 1992). By contrast, proteolysis will negatively affect the yield. Proteolytic enzymes display several crucial functions, such as breakdown of misfolded proteins and processing to form functional proteins from precursors (Gottesman 1989). However, amino acid deficiencies during cultivations may cause proteins to be degraded by host cell proteases in order to replenish amino acid resources. The free amino acids may then be redirected for incorporation into essential growth-associated proteins and ultimately results in increased protease activity against recombinant proteins (Harcum and Bentley 1993a).

In the current investigation, the feed-strategy of Åkesson et al. (2001) was used in high cell density cultures of *E. coli* for production of thermostable enzymes. The enzymes produced were isolated modules of a thermostable xylanase (EC 3.2.1.8) (Nordberg Karlsson et al. 1998b) and an extremely thermostable cellulase (EC 3.2.1.4) (Wicher et al. 2001), all originating from the thermophilic bacterium *R. marinus* (Alfredsson et al. 1988). This report describes how cell-mass concentration in the feed phase at the time of induction influences the production of the proteins. The results showed that a certain feed composition was required to avoid decreases in the measured activity of the two xylanase variants in the late production phase at high cell-mass concentrations. The reason for this is discussed in relation to post-induction protease activity and deficiency of amino acids, as metabolite-supplemented nutrient feed solutions (including either tryptone soy broth (TSB) or glutamic acid) circumvented decreases in measured specific activity during the post-induction phase.

Materials and methods

Bacterial strain, plasmid and inoculum preparation

Escherichia coli strain BL21(DE3) was used as the host microorganism. All plasmids were derived from the vector pET25b(+)

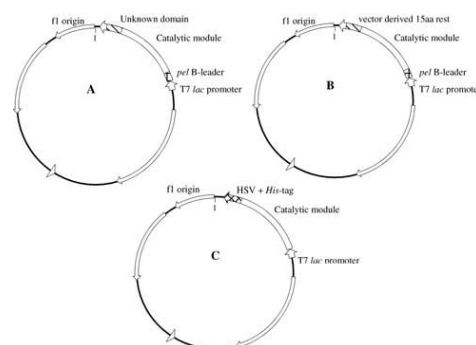


Fig. 1A–C Plasmids encoding the glycoside hydrolases. The plasmids encoding the xylanases **A** (Xyn10AΔN) and **B** (Xyn10AΔNC), and the cellulase **C** [Δ(SP,L)Cel12A] are derived from the vector pET25b. Expression is in all cases under the control of the T7/lac promoter. Xyn10AΔN is composed of a vector-derived signal peptide (21 amino acids), a catalytic module (322 amino acids) connected by a linker to a C-terminal 84-amino-acid domain. Xyn10AΔNC consists of signal peptide and the catalytic module but has a short vector-derived stretch of 15 amino acids in the C-terminus. [Δ(SP,L)Cel12A] contains a cellulase catalytic module of 225 amino acids connected to a HSV+His-tag of 31 amino acids

Table 1 Medium composition (mAT) and initial concentration in batch and feed-solution. IPTG Isopropyl-β-D-thiogalactopyranoside

Ingredient	Concentration
Medium	
(NH ₄) ₂ SO ₄	2 g l ⁻¹
K ₂ HPO ₄	14.6 g l ⁻¹
NaH ₂ PO ₄ 2H ₂ O	3.6 g l ⁻¹
(NH ₄) ₂ H-citrate	0.5 g l ⁻¹
Glucose	10 g l ⁻¹
1 M MgSO ₄	2 ml l ⁻¹
Trace elements ^a	2 ml l ⁻¹
Ampicillin	0.1 g l ⁻¹
Antifoam (Adekanol)	0.1 ml l ⁻¹
IPTG	1 mM
Feed 1 (F1)	
Glucose	500 g l ⁻¹
1 M MgSO ₄	50 ml l ⁻¹
Trace elements	10 ml l ⁻¹
Feed 2 (F2)	
Feed 1+tryptone soy broth (TSB)	30 g l ⁻¹
Feed 3 (F3)	
Feed 1+glutamate	2 g l ⁻¹

^a According to Holme et al. (1970)

(Novagen, Madison, Wis.) (Fig. 1). The construction of plasmids encoding the xylanase (Xyn10AΔN) and (Xyn10AΔNC) is described in Nordberg Karlsson et al. (1998a) and Nordberg Karlsson et al. (1998b) respectively, and the cellulase [Δ(SP,L)Cel12A] in Wicher et al. (2001).

Inoculum was prepared using 100 ml of defined mAT medium [Table 1 (excluding IPTG and antifoam)]. The salts, glucose and trace elements were sterilized separately at 121 °C for 20 min and aseptically pooled into a 1 L baffled Erlenmeyer flask. Mid-log

cultures (1 ml), in 20% glycerol (stored at -80°C), each containing the respective *E. coli* clone, were used to inoculate the flasks which were subsequently incubated at 30°C for 12 h at 125 rpm on a rotary waterbath shaker (Heto, Allerød, Denmark).

Experimental set-up and cultivation conditions

Fed-batch cultivations were performed using a 3 L fermentor (Chemoferm FLC-B-3, Hågersten, Sweden) with an initial medium volume of 2 L. The salts were sterilized in the vessel at 121°C for 45 min; thereafter, sterile MgSO_4 , glucose and trace elements were aseptically added. Ampicillin (see Table 1) was added by sterile filtration. Data capturing of dissolved oxygen concentration (%DO), feed-pump control, stirrer speed (rpm) and off-gas were conducted with the SattLine control system (Alfa Laval Automation AB, Malmö, Sweden). Cultivation temperature was controlled at 37°C and the pH was maintained at 7.0 by titration with 6.7 M aqueous ammonia. DO concentration was measured using a polarographic electrode calibrated to 100% at 1,100 rpm at 37°C and zeroed by sparging sterile nitrogen into the vessel. The DO was controlled at 30% saturation using a gain-scheduled PID controller connected to stirrer speed, as described in Åkesson and Hagander (1999). A pulse feeding strategy (Åkesson et al. 2001) was employed using the nutrient feed solutions defined in Table 1. A Tandem dual gas system (Adaptive Biosystems, Luton, UK) was used for off-gas analysis.

Statistics on the cultivation data was calculated using Microsoft Excel 97, and are reported as mean \pm standard deviation.

Analytical Methods

Sampling and sample treatment

Samples were withdrawn through a rubber septum at the bottom of the fermentor using 3-ml Venoject evacuated blood-collecting tubes (Terumo, Madrid, Spain). The medium fractions (for extracellular enzyme activity determination) were collected after centrifugation (11,000 g, 15 min, at room temperature) and kept frozen (-20°C) until analysis. The cell pellets (for intracellular enzyme activity and total protein determination) were dissolved to the original volume in 20 mM Tris-HCl, pH 7.5, and disrupted by ultrasound (90 s, cycle 0.5) with a sound intensity of 230 W/cm^2 using UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany), equipped with a 3-mm titanium probe. After sonication, the samples were centrifuged (11,000 g, 15 min, at room temperature) and the supernatants were stored on ice or kept frozen (-20°C) until analysis.

Samples for glucose and acetic acid determinations were collected in tubes half filled with 0.132 M perchloric acid (ice cold) and centrifuged (1,400 g, 10 min, at room temperature). The supernatant was dispensed (1 ml portions) into Eppendorf tubes and neutralized with 3.6 M K_2CO_3 (15–20 μl) and kept frozen (-20°C). Prior to analysis, samples were thawed, heated (80°C , 15 min) and centrifuged (11,000 g, 3 min, at room temperature).

Optical density

The OD was determined at 620 nm. Samples were appropriately diluted with 0.9% (w/v) NaCl at OD values exceeding 0.5.

Cell dry weight determination

Cell dry weight (CDW) was determined after centrifuging (1,400 g, 15 min, at room temperature) triplicate samples (4 ml) in pre-weighed glass tubes. The pellets were washed with 0.9% (w/v) NaCl (4 ml), re-centrifuged, then dried overnight (105°C) and subsequently weighed to determine CDW.

Estimation of glucose and acetic acid concentration

Glucose and acetic acid concentrations were determined enzymatically using test kits (no. 716251 and 148261 respectively, Roche, Darmstadt, Germany).

Enzyme activity

Xylanase and cellulase activities were determined using the DNS (3,5-dinitrosalicylic acid) method (Bailey et al. 1992). Xylanase activity was determined under the conditions described by Nordberg Karlsson et al. (1998a) using xylose as standard (2–10 $\mu\text{mol/ml}$). Enzyme blanks were prepared for each sample by incubating the substrate, 1% (w/v) birch xylan (Birch 7500, Roth, Karlsruhe, Germany), at 65°C for 5 min, then adding the DNS-reagent and immediately thereafter the enzyme. Cellulase activity was determined under similar conditions, but with glucose as standard (2–10 $\mu\text{mol/ml}$), and 1% (w/v) carboxymethyl cellulose (medium viscosity, Sigma, St. Louis, Mo.) as substrate. Xylanase and cellulase activity was expressed in units (U) defined as the amount catalyzing the transformation of 1 μmol of the substrate per minute under standard conditions.

Estimation of total protein

Protein concentration was determined using the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin (0.2–1.0 mg/ml) as the standard.

Electrophoresis

Protein production was analyzed by the SDS-PAGE according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany). Enzyme activity was detected by an overlayer agarose 1% (w/v) gel, containing either 0.05% (w/v) xylan or carboxymethyl cellulose. The SDS-PAGE gel was washed with 20 mM Tris-HCl, pH 7.5, containing 2.5% Triton X-100 (Merck) for 20 min. Thereafter, the gel was washed with 20 mM Tris-HCl, pH 7.5, covered with the overlayer gel, and incubated for 15–25 min at 65°C . The agarose gel was stained in 1% (w/v) Congo Red solution and destained with 1 M NaCl.

Densitometric measurements

The relative amounts of protein, separated by electrophoresis, were estimated by laser densitometry using a BioImage system (BioImage, Ann Arbor, Mich.) equipped with a Megaplus Videk camera (model 1400, Kodak, Rochester, N.Y.).

Qualitative detection of protease activity and incubation of xylanase with protease positive extracts

Qualitative detection of protease was visualized by a casein polyacrylamide gel electrophoresis (CPAGE) [1% (w/v) casein] with the same gel compositions and sample preparation method as presented in Harcum and Bentley (1993b). Gels were cast in a Mini-Protean 3 apparatus (Bio-Rad, Richmond, Calif.) with 1 mm spacers and run at 120 V at 4°C with (0.025 M Tris-HCl, 0.2 M glycine, 0.1% SDS, pH 8.3) as running buffer. The sample buffer consisted of 0.3 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 20% (w/v) glycerol, 1.5% (w/v) bromophenol blue. A protease positive control from *Bacillus thermoproteolyticus* rokko (Sigma) was included. The positive control (2.5 $\mu\text{g/ml}$) and sample supernatants were mixed with sample buffer (1:1) and incubated at 37°C for 1 h. The wells were then loaded with 15 μl sample supernatants, 8–12 μl prestained, low molecular mass standard (Bio-Rad) and 10 μl

positive control. After electrophoresis, the gels were first washed with 2.5% (v/v) Triton X-100 in 20 mM Tris-HCl, pH 7.5, for 1.5 h at room temperature and then with 20 mM Tris-HCl, pH 7.5, for 30 min. The washing step was followed by incubation at 37 °C for 15 h in 150 mM glycine, 3 mM ATP, 3 mM MgCl₂, pH 7.5 buffer. The gels were stained with 0.2% amido black in water:methanol:acetic acid (20:5:2) for 45 min and destained in water:metanol:acetic acid (47:5:2) for approximately 3 h to reveal clear zones.

For in vitro analysis of possible proteolysis of the xylanase, whole cells from the post-induced phase of cellulase producing cultivations (3 h after induction) were exposed to mild sonication (40 s, 0.5 cycle) using the equipment mentioned above. The resulting cell extracts (800 µl) were incubated at 37 °C together with 800 µl buffer (150 mM glycine, 3 mM ATP, 3 mM MgCl₂, pH 7.5) and 400 µl of partially purified (heat-treated) xylanase (Xyn10AΔN). Samples were withdrawn at the start of incubation and hourly for 4 h and accordingly assayed for xylanase activity.

Amino acid composition

The amino acid composition of the enzymes was calculated using the ProtParam tool available on the ExPASy Molecular Biology Server (<http://www.expasy.ch>).

Results

Feed strategy for high cell density cultures

Fed-batch cultivations were carried out in order to achieve high cell densities of the recombinant *E. coli* strains. With glucose being the limiting parameter during the batch phase, a specific growth rate of $(0.68 \pm 0.02) \text{ h}^{-1}$ ($n=15$) and a yield coefficient for glucose ($Y_{x/s}$) of $(0.46 \pm 0.03) \text{ g g}^{-1}$ ($n=15$) was reproducibly attained. During the feed-phase, the feed strategy described in Åkesson et al. (1999) was selected. In all experiments the feed start commenced upon depletion of the initial glucose, detected as a peak in the DO signal (Fig. 2). After the feed start, the control algorithm increased the feed rate gradually until maximum stirrer speed was obtained. Thereafter, the feed rate was mainly limited by cell metabolism as seen in the oxygen response to feed pulses. In the later phase of the fed-batch, a safety net gradually decreased the feed rate to maintain aerobic conditions, in effect acting as a DO-stat (Konstantinov et al. 1990). The stirrer speed was then kept at its maximum. This feed strategy led to good reproducibility and the final CDW concentrations from cultivation with a feed-phase duration of $6.5 \pm 0.5 \text{ h}$ ($n=11$) were $33.9 \pm 0.2 \text{ g l}^{-1}$ ($n=6$) using feed F1 (without metabolite additions), and $39.0 \pm 0.3 \text{ g l}^{-1}$ ($n=5$) using feed F2 or F3 (with TSB or glutamic acid, respectively). Offline analysis indicated neither glucose nor acetate accumulation with concentrations maintained below 0.2 g l^{-1} , and 30 mg l^{-1} , respectively, during the feed-phase even when employing metabolite-supplemented feeds (F2 and F3). Differences found between the cultivations were mainly observed in connection with the addition of antifoaming agent, which caused a decrease in the oxygen transfer rate (Fig. 2);

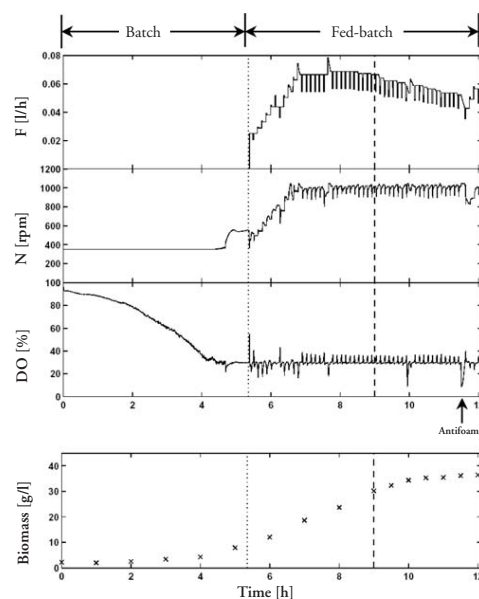


Fig. 2 High cell density fed-batch cultivation (HCDC) of recombinant *Escherichia coli* strain BL21(DE3) encoding thermostable xylanase (Xyn10AΔN). Nutrient feed F1 started at 5.3 h (dotted line) and induction by isopropyl-β-D-thiogalactopyranoside (IPTG) (dashed line) at 9 h. *F* Nutrient feed flow, *N* stirrer speed, *DO* dissolved oxygen concentration, *biomass* cell dry weight

therefore, the feeding strategy reduced the feed rate to maintain aerobic conditions.

Induction at varying cell densities using defined feed

In order to evaluate the efficiency of the expression, the cultures were induced with IPTG at two different cell-mass concentrations (19 and 26 g l^{-1}). The protein production phase of the cultivation was limited to a time period of 3 h based on previous results (Nordberg Karlsson et al. 1999).

Induction of xylanase and cellulase at the lower cell-mass concentration ($19.1 \pm 0.3 \text{ g l}^{-1}$, $n=3$) showed efficient expression of both proteins, with increasing activity per gram CDW during the entire production phase (Fig. 3A, C). A slightly different picture emerged when induction was initiated at a higher cell-mass concentration ($26.1 \pm 0.3 \text{ g l}^{-1}$, $n=10$) which initially was more favorable. As depicted in Fig. 3C, the cellulase activity per gram CDW was, under these conditions, slightly higher than that obtained after the earlier induction. This is indicative of a concomitant increase in the total amount of target protein with increasing cell-mass concentration after late induction, and hence more favorable from a production

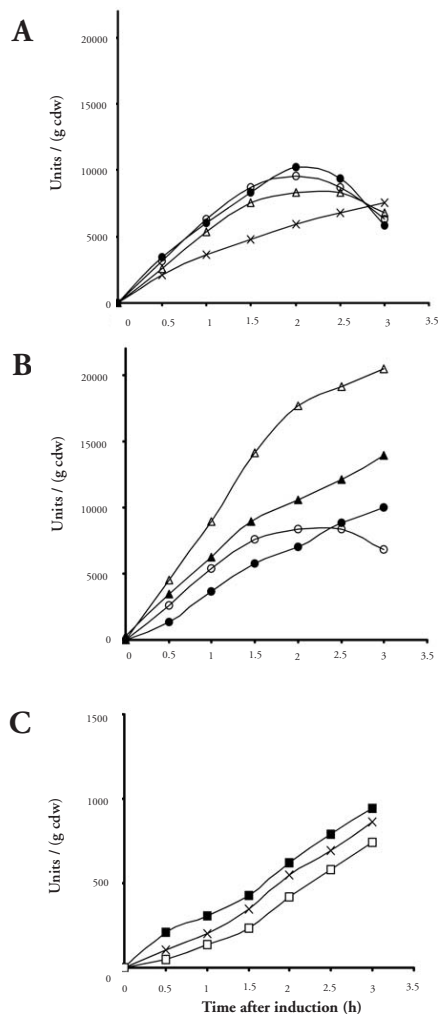


Fig. 3A–C Specific enzyme activities expressed in units (U) per gram cell dry weight. **A** Xylanase variants induced at varying cell-mass concentrations employing feed F1: ●, ○ Xyn10AΔNC induced at 26 g l⁻¹; Δ Xyn10AΔN induced at 26 g l⁻¹; × Xyn10AΔNC induced at 19 g l⁻¹. **B** Xylanase (Xyn10AΔNC) induced at 26 g l⁻¹ with varying nutrient feed compositions: Δ F2 (TSB supplemented); ▲ F3 (glutamic-acid-supplemented); ○ F1 (unsupplemented); ● F1 (glutamic acid spike). **C** Cellulase [Δ(SP,L)Cel12A] induced at varying cell mass and nutrient feed compositions: ■ induced at 26 g l⁻¹ employing feed F3 (glutamic-acid-supplemented); × induced at 26 g l⁻¹ employing feed F1 (unsupplemented); □ induced at 19 g l⁻¹ employing feed F1 (unsupplemented)

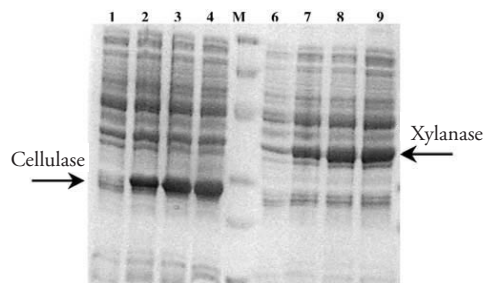


Fig. 4 SDS-PAGE from post-induction phase during HCDC of recombinant *E. coli* strains expressing thermostable cellulase and xylanase. Lanes 1–4 Induction time 30, 60, 90, 120 min, respectively, of [Δ(SP,L)Cel12A]; lanes 6–9 induction time 30, 60, 90, 120 min of (Xyn10AΔNC); lane 5 marker (M). The theoretical molecular masses of [Δ(SP,L)Cel12A] and (Xyn10AΔNC) are 28.5 kDa and 39 kDa, respectively

point of view. For the xylanases, the specific activity (Fig. 3A) was significantly higher during the first 2 h after induction. The increase in production level with time did, however, cease after approximately 2 h and was followed by a substantial decrease in xylanase activity per gram CDW, during the next hour. This resulted in a final specific activity (3 h after induction) approximately at the level observed in cultures induced at 19 g l⁻¹. This pattern was reproducible in several cultivations for production of each of the two xylanases (Xyn10AΔ N, *n*=2; Xyn10AΔNC; *n*=3) (Fig. 3A).

Judging from SDS-PAGE (Fig. 4), the xylanase (Xyn10AΔNC), which has a higher specific activity than the cellulase, constituted approximately 30% of the total protein, while the cellulase constituted up to 40% of the total protein at the end of the respective cultivation. However, it was not possible to monitor any decrease in the ratio xylanase/total protein corresponding to the observed activity decrease towards the end of the late production phase.

Induction at high cell density using supplemented feeds F2 and F3

In order to evaluate whether recombinant enzyme activity was significantly affected as a result of nutritional deficiencies such as amino acids, nutrient feeds F2 or F3, containing TSB and glutamic acid, respectively (Table 1), were employed when cultures were induced at high cell densities (26 g l⁻¹).

TSB (30 g l⁻¹) supplemented feed (F2) completely prevented the previously observed decrease in xylanase production (Fig. 3B); in addition, no protease activity was detected (see below). In order to follow up on what types of amino acids were possible bottle-necks in the xylanase activity, the amino acid composition of the xylanase variants and of the cellulase were compared. Calculations

Table 2 Amino acid composition of the two produced xylanase variants (Xyn10AΔN, 436 amino acids); (Xyn10AΔNC 346 amino acids) and cellulase (Δ(SP,L)Cel12A, 256 amino acids). Calculations by ProtParam (<http://www.expasy.ch>). Amino acid groups defined from Stanier et al. (1998)

Amino acid	Δ(SP,L)Cel12A (% of total)	Xyn10AΔN (% of total)	Xyn10AΔNC (% of total)
Glutamate group	18.7	27.3	26.9
Arg	6.2	6.7	5.5
Gln	1.6	5.7	7.2
Glu	7.0	8.9	8.4
Pro	3.9	6.0	5.8
Aspartate group	25.1	17.9	20.4
Asn	5.1	4.4	4.6
Asp	5.5	3.9	4.6
Ile	4.3	3.4	4.3
Met	1.2	2.3	2.0
Thr	7.8	3.9	4.9
Lys	1.2	0.9	1.2
Pyruvate group	24.5	26.8	25.1
Ala	10.9	8.9	9.2
Leu	6.2	10.6	9.8
Val	7.4	7.3	6.1
Serine group	16.5	13.8	13.3
Cys	1.6	0.5	0.9
Gly	9.8	7.8	7.8
Ser	5.1	5.5	4.6
Aromatic group	11.7	12.0	11.9
Phe	2.3	4.4	4.3
Trp	5.5	3.2	3.8
Tyr	3.9	4.4	3.8
His	3.5	1.4	1.2

revealed that the glutamate group was the only group of which the two xylanases (the major part of the two enzymes is identical) had a markedly higher content than the cellulase. The amino acids from this group constituted 27% of the xylanase polypeptides, an 8.6% (Xyn10AΔN) and 8.2% (Xyn10AΔNC), respectively, higher amount than found in the cellulase (Table 2).

Subsequently, glutamic acid (2 g l⁻¹) supplementation during fed-batch cultivations of one of the xylanase-encoding strains (Xyn10AΔN) was evaluated. Two different strategies for glutamic acid addition were investigated. The first involved addition of glutamic acid into the fermenter via a septum (glutamic acid spike) concomitantly with inducer. However, this resulted in a pronounced decrease in both DO concentration and feed rate. Consequently the cell culture experienced oxygen limitation for approximately 20 min before recovery (data not shown). The other, more efficient method allowed glutamic acid to be added in the feed (feed F3). Thus, the cell culture was supplied with glutamic acid from the start of the fed-batch phase, lowering the metabolic burden with respect to anabolism of the glutamate group of amino acids. This preconditioning of the cell culture with glutamic acid circumvented the decrease in xylanase activity during the late post-induction phase, and also in this case no protease activity was detectable (see below).

Cultivation conditions under which host cell proteases were detectable and their impact on xylanase production

An investigation into the presence of protease activity during the post-induction phase was carried out based on a first hypothesis that the observed decrease in xylanase activity in both the variants was caused by *in vivo* proteolysis. Qualitative analysis of samples for protease activity was conducted using the CPAGE technique. Furthermore, samples from different phases (post- and pre-induction phase, uninduced) of cultivations using xylanase- or cellulase-encoding constructs, as well as those obtained from supplemented nutrient feeds (Table 1, F2 and F3) were analyzed in order to ascertain whether protease-active bands could be detected as a consequence of an induction-related stress response.

Evaluation of proteases activity in cultivations employing nutrient feed F1

Qualitative CPAGE analysis revealed the presence of 90-, 50- and 30-kDa protease-active bands during the post-induction phase. These were present in both the xylanase- and cellulase-producing cultures 2 h after induction at cell-mass concentrations of 26 g l⁻¹ (Fig. 5 B). No protease activities were noted with the CPAGE assay either during the pre-induction phase (Fig. 5C) or in uninduced cultivations grown to high cell densities [(40 g l⁻¹, (Fig. 5A)], showing that detectable protease activity was a consequence of production of induced recombinant protein. Induction at lower cell density (19 g l⁻¹) (Fig. 5D)

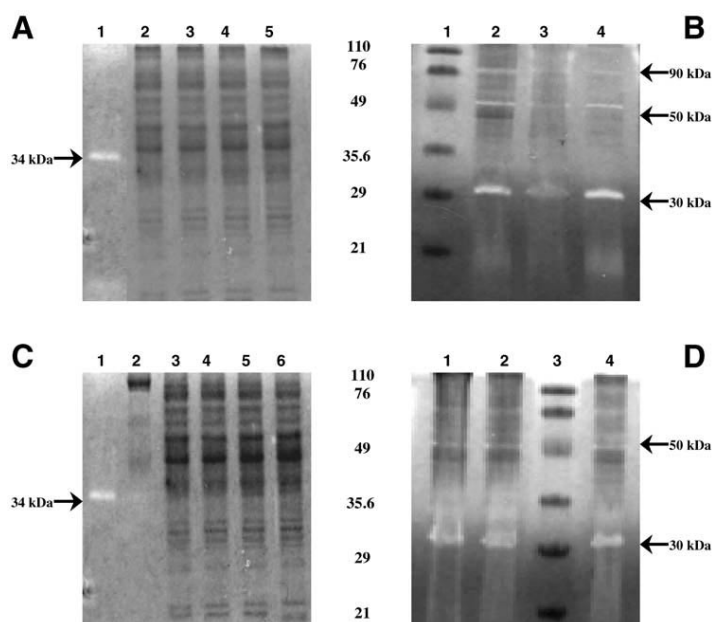


Fig. 5A–D Qualitative analysis for protease activity using CPAGE. Samples from various stages of different recombinant *E. coli* cultivations are presented and a supplemented nutrient feed was employed as stated. Xylanase and Cellulase refers to recombinant strains with plasmids [Xyn10AΔNC] and [Δ(SP,L)Cel12A] respectively. **A** Samples from uninduced and induced cultures (26 g l^{-1}): 1 Positive control, 2 xylanase (uninduced), 3 cellulase (uninduced), 4 xylanase (F2: TSB-supplemented; 3 h after induction), 5 xylanase (F3; glutamate supplemented; 3 h after induction). **B** Samples from

post-induction phase (26 g l^{-1}): 1 Molecular mass standard, 2 xylanase (2 h after induction), 3 cellulase (3 h after induction), 4 xylanase (3 h after induction). **C** Samples from pre induction phase: 1 Positive control, 2 molecular mass standard, 3 xylanase (1 h before induction), 4 xylanase (2 h before induction), 5 cellulase (1 h before induction), 6 cellulase (2 h before induction). **D** Post-induction phase (19 g l^{-1}): 1 Xylanase (3 h after induction), 2 xylanase (3 h after induction), 3 molecular mass standard, 4 cellulase (3 h after induction)

also resulted in detectable protease activity, but in this case no cease or decrease of the xylanase production was observed.

Evaluation of proteases activity in cultivations employing supplemented feeds

Protease activities were undetected during the post- and pre-induction phases of the cultivations when feeds F2 and F3 (nutrient feed supplemented with TSB or glutamic acid, respectively) were employed (Fig. 5A). This indicates that the protease-encoding genes are only expressed as a consequence of nutritional deficiencies, such as amino acid depletion.

In vitro xylanase incubations

The resistance of the xylanase to proteolytic attack was demonstrated by in vitro incubations. Active xylanase

(Xyn10AΔ N) incubated with the above identified protease-active cell extract (from a production of cellulase) did not result in decreased xylanase (Xyn10AΔ N) activity (data not shown).

In summary, this means that it is unlikely that the detected proteolytic activities cause the observed decrease in xylanase activity; hence, their presence can not be concluded to be a factor causing significant degradation of the thermostable xylanase. Instead, proteases should be looked upon as a part of the cellular stress response and an indicator of amino acid depletion in cell culture.

Discussion

The current findings show that metabolic deficiencies in the medium significantly influence expression of recombinant proteins. Furthermore, it is evident that an induction-related stress response [here shown as detectable protease activity, in line with results presented by Ramirez and Bentley (1995) and Harcum and Bentley

(1993b)] was, in this investigation, associated with recombinant protein production in a glucose-based medium. The metabolic burden imposed on the cells to produce the recombinant proteins leads to induction of host cell proteases in order to release amino acids from existing proteins. This phenomenon is similar to the stringent response, defined as an amino acid deficiency leading to reduced tRNA levels during transcription and ultimately resulting in increased protease activity in order to replenish amino acid resources for incorporation into essential proteins (Harcum and Bentley 1993a).

Previous studies have indicated that IPTG alone (independent of protein production) could cause such a stress response in *E. coli*, observed as reduced growth rates and/or elevated expression levels of stress response proteins (Kosinski et al. 1992; Andersson et al. 1996). However, results from our lab employing IPTG and the alternative inducer lactose led to similar growth rate reductions after induction under comparable conditions (Nordberg Karlsson et al. 1999). Furthermore, absence of detectable protease activity during production in metabolite-supplemented cultivations in this investigation suggests that metabolic stress is not caused by the presence of the inducer, but rather it is the production of proteins under certain conditions that is the major cause of the stress response.

A relevant strategy for efficient production of these thermostable glycoside hydrolases is thus based on the use of metabolite-supplemented feeds (e.g. TSB, or glutamic acid). This strategy suppressed host protease activity after induction and allowed increases in the production of specific heterologous proteins over a longer period of time (in this work exemplified by xylanase production). Selection of glutamic acid as the supplementing metabolite was, in this work, based on the amino acid composition of the xylanase variants. Ramirez and Bentley (1993) described a similar approach when adding phenylalanine prior to induction, based on the amino acid composition of chloroamphenicol-acetyl-transferase (compared to that of average *E. coli* proteins), which was found to enhance recombinant production. It is, however, important to stress that from our current results we can not distinguish whether improvements in production are due to a balancing (based on amino acid composition), or a relieving effect caused by the presence of any precursor metabolite that lowers the metabolic burden generated by production.

No direct correlation between protease presence and the reduction of xylanase activity could be drawn since protease-containing extracts failed to decrease the activity of previously produced xylanase (Xyn10AΔN). Further support for the resistance of the xylanase to proteolytic attack is that no decrease in specific activity was observed in cultures induced at a lower cell-mass concentration (19 g l⁻¹), despite visible protease activity. The fact that the target proteins in this investigation are thermostable could very well explain their resistance to proteolysis, and it has previously been shown that protein thermostability appears to be correlated to resistance to proteolysis

(Daniel et al. 1996). This can be attributed to their rigidity at the *E. coli* production temperature, due to their high temperature optimum (approx. 80 °C for Xyn10AΔN, Nordberg Karlsson et al. 1998b) and thermostability ($T_m=86$ °C for Xyn10AΔN; Pfabigan et al. 2002).

If the xylanase is resistant to proteolytic attack, another explanation has to be found to understand the decrease in production during the late post-induction phase. Schweder et al. (2002) indicated that it may be competition for sigma factor that leads to down-regulation of strong expression systems. This explains a ceased but not a decreased production of heterologous protein. An alternative explanation (again involving effects of induction-related stress responses) is aggregation. Protein aggregation has been reported to occur in bacteria under stress conditions such as heat shock (Rosen et al. 2002; Tomoyasu et al. 2001). If the metabolic stress developed during heterologous protein production induces aggregation of the recombinant proteins produced in the late production phase, for instance due to a combination of lack of chaperones and resistance of misfolded products to proteolysis, these products would be non-functional and lead to ceased target enzyme activity. Moreover, already produced and folded proteins are likely to be involved in the aggregates, which will lead to activity losses. Incorrect folding of the target protein could, in turn, be induced by misincorporation of amino acids upon depletion of amino acid pools, previously reported as a drawback when using minimal medium (Tsai et al. 1988). The presence of non-functional proteins would also explain the absence of a detectable decrease of the recombinant protein on SDS-PAGE.

When comparing results from the production of thermostable model proteins in the current investigation, larger variations in activity of produced xylanase (both variants) than in cellulase productions are seen. There are also several differences between the xylanase (both variants) and the cellulase that could be taken into account. Firstly, they display different molecular masses [Xyn10AΔ N (49 kDa), Xyn10AΔNC (39 kDa), and Δ(SP,L)Cel12A (29 kDa)], but these differences are rather small. Secondly, they display different structural folds, with the xylanase being folded as a TIM-barrel and the cellulase having a β-jellyroll fold. Such differences are, however, very hard to translate into differences in protein production, and both folds are common in nature. Thirdly, the xylanase is, in both cases, preceded by a signal peptide (*pelB*) for export, while the cellulase construct lacks any type of leader peptide. Previous investigations have shown that the xylanase is in part exported to the periplasm, but that a fraction also remains intracellularly (Nordberg Karlsson et al. 1999). Whether export is the reason for increased metabolic stress is, however, from the present results hard to judge and would require construction and investigation of productions of the same polypeptide with and without the signal peptide. Finally, and important for the current comparison, is the approximately hundred-fold higher specific activity of the xylanase [120 U/mg on birch xylan for purified

Xyn10AΔ N (Nordberg Karlsson et al. 1998b)] compared to that of purified cellulase [1 U/mg on CMC (Halldorsdottir et al. 1998; Wicher et al. 2001)]. This makes xylanase activity a more sensitive indicator of small changes in protein concentration, which could explain the larger variations observed for xylanase activity at the cultivation conditions tested.

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PAPER III

Effect of post-induction nutrient feed composition and the use of lactose as inducer during production of thermostable xylanase in *Escherichia coli* glucose limited fed-batch cultivations

Santosh O Ramchuran, Olle Holst¹ and Eva Nordberg Karlsson

¹*Dept. Biotechnology, Centre for Chemistry and Chemical Engineering, Lund University, P.O.Box 124, SE-22100 Lund, Sweden. E-mail: olle.holst@biotek.lu.se*

ABSTRACT

Escherichia coli is a microorganism routinely used for the production of heterologous proteins. Overexpression of a xylanase gene (Xyn10A Δ NC), originating from the thermophile *Rhodothermus marinus*, cloned under the control of the strong T7/lac-promoter, in a defined medium (mAT) using a substrate limiting feed strategy, was however shown to impose a significant metabolic burden on the host cells. This resulted in a decrease in cell growth and ultimately also in decreased target protein production. The investigation hence centres on the effect of some selected nutrient feed additives [amino acid (Cys) or TCA-intermediates (citrate, succinate, malate)] as a strategy to relieve the metabolic burden imposed during the feeding and post-induction phases of these substrate (glucose) limited fed-batch cultivations. Use of either succinic acid or malic acid as feed-additives resulted in an increase in production of approximately 40% of the heterologous thermostable xylanase. Furthermore, use of lactose as an alternative inducer of the T7/lac-promoter, was also proven to be a suitable strategy that significantly prolonged the heterologous protein production phase as compared to when induced by isopropyl β -D-thiogalactopyranoside (IPTG).

[**Keywords:** thermostable, xylanase, lactose, pulsed feeding strategy]

INTRODUCTION

Heterologous protein production in *Escherichia coli* is widely used to obtain large amounts of proteins, which are not easily available from the wild-type organism. The production levels are affected by numerous process factors, such as cultivation mode, composition of the medium, time of induction (with respect to cell mass concentration) and duration of the production phase (1,2). Due to the lack of a natural secretion mechanism in *E. coli*, concentration of the produced protein is to a great extent proportional to the cell concentration, and processes designed to yield high cell densities are therefore beneficial. In addition other factors, such as host cell-vector interactions, plasmid stability and cellular stress responses (3-5) need to be considered. As a result, research to optimize the protein production is complex and encompasses the disciplines of both molecular biology and engineering, and numerous strategies are thus applied to maximize the production of recombinant proteins in *E. coli* (6-8).

Metabolic stress in *E. coli* is often proposed to be a reason for decreased yields of the target protein during its gene expression, which can result in the redirection of the cellular metabolism (9) and a decreased growth rate (6-10). This has in some cases been reported to occur due to the addition of the inducer IPTG (11,12) but is also more generally found during post induction phases especially when induction is initiated after a certain critical cell-mass concentration, possibly due to absence of necessary metabolites. It has for example previously been established that depletion of certain amino acids in the medium significantly influenced the expression of recombinant proteins (2,13). This lack of resources in the cell are likely due to the increased protein production rate, in combination with the fact that

the heterologous protein often has a different amino acid composition compared to that of average *E. coli* proteins. This can result in limitations in certain key amino acids that hence act as bottlenecks and control the protein production rate. Consequently, addition of the deficient amino acids to the growth medium has been reported to circumvent a decreased production of the recombinant protein in the late post-induction phase (2,13). Such additions can also circumvent reduced tRNA levels during translation, a factor that has previously been observed during amino acid limitation (14) and resulted in conditions with amplified proteolytic activity in the cell to replenish amino acid resources (15). Released amino acids from nonessential proteins may in this way be incorporated into essential proteins that are required for the cell survival (16).

This investigation hence centers on the effect of nutrient additives from the TCA-cycle (present during the fed-batch and post-induction phase) on recombinant protein production in *E. coli* grown using a substrate limited fed-batch strategy, designed to control the feed in order to keep glucose below the critical value for overflow metabolism (17). By analyzing protease activity, a measure on the induction related stress response is also collected. In addition, the effect of the choice of inducer (IPTG vs. lactose) on both the recombinant protein production and on the induction related stress response in cells grown by this fed-batch strategy is investigated. For these purposes high cell density fed-batch cultures of *E. coli* producing thermostable xylanase (18), originating from the thermophilic bacterium *Rhodothermus marinus* (19), were analyzed. The results are expected to give new complementary information regarding the protein-synthesizing machinery of the cell in order to explain the cell behavior as the recombinant protein-production proceeds.

MATERIALS & METHODS

Bacterial strain, plasmid and inoculum preparation

Escherichia coli strain BL21(DE3) was used as the host microorganism. The plasmid was derived from the vector pET25b(+) (Novagen, Madison, WI). The construction of plasmid encoding the xylanase (Xyn10A Δ NC) is described in (20). Xyn10A Δ NC consists of signal peptide (21aa) and the catalytic module (322aa) and has a short vector derived stretch of 15 amino acids in the C-terminus. Expression is in all cases under the control of the T7/lac promoter. Inoculum was prepared using 100 ml of defined mAT-medium [Table 1 (excluding IPTG & antifoam)]. The salts, glucose and trace elements were sterilized separately at 121 °C for 20 minutes and aseptically pooled into a 1 L baffled Erlenmeyer flask. Mid-log cultures (1 mL), in 20% glycerol (stored at -80 °C), containing the *E. coli* clone, was used to inoculate the flask which was subsequently incubated at 30 °C for 12 h at 125 rpm on a rotary, water bath shaker (Heto, Allerød, Denmark).

Experimental set-up and cultivation conditions

Fed-batch cultivations were performed using a 3 L fermentor (Chemoferm FLC-B-3, Hägersten, Sweden) with an initial medium volume of 2 L. The salts were sterilised in the vessel at 121 °C for 45 minutes, thereafter sterile MgSO₄, glucose and trace elements were aseptically added. Ampicillin (see Table 1) was added by sterile filtration. Data capturing of dissolved oxygen concentration (%DO), feed-pump control, stirrer speed (rpm) and off-gas were conducted with the SattLine control system (Alfa Laval Automation AB, Malmö, Sweden). Cultivation temperature was controlled at 37 °C and the pH was maintained at 7.0 by titration with 6.7 M aqueous ammonia. Dissolved oxygen concentration was measured using

a polarographic electrode calibrated to 100% at 1100 rpm at 37 °C and zeroed by sparging sterile nitrogen into the vessel. The DO was controlled at 30% saturation using a gain-scheduled PID controller connected to stirrer speed, as described in (17). A pulse feeding strategy (21) was employed using the varying nutrient feed solutions defined in Table 1. A Tandem dual gas system (Adaptive Biosystems, Luton, UK) was used for off-gas analysis. Induction was carried out by IPTG or lactose. Fermentations and assay conditions were standardised to avoid any bias and to allow accurate and reproducible comparison. Furthermore, selection of appropriate precursor supplementation concentrations was based on the highest concentrations of organic acids obtained during uninduced control cultivations.

Sampling and sample treatment

For intracellular enzyme activity, total protein estimation and SDS-PAGE, samples were centrifuged (11,000 × g, 15 min, at room temperature) and the resulting cell pellets were dissolved to the original volume in 20 mM Tris-HCl pH 7.5 and disrupted by ultrasound (90 s, cycle 0.5) with a sound intensity of 230 W/cm² using UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany), equipped with a 3 mm titanium probe. After sonication the samples were centrifuged (11,000 × g, 15 min, at room temperature) and the supernatants were stored on ice or kept frozen (-20 °C) until analysis.

For extracellular enzyme activity (i.e. analysis of enzyme activity in the fermentation broth as a result of lysis or cell disruption), samples were centrifuged (11,000 × g, 15 min, at room temperature) and the resulting supernatants were stored on ice or kept frozen (-20 °C) until analysis.

For HPLC analysis of organic acids and glucose, samples were centrifuged ($11\ 000 \times g$, 5 min, at room temperature) and the resulting supernatants were filtered (0.2 μm) into vials and stored ($-20\ ^\circ\text{C}$) until analysis.

Analytical Methods

Optical density and Cell dry weight determination

The optical density was determined at 620 nm. Samples exceeding an OD value of 0.5, were appropriately diluted with 0.9% (w/v) NaCl before determination.

Triplicate cell dry weight (CDW) determinations (4 ml sample each) were made after drying overnight at $105\ ^\circ\text{C}$ as described in (13).

Enzyme activity

Xylanase activity was determined using the DNS (3,5-dinitrosalicylic acid) method (22) under the conditions described in (13). Xylanase activity was expressed in units (U) which is defined as the amount xylanase required to liberate 1 μmol of reducing sugar equivalents per min under standard conditions.

Estimation of total protein

Protein concentration was determined using the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin (0.2-1.0 mg/ml) as a standard.

Organic acids

The concentration of organic acids was determined by HPLC using an Aminex HPX-87H column (Bio-Rad, CA, USA). The column temperature was set at $30\ ^\circ\text{C}$ at a wavelength of 210 nm and a mobile phase (0.005 M H_2SO_4) using a flow rate of 0.5 ml/min. An organic acid standard (Bio-

Rad: Catalog no. 125-0586) which enabled the quantification of citric acid, succinic acid, and malic acid was used for calibration.

Glucose estimation

The residual glucose concentration was determined using a Waters Breeze HPLC System equipped Breeze Software and a Water 2410 refractive index detector (Waters Corporation, Milford, MA). The internal temperature of the RI-detector was 45°C and the external temperature was 60°C using 0.005 M H₂SO₄ as elute. Glucose standards (0.1-10 g/L) were used for calibration.

TABLE 1: Medium composition for batch and nutrient feed-solutions

Ingredient	Concentration	
(NH ₄) ₂ SO ₄	2	g L ⁻¹
K ₂ HPO ₄	14.6	g L ⁻¹
NaH ₂ PO ₄ 2H ₂ O	3.6	g L ⁻¹
(NH ₄) ₂ H-citrate	0.5	g L ⁻¹
Glucose	10	g L ⁻¹
1M MgSO ₄	2	ml L ⁻¹
*Trace elements	2	ml L ⁻¹
Ampicillin	0.1	g L ⁻¹
Antifoam (Adekanol)	0.1	ml L ⁻¹
Inducer	1 mM (IPTG) or 13 mmol/g CDW(Lactose)	
Feed (F)		
Glucose	500	g L ⁻¹
1M MgSO ₄	50	ml L ⁻¹
Trace elements	10	ml L ⁻¹
Feed (F_{cit})		
Feed F + Citric acid	2	g L ⁻¹
Feed (F_{suc})		
Feed F + Succinate	2	g L ⁻¹
Feed (F_{mal})		
Feed F + Malate	2	g L ⁻¹
Feed (F_{cys})		
Feed F + Cystein	2	g L ⁻¹

SDS-PAGE

Protein production was analysed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to (23) and stained with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany). Densitometric measurements to estimate the percentage of recombinant protein were performed using a Gel Doc 2000 system (Bio-Rad). The sum of the light intensity from individual bands in the lane (last hour of induction) was divided by the light intensity of the band corresponding to recombinant xylanase.

Qualitative detection of protease activity by CPAGE

Detection of protease by *CPAGE* (14) was performed using the conditions described previously (13).

RESULTS

Previous results (13), using the same host-vector system, the same fed-batch strategy, and a feed consisting solely of glucose, magnesium and trace elements (F in Table 1) were indicative of a nutrient limitation during the post induction phase as xylanase activity significantly decreased 2 h after induction. This decrease was avoided when the amino acid Glu (over-represented in the xylanase compared to average *E. coli* proteins) was added to the nutrient feed and it was concluded that this addition alleviated limitation of building blocks needed for the production of the recombinant protein. In this work, the cause of the post-induction nutrient limitation (monitored by following xylanase production, detectable protease activity, or the level of TCA-cycle intermediates) is further investigated using feed supplementation with: I) an amino acid (Cys) not over represented in the target protein, to analyse if addition of any amino acid affects target protein production and II) selected TCA-cycle intermediates (Table 1), which are

organic acids and precursor molecules with a potential to affect recombinant protein production. In addition the effect of the inducer-choice (IPTG vs. lactose) at glucose limited conditions was evaluated.

TABLE 2: Protease detection at two stages of the fed-batch cultivation, in the pre-induction feeding phase, and in the post-induction phase 2 h after induction. Detected protease activity is indicated (+) (-) = not detected

Type of feed (Inducer)	Protease detection by CPAGE	
	Pre-induction phase	Post-induction phase
F (IPTG)	-	+
F (uninduced)	-	-
F (Lactose)	-	-
Fcit (IPTG)	-	+
Fsuc (IPTG)	-	-
Fmal (IPTG)	-	-
Fcys (IPTG)	-	-

Feed strategy and feed-supplementation

The substrate limiting feeding strategy (17) was applied in all cultivations. The start of the feeding phase was commenced upon depletion of the initial glucose, detected as a peak in the DO signal, and the feed rate was gradually increased by the control algorithm until maximum stirrer speed was obtained. Thereafter, the feed rate was mainly limited by cell metabolism as seen in the oxygen response to feed-pulses, and in the later phase a safety net gradually decreased the feed rate to maintain aerobic conditions, keeping the stirrer speed at its maximum (21). Totally seven fed-batch cultivations, all controlled by the feeding strategy described above were run. The batch phase conditions were similar in all cultivations, and the cell dry weight obtained at the transition from batch to fed-batch

phase did not significantly vary between the cultures (6.5 ± 0.7 g/L). At this stage, the substrate-limiting feed-control strategy was initiated, and the variation in feed-composition introduced. This consisted of feed-supplementation (2 g L^{-1}) with one of the following components: the amino acid cystein or one of the organic acids: citric acid, succinic acid, malic acid, in each of four cultivations (where stated). The remaining three cultivations were fed with the originally defined feed (F in table 1), and variation was instead introduced at the point of induction (one induced by IPTG, one by lactose, and one uninduced).

Six of the seven cultures were hence induced 2.5 h after the feed-start: five (four with feed-supplementation's and one with the originally defined feed, F) by a single addition of the inducer IPTG (1 mM), and one (fed with the originally defined feed, F) with the alternative inducer lactose (13 mmol/g CDW). The seventh cultivation was an uninduced control cultivation (also fed with the originally defined feed F in table 1), run to establish a baseline comparison for the induced cultivations mentioned above. Induction was initiated at approximately the same cell mass concentration (21.4 ± 1.2 g/L), in all six cultivations. The protein production phase in the cultivations induced by IPTG was limited to a time-period of 3 h, based on previous results (6,13), while the lactose induced production proceeded for 6 h. Lactose induction was analysed as a low cost alternative, suitable with this type of substrate-limited feed control strategy, as the concentrations of glucose are proven to be low throughout the fed stages of the cultivation (6,13,17).

Analysis of organic acids, and biomass production

HPLC analysis allowed the quantification of, citric acid, succinic acid, and malic acid in the fermentation samples [from unsupplemented

cultivations and from those supplemented with the above organic acids (Table 1)]. The results from the sample analysis were subsequently evaluated in order to ascertain the effect of the limited substrate feeding on their concentration levels during cultivation (Fig. 1A-C). A comparison between the uninduced cultivation and cultivations induced with IPTG (both fed with the originally defined feed) showed an almost complete exhaustion of the concentration of both citric acid (Fig. 1A) and malic acid (Fig. 1B) during the post induction phase. Succinic acid was not exhausted, but concentrations (Fig. 1C) were reduced to approximately 60% during the post induction phase compared to the uninduced culture. In the lactose-induced cultivation, the complete exhaustion was avoided, which could be a consequence of the lactose addition, and hence higher level of available carbon-source. As expected (Fig. 1B & C), the residual concentrations of succinic acid and malic acid were higher during the pre-induction (fed-batch starts at 5.5 h) and post induction phases when nutrient feeds F_{suc} and F_{mal} are employed, respectively, as opposed to use of an unsupplemented feed (F). Although the citric acid concentration decreased during the post-induction phase also when using feed-supplementation (F_{cit}) it only amounted to 30% compared to the 100% reduction using feed F. Thus, the previously observed depletion during the post-induction phase was circumvented. Accumulation of the added organic acid towards the end of the post-induction phase, was only observed using succinic acid supplementation, indicating direct use in metabolism, providing an increased flux through the TCA cycle or use as precursors for transamination reactions. It was also observed that there was an increase in cell mass production rate when either F_{cit} or F_{cys} (Fig. 2B) was used as feed as compared to using feed F_{mal} or F_{suc} (Fig. 2A). This was an indication that citrate and the amino acid cystein were metabolites used

directly for host specific biosynthesis (cellular components) due to a final cell dry weight concentration of approximately 12.3% higher being observed when feeds F_{cit} and F_{cys} were employed (Fig. 2 B). In line with previous results using the substrate limited control strategy (13,17), offline analysis indicated no glucose accumulation with concentrations maintained below 0.2 g L^{-1} during the feed-phase even when employing metabolite supplemented feeds.

The effect of nutrient feed supplementation's on xylanase activity and on detectable protease activity

The presence of either succinic acid or malic acid increased xylanase production levels by approximately 41.6% [compared to the highest xylanase activity attained using unsupplemented feed, F (Fig. 3)]. Moreover, the previously observed decrease in xylanase activity (using unsupplemented feed, F) was alleviated when these precursors were present in the nutrient feed. In contrast, the presence of citric acid or the amino acid cystein in the feed did not show any effect on enzyme production levels and displayed an enzyme activity curve similar to cultivations employing the unsupplemented feed (Fig. 3). Xylanase activity measurements of the fermentation broth revealed an average of 9.1 % of the total measured intracellular activity irrespective of the nutrient feed used. A previous investigation (20) showed that the xylanase activity was located both in the periplasm and intracellular, but that the major part of the activity was intracellular despite the presence of the signal peptide. Our current results also show that a similar degree of either cell lysis or leakage of the enzyme occurs during the cultivation and is independent of the nutrient feed composition. Furthermore, protease was detected in the citric acid supplemented fed culture (F_{cit}) at a similar point (2 h) as in the unsupplemented culture (F) during the post induction phase (Table 2). This

further demonstrates that the presence of citric acid or cystein was not effective for recombinant xylanase production in this study, but that it may be metabolised for host specific biosynthetic reactions.

The effect of the alternative inducer lactose on xylanase activity and on detectable protease activity in substrate limited fed-batch cultivations

The applicability of lactose for gene expression control was assessed in this type of glucose limited fed-batch cultivations employing unsupplemented feed (F). In order for the induction efficiency of lactose to be equivalent to that of IPTG, both the lactose uptake and the conversion to the true inducer allolactose, needs to be efficient. The first requirement was considered fulfilled by the low levels of glucose, kept as a consequence of the control strategy, which should allow uptake. Therefore, a one-point induction strategy involving the introduction of lactose to the fermentor was performed. This resulted in a metabolic shift (Fig. 4) decreasing the glucose nutrient feed rate during the post-induction phase in order to maintain 30% dissolved oxygen saturation, and was indicative of an increased lactose uptake rate. It was shown that, although the recombinant protein production start is delayed approximately 30 min as compared to the IPTG-induced culture, the rate of product formation appears to be approximately the same once the production proceeds. This is in accordance with previous data collected from lactose induced fed-batch cultures fed using an exponential scheme but assuming a low specific growth rate (6), and may be a reflection of initial conversion time from lactose to allolactose. Moreover, the specific xylanase activity (Fig. 5A) continually increased during the entire post-induction phase (6 h). Judging from SDS-PAGE (Fig. 5B), the recombinant xylanase constituted approximately 30 % of the total protein at the end of the cultivation. Also,

protease activity was undetected during the pre- and post-induction phases (Table 2). Finally, the pronounced reductions in citric acid, malic acid and succinic acid (Fig. 1A-C) were avoided during the post induction phase. Taken together, this indicates a significant reduction in the metabolic burden imposed during recombinant protein expression when lactose is employed as an inducer under otherwise substrate limited conditions.

DISCUSSION

In this work we have explored the effect of selected organic acids from the TCA-cycle on the production of a recombinant protein in *E. coli*, grown using a substrate (glucose) limited fed-batch strategy. In addition supplementation with an amino acid (not over-represented in the recombinant protein compared to average *E. coli* proteins) was investigated. Expression of recombinant proteins has previously been shown to be strongly affected by amino acid deficiencies (2,8,13). The amino acid deficiencies has in turn been suggested to lead to reduced tRNA levels during translation, creating metabolic conditions that ultimately result in induction-related stress responses, such as increased protease activity, which (24) towards already synthesized recombinant proteins (in order to supply amino acids for the host cell metabolism) ultimately decreases productivity. Over-expression of recombinant proteins will hence cause a diminished flow in the TCA cycle through the withdrawal of intermediates that serve as precursors for further biosynthesis (25). Ten amino acids (4 of the Glu-family, and 6 of the Asp-family) are biochemically derived from TCA cycle intermediates (Fig. 6), which imposes an additional metabolic burden during recombinant protein expression that may result in a decreased growth of the host cell or poor expression of the desired protein due to the increase in protein synthesis rate redirecting the carbon flux

from anabolic to energy-generating catabolic pathways (26). Our results showed that the metabolic load caused by expression of heterologous xylanase in minimal medium exceeded the host's capacity thereby resulting in a loss of final enzyme yield, which was likely caused by amino acid misincorporation and protease degradation (eventually in combination). Another indication of metabolic load was the decrease in key metabolic intermediates during the post-induction phase accompanying the decrease in xylanase activity. Therefore, in order to better synchronize the metabolic need the feed strategy was modified to include both control of the nutrient feed rate, and supplementation of the feed with amino acids/other metabolites in order to reduce biosynthetic precursor demand. This was the reason for selecting 3 intermediates (citrate, succinate and malate) from the TCA-cycle as feed supplements in order to establish a balance between heterologous and host specific protein biosynthesis in order to enable the production phase to be extended thus concomitantly increasing the amount of heterologous protein produced. It has previously been shown that supplementation with the amino acid Glu, (which is over-represented in the xylanase produced in this work) resulted in improved production yield during the late production-phase (>2 h production) (13). It was however unclear if the production improvement was due to a balancing (based on the amino acid residue composition of the xylanase), or if it was a relieving effect caused by the presence of any precursor metabolite. During this investigation it was found that not all post-induction nutrient supplementation's directly ensured an increased expression of the gene encoding the heterologous protein. This could be seen with regards to addition of the amino acid cystein, an amino acid with a metabolic precursor (3-phosphoglycerate) in the Embden-Meyerhof pathway, but which opposed to glutamate is not over-represented in the heterologous

xylanase. Use of this supplementation, although it did not improve recombinant protein production, instead led to an increased biomass production. A similar effect was also seen using the TCA-intermediate citrate. So despite lacking an effect in recombinant protein production, these additions appeared to have been utilized for host specific biosynthesis. On the other hand the presence the TCA cycle intermediates malate or succinate, respectively, resulted in a pronounced increase of the heterologous xylanase during the late (> 2 h) production phase. Thus it can be seen that during the post induction phase low levels of TCA cycle intermediates have a direct influence on heterologous protein production levels and host specific biosynthesis. However, these limitations may be overcome by using an optimized nutrient feed during the post-induction phase.

Metabolic balancing during the production phase to circumvent loss of produced protein can be achieved in different ways. The nutrient supplementation way described above is affecting the translational level, as it involves manipulation of the amount available nutrients in the feed in order to achieve metabolic balancing. Alternative strategies can however also be used, such as modulation of the gene dosage (on replication level) by use of vectors with varying copy numbers, or modulation of the transcription either by use of promoters of different strength, or by inducer tuning of a strong promoter (27, 28). The latter offers an easier alternative from a process-engineering point of view, as tuning of the expression rate is also possible for example by use of lactose as an inducer. The T7/*lac* promotor using IPTG to induce protein expression is one of the most powerful and commonly used systems employed for heterologous protein production due to its specificity and control (29). Although it's a strong promotor, use of IPTG is however not cost effective for industrial scale

and the inducer has in some cases been reported to be toxic to the cells (30). The use of lactose may hence provide an inexpensive alternative for the induction of *lac*- (and related) promoters, and it can simultaneously serve as an inducer and as a carbon source (6, 31-34).

In conclusion, the findings of this study suggest that there is a strong dependence of nutrient feed composition in the post induction phase for efficient protein synthesis. Furthermore, lactose is proven to be a suitable inducer for expression under the control of the *lac* promoter using the substrate limited fed-batch strategy, indicating the potential for production of thermostable xylanase from *R. marinus* by an *E. coli* expression system.

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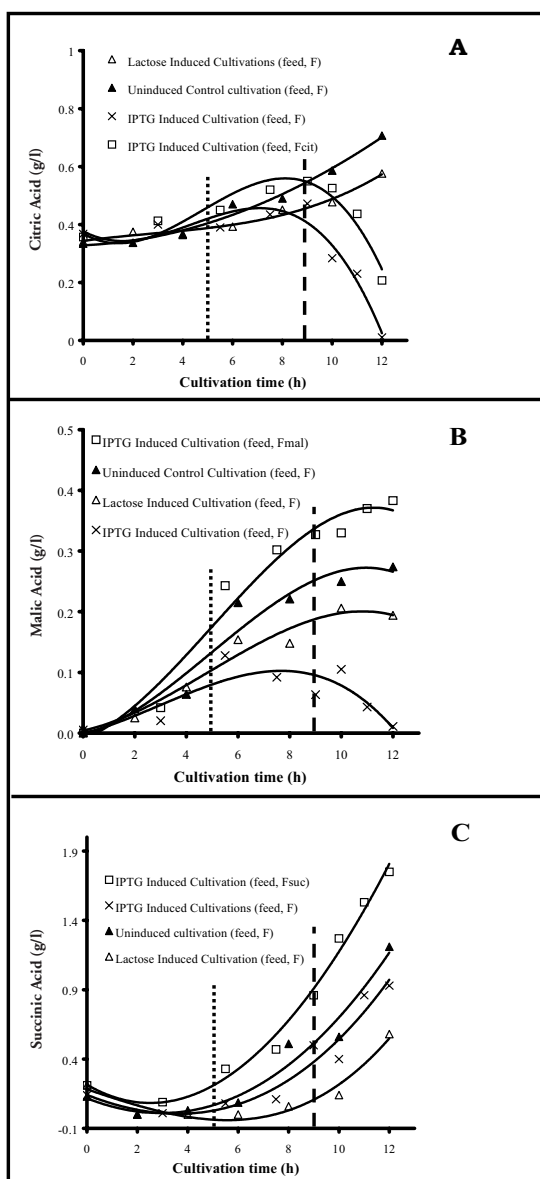


FIG. 1A-C. HPLC analysis of fermentation samples for the quantification of organic acids. A) measured citric acid concentration. B) measured malic acid concentration. C) measured succinic acid concentration. Nutrient feed started at 5 h (dotted line) and induction at 9 h (dashed line).

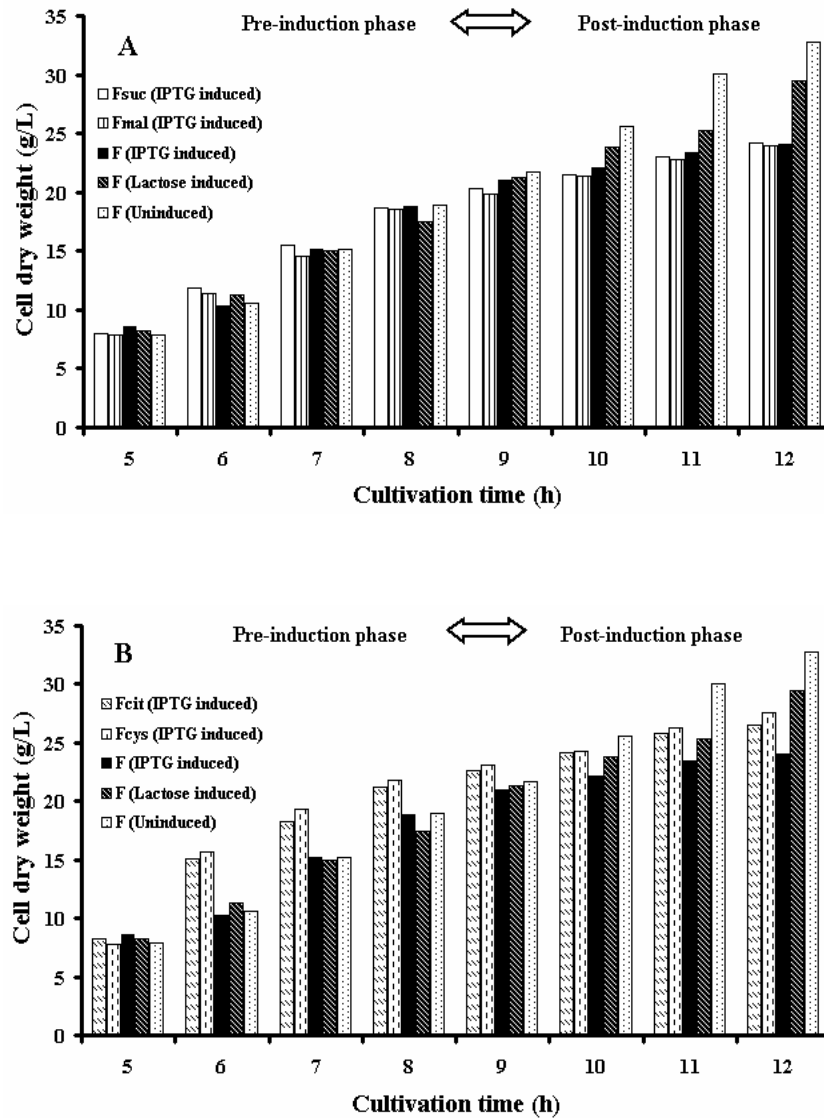


FIG. 2. Cell dry weights attained in fed-batch cultivations shown from the start of the feeding phase employing varying nutrient fed composition. A) demonstrates the effect of malic and succinic acid on cell mass production. B) demonstrates the effect of citric acid and the amino acid cystien on cell mass production. For comparative purposes only the first 3 hours using lactose as an inducer is shown. Expression of the xylanase gene is induced at cultivation time 9 h.

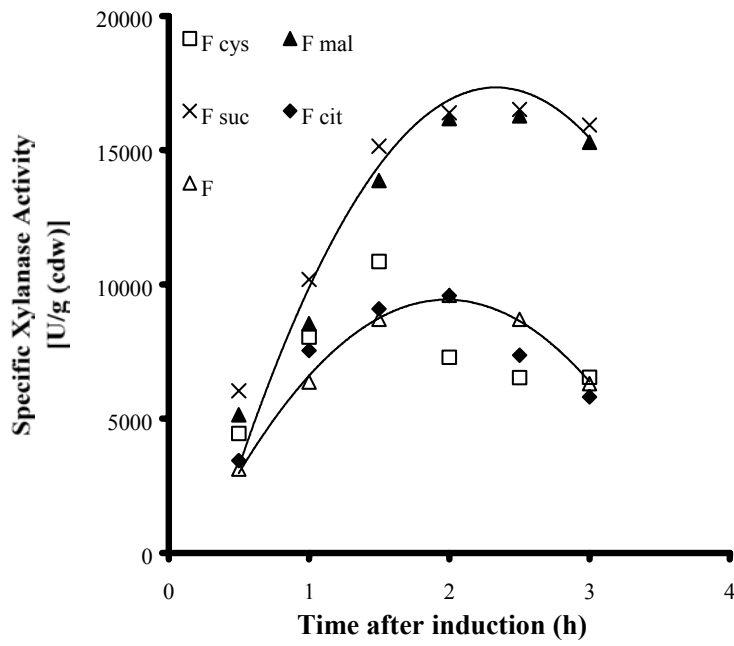


FIG. 3. The effect of varying nutrient feed composition on heterologous thermostable xylanase production during the 3 h production phase in the substrate (glucose) limited high cell density fed-batch cultivations using IPTG as an inducer. Trend-lines were fitted using data points of F_{suc} and F_{mal}.

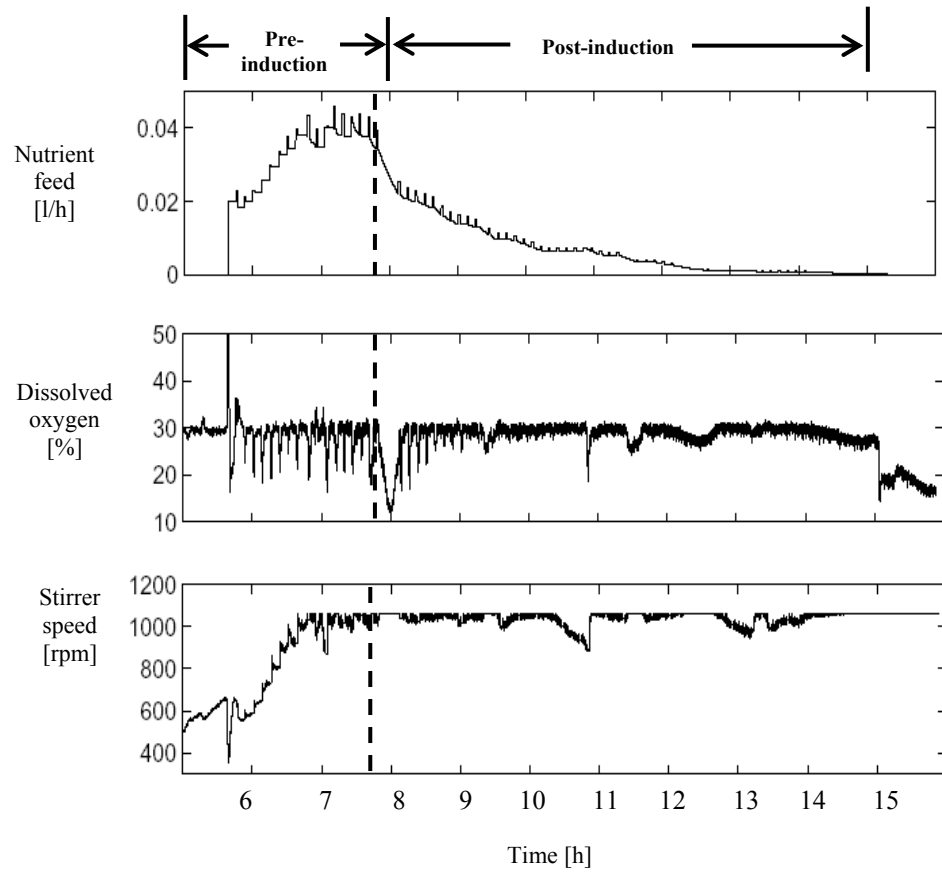


FIG. 4. High cell density fed-batch cultivation (HCDC) of recombinant *Escherichia coli* strain BL21(DE3) encoding thermostable xylanase (Xyn10A Δ NC). Nutrient feed started at 5 h after inoculation and lactose was used as an inducer (dashed line) at 7.8 h.

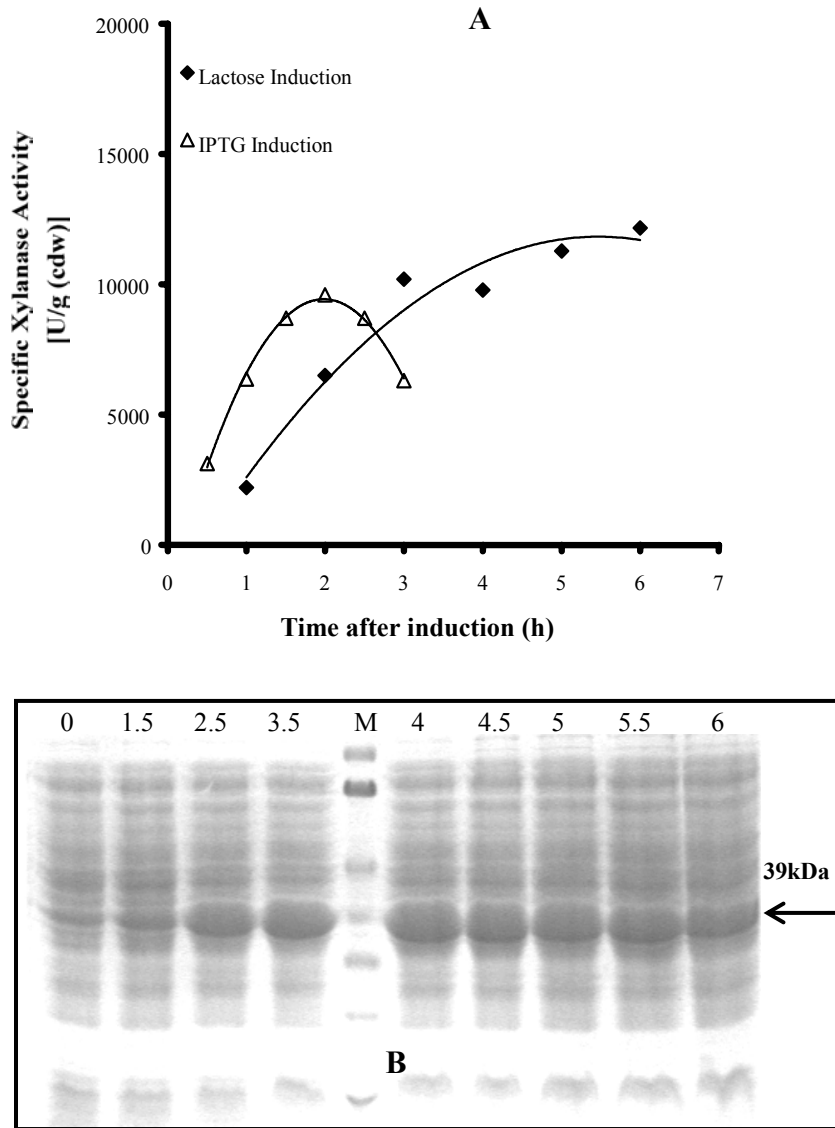


FIG. 5. Specific xylanase activities expressed in units (U) per gram cell dry weight. A) comparison of xylanase activity using IPTG or lactose as an inducer. B) SDS-PAGE from post induction phase of the cultivation using lactose as an inducer. The arrow indicates the 39 kDa (Xyn10AΔNC) xylanase produced at various times after induction (0-6 hours). The position of the xylanase active band was confirmed by activity staining using a substrate containing overlayer gel. (M) Corresponds to the molecular mass marker (113, 92, 53, 35, 29 and 22kDa).

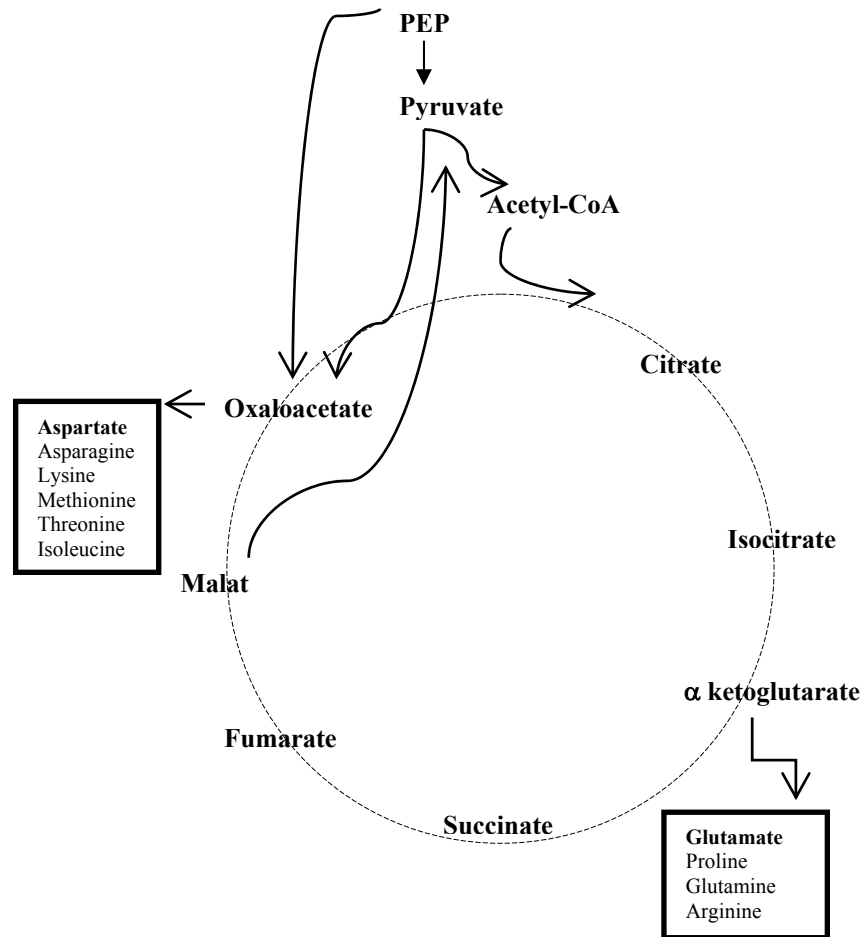


FIG. 6. Schematic representation of TCA cycle showing precursors used directly in the synthesis of amino acids.

PAPER IV

Production of two glycoside hydrolases of thermophilic origin in *E. coli* and analysis of released endotoxins using substrate limited and temperature limited fed-batch cultivation strategies.

Eva Nordberg Karlsson¹, Santosh O. Ramchuran¹, Pernilla Turner¹, Lena de Maré², Christian Cimander³, Stephane Velut², Åsa Ekman¹, Christina Wennerberg¹, Per Hagander² and Olle Holst¹

¹Dept. Biotechnology, Center for Chemistry and Chemical Engineering, Lund University,
P.O.Box 124, SE-22100 Lund, Sweden

²Automatic Control, Lund University, Lund, Sweden

³Novozymes, Lund, Sweden

Corresponding author: eva.nordberg_karlsson@biotek.lu.se

Abstract

Substrate limited and temperature limited feed control strategies were used to produce two glycoside hydrolases (a cyclodextrinase (CDase) and a xylanase) of thermophilic origin from genes cloned under the control of the T7/*lac* promoter in the *E. coli* production strains Tuner(DE3) and BL21(DE3). It was shown that the control-strategies applied led to produced enzyme activities in the same range in the first part of the production phase (0 - 2 h), but that the temperature limited-strategies appeared beneficial when a longer production-phase was applied. The CDase (GH13) which is a 4-domain enzyme, prone to aggregation was produced with optimized inducer concentration in strain Tuner(DE3), yielding final concentrations of 0.31 g CDase/L in substrate limited fed batch (SLFB) at 37 °C, and 0.27 g/L in temperature limited fed batch (TLFB). The xylanase (GH10), a highly soluble 5 module enzyme, was

produced in equal amounts up to 2 h after induction in BL21(DE3), but due to cell lysis and loss of activity in the later stage of the SLFB, 2.8 g xylanase/L was produced in the final sample compared to 4.8 g xylanase/L using a TLFB strategy with a probed feeding control. Release of endotoxins was monitored at the start and end of the production phases in all cultivations. The levels released did not vary significantly as a consequence of the feeding strategy applied, and higher amounts of endotoxin ($2\text{-}6 \times 10^8$ EU/g cdw) were always released per produced cell mass at the end of the production-phase compared to at the time of induction ($0.8\text{-}3 \times 10^8$ EU/g cdw).

[Keywords: cyclodextrinase, cyclomaltodextrinase, CDase, xylanase]

Introduction

Carbohydrates are essential components in biomass, which contains an array of structural and storage polysaccharides, and is estimated to be produced in a quantity of about 60 Gt/year (Cox *et al*, 2000). This resource has a potential for utilization in a number of applications, and with the increasing legislation demand on industry to limit the use of harmful chemicals, renewable biomass as a raw material for environmentally friendly technologies has received considerable attention over the past few years (Lichtenteiler & Peters, 2004). The carbohydrate material can be specifically modified/hydrolysed by carbohydrate hydrolysing/modifying glycoside hydrolases(GH)/glycosidases. Enzymes from extremely thermophilic microorganisms have in this aspect received attention, as their thermostability opens up the possibility of biocatalysis in a completely different window of conditions, allowing for instance process temperatures

previously not believed to be possible for biological systems (Kristjansson *et al.*, 1989, Niehaus *et al.*, 1999). Their possibility of use is however, dependent on recombinant DNA technology, allowing expression of the genes encoding these enzymes in host organisms selected for their ability to grow in cultivation-processes that enable production in quantities that otherwise would have been difficult or impossible to obtain from natural resources.

Escherichia coli is a well-studied and extensively used host for recombinant protein production. A wide spectrum of vectors has been developed and many are commercially available, which in many cases makes *E. coli* the first choice when a novel protein is to be heterologously produced. The organism however lacks a natural secreting pathway to the medium, making high cell density cultivations the most used strategy for producing the recombinant target proteins (Yee and Blanche, 1992). Fed-batch processes are most widely used to gain sufficient cell mass concentrations, by consecutive additions of a selected growth-limiting variable. The growth limiting substrate is typically used for this purpose, with feeding rates set to control the specific growth rate (μ) at values lower than μ_{\max} . Such limitations in substrate feed-rate are crucial in order to prevent overflow metabolism, (i.e. production of acetate under glucose excess conditions) (Yee and Blanch 1992; Riesenber *et al.* 1991), which otherwise (due to metabolic by-product accumulation) could inhibit cell growth, and reduce the yield of heterologous protein, as well as to control the oxygen consumption rate to avoid oxygen limitations. Åkesson *et al.* (1999a) has proposed a substrate limited fed-batch (SLFB) strategy based on superimposed pulses in the substrate feed, followed by evaluation of the responses in the dissolved oxygen signal, which is then in turn used to feed back control the flow rate of the nutrient feed. The dissolved oxygen is in

this strategy regulated by the stirrer speed around a fixed set-point (Åkesson *et al.*, 1999a, Åkesson *et al.*, 1999b). An alternative strategy is to use the temperature as a limiting factor during the fed-batch and production phase. In the temperature limited fed-batch (TLFB) strategy, the oxygen demand is instead controlled by the cultivation temperature, and alterations in temperature will hence be used to avoid fall of the dissolved oxygen (DO) below the set-point, while substrate levels in this strategy are not controlled and substrate generally available in excess (Svensson *et al.*, in press). The TLFB strategy used in this work has also been further developed, in order to be able to reach a controlled excess of substrate (in this case glucose), thereby avoiding overflow acetate accumulation (de Maré *et al.*, 2004). A drawback using *E. coli* as a production host is the presence of endotoxin, a lipopolysaccharide (LPS), from the cell wall present in most Gram-negative bacteria. The LPS:s cause inflammation when accessing the bloodstream of a mammal and nanogram quantities can affect biochemical events in certain cells or cell-systems (Liu *et al.*, 1997), thus leading to a desire to avoid strategies that promotes its release during the cultivation. Svensson *et al.* (in press) showed that the TLFB- technique strongly repressed the extensive release of endotoxin observed in *E. coli* fed-batch cultivations at high cell density (using e.g. substrate limiting techniques) (Han *et al.*, 2003). In this study, both the heterologous enzyme production, and the release of endotoxin were hence analysed during the production phase of cultivations fed by either substrate or by the temperature limited strategies. Two glycoside hydrolases of thermophilic origin were produced: a cyclodextrinase isolated from an *Anoxybacillus flavithermus* strain (A/Cda13) (Turner *et al.*, submitted manuscript) and a modular thermostable xylanase originating from *Rhodothermus marinus* (RmXyn10A) (Nordberg Karlsson *et al.* 1997). The paper describes the

production profiles of the respective enzyme and the amount released endotoxin using the different feeding strategies.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli strains BL21(DE3) or Tuner(DE3) were used as the host microorganisms. Both genes were cloned under the control of the T7/lac promoter, in the vector pET22b(+) (Novagen, Madison, WI). The sequence encoding the complete native xylanase was inserted into the multicloning site of the vector, using the restriction sites *NdeI* and *XbaI* as described in Abou Hachem *et al*, (2003a), and transformed to the producer strain BL21(DE3). The cyclodextrinase encoding gene was inserted using the restriction site *NcoI* and *NoaI* as described in Turner *et al*, (2005), and transformed to the expression strain Tuner(DE3).

Inoculum preparation

Inoculum was prepared using 100 ml of defined mAT-medium [Table 1 (excluding antifoam)]. The salts, glucose and trace elements were sterilised separately at 121 °C for 20 minutes and aseptically pooled into a 1 L baffled Erlenmeyer flask. Mid-log cultures (1 ml), in 20 % glycerol (stored at -80 °C), each containing the respective *E. coli* clone, were used to inoculate the flasks which were subsequently incubated at 30 °C for 12 h at 125 rpm on a rotary, water bath shaker (Heto, Allerød, Denmark).

Experimental set-up and cultivation conditions

Fed-batch cultivations were performed using a 3 L fermentor (Chemoferm FLC-B-3, Hägersten, Sweden) with an initial medium volume of 2 L. A defined medium was used (mAT), and the salts of the medium were sterilised in the vessel at 121 °C for 45 minutes, thereafter sterile

MgSO₄, glucose and trace elements were aseptically added. Ampicillin (see **Table 1**) was added by sterile filtration. The feed solution (**Table 1**) was sterilised at 121 °C for 20 min in a separate vessel and thereafter aseptically connected to the reactor. Data capturing of dissolved oxygen concentration (%DO), feed-pump control, stirrer speed (rpm) and off-gas were conducted with the SattLine control system (ABB Automation Technologies AB, Malmö, Sweden). The DO was controlled at 30 % saturation using a gain-scheduled PID controller connected to stirrer speed, as described in Åkesson *et al* (1999b). Cultivation temperature was controlled at 37 °C (unless otherwise stated) and the pH was maintained at 7.0 by titration with 6.7 M aqueous ammonia. Dissolved oxygen concentration was measured using a polarographic electrode calibrated to 100% at 1100 rpm at 37 °C and zeroed by sparging sterile nitrogen into the vessel.

Table 1. Medium and feed-solution composition

Ingredient	Concentration	
Medium (mAT)		
(NH ₄) ₂ SO ₄	2	g L ⁻¹
K ₂ HPO ₄	14.6	g L ⁻¹
NaH ₂ PO ₄ 2H ₂ O	3.6	g L ⁻¹
(NH ₄) ₂ H-citrate	0.5	g L ⁻¹
Glucose	10	g L ⁻¹
1M MgSO ₄	2	ml L ⁻¹
^a Trace elements	2	ml L ⁻¹
Ampicillin	0.1	g L ⁻¹
Antifoam (Adekanol)	0.1	ml L ⁻¹
Feed		
Glucose	500	g L ⁻¹
1M MgSO ₄	50	ml L ⁻¹
Trace elements	10	ml L ⁻¹

^a According to Holme *et al.* 1970

Feeding strategies

Three types of feeding strategies were used in the cultivations in this work: substrate limited fed-batch (SLFB), temperature limited fed-batch (TLFB), and a developed strategy that combines the control of the two previous strategies, here termed probed temperature limited fed-batch (pTLFB).

The substrate limited pulse feeding strategy was employed using the conditions described in Ramchuran *et al* (2002) in which the dissolved oxygen response to pulses in the feed-rate is used for feed-back control of the feed (Åkesson *et al*, 1999b). This probing strategy was developed to avoid acetate formation, and thereby improve recombinant protein yield. In this strategy, pulses are superimposed in the glucose feed (*up-pulses*) and the cellular response detected using a dissolved oxygen sensor (**Fig. 1**). The detected response is then utilized in a feed-back algorithm that adjusts the feed rate to keep the glucose below the critical value for acetate production (Åkesson *et al*, 1999). When the maximum stirrer speed is reached the feed rate is decreased to maintain a constant oxygen level.

In the TLFB-strategy, the cultivation temperature is instead changed (in this case when the maximum stirrer speed is reached) by modulation of the cold and hot water flows (de Maré *et al*, 2004) in order to decrease the oxygen consumption rate, and avoid oxygen limitations. To avoid high substrate excess, the feed-rate is manually adjusted after evaluation of *down-pulses* (A down-pulse gives a response in the dissolved oxygen at higher glucose-values than an up-pulse [Åkesson *et al*, 1999, de Maré *et al*, 2004] **Fig. 1**).

The pTLFB-strategy can be described as a combination of the two above used strategies. In this case, during the first part of the feeding-phase, the cellular response (measured by the dissolved oxygen sensor) to

up-pulses in the feed is used to feed-back regulate the feed-rate until maximum stirrer speed is reached. Thereafter, temperature instead of feed-rate is manipulated to decrease the oxygen demand in the reactor. In this way the oxygen demand does not exceed the oxygen transfer capacity of the reactor. A controlled excess is automatically achieved by the probing strategy with *down-pulses*.

Calculations

Production of recombinant enzyme (P) in (g/L) was calculated as follows: $P = (A_{\text{sol}}/A_{\text{pure}}) \times P_{\text{tot}}$, where A_{sol} = specific activity in the soluble fraction after sonication, A_{pure} = specific activity of the pure enzyme (for Cda13 $A_{\text{pure}} = 260$ U/mg, and for Xyn10A $A_{\text{pure}} = 56$ U/mg), P_{tot} = total protein content (g/L) in the soluble fraction after sonication.

The volume in the reactor (V_r in L) was during the feeding (and production) phase: $V_r = V_0 + V_f + V_b - V_s$, where V_0 = initial volume (2 L), V_f = volume added feed, V_b = volume added base, V_s = accumulated sampling volume. Consumed glucose (S_c in g) in the reactor during feeding (and production) phase was: $S_c = (s_0 \times V_0) + (s_f \times V_f) - (s_r \times V_r)$, where s_0 = initial substrate added in the batch (g/L), s_f = glucose added during feeding (g/L), s_r = glucose in the reactor at the relevant cultivation time (g/L). Yield coefficients ($Y_{X/S}$, $Y_{P/S}$, and $Y_{P/X}$ in g/g) were calculated for produced biomass (X) per consumed glucose (S), produced recombinant protein-product (P) per consumed glucose and produced recombinant protein-product per produced biomass (**Table 2 and 3**) and were only taking the production-phase into account. Volumetric productivity of recombinant protein was also calculated for the production-phase.

Analytical Methods

Sampling and sample treatment

Samples were withdrawn through a sampling port at the bottom of the reactor, using 25 ml pre-sterilised metal-capped glass tubes. The medium fractions (for extracellular enzyme activity, total protein and endotoxin determination) were collected after centrifugation ($11\,000 \times g$, 15 min, at room temperature in 1 mL portions) and kept frozen ($-20\text{ }^{\circ}\text{C}$) until analysis. The cell pellets (for intracellular enzyme activity, protease activity and total protein determination) were dissolved to the original volume in 20 mM Tris-HCl pH 7.5 and sonicated (5×60 s, cycle 0.5) with a sound intensity of $230\text{ W}/\text{cm}^2$ using a UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany), equipped with a 3 mm titanium probe. After sonication the samples were centrifuged ($11\,000 \times g$, 15 min, $4\text{ }^{\circ}\text{C}$) and the supernatants (soluble protein fraction) and pellets (insoluble protein fraction) were stored on ice or kept frozen ($-20\text{ }^{\circ}\text{C}$) until analysis.

Samples for glucose and acetic acid determinations were collected in tubes half filled with 0.132 M perchloric acid (ice cold) and centrifuged ($1400 \times g$, 10 min, at room temperature). The resulting supernatant was dispensed (1 mL portions) into eppendorf tubes, neutralised with 3.6 M K_2CO_3 (15-20 μL) and kept frozen ($-20\text{ }^{\circ}\text{C}$). Prior to analysis samples were thawed, heated ($80\text{ }^{\circ}\text{C}$, 15 min) and centrifuged ($11\,000 \times g$, 3 min, at room temperature).

Optical density

The OD was determined at 620 nm. Samples were appropriately diluted with 0.9% (w/v) NaCl at OD values exceeding 0.5.

Cell dry weight determination

Cell dry weight (cdw) was determined after centrifuging ($1400 \times g$, 5 min, at room temperature) triplicate samples (5 mL) in pre-weighed glass tubes. The pellets were dried overnight ($105\text{ }^{\circ}\text{C}$) and subsequently weighed to determine cdw.

Estimation of glucose and acetic acid concentration

Glucose and acetate were analyzed with a high performance liquid chromatography (HPLC) system (Agilent 1100 series). An organic acid and alcohol analysis ion-exclusion column (Micro-Guard precolumn cation H-cartridge (30 x 4.6 mm) followed by an Aminex HPX-87-H (300 x 7.8 mm), BioRad, Hercules, USA) was used at $50\text{ }^{\circ}\text{C}$ with 80 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min followed by refractive index detection.

Enzyme activity

Xylanase and cyclodextrinase activities were determined using the DNS (3,5-dinitrosalicylic acid) method (Bailey *et al.* 1992). Xylanase activity was determined under the conditions described by Nordberg Karlsson *et al.* (1998) using xylose as standard (2-10 $\mu\text{mol/ml}$). Enzyme blanks were prepared for each sample by incubating the substrate 1 % (w/v) birch xylan (Birch 7500, Roth, Karlsruhe, Germany) at $65\text{ }^{\circ}\text{C}$ for 5 min, then adding the DNS-reagent and immediately thereafter the enzyme. Cyclodextrinase activity was determined using a microtitre plate based assay described in (Turner *et al.*, 2005), with glucose as standard (2-15 $\mu\text{mol/mL}$), and 1.25 % (w/v) beta-cyclodextrin (Sigma, St. Louis, MO) as substrate. Xylanase and cyclodextrinase activity was expressed in units (U) which is defined as the amount which will catalyse the formation of 1 micromole of reducing sugar equivalents per minute under standard conditions.

Estimation of total protein

Protein concentration was determined using the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin (0.2-1.0 mg/mL) as a standard.

Electrophoresis

Protein production (soluble and insoluble protein fraction) was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli 1970) and stained with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany).

Xylanase activity was also detected by an over-layer agarose 1 % (w/v) gel, containing 0.05 % (w/v) xylan. The SDS-PAGE gel was washed with 20 mM Tris-HCl pH 7.5 containing 2.5 % Triton X-100 (Merck) for 20 min. Thereafter, the gel was washed with 20 mM Tris-HCl pH 7.5 covered with the overlay gel, and incubated for 15-25 min at 65 °C. The agarose gel was stained in 1 % (w/v) Congo Red solution and destained with 1 M NaCl.

Protease detection

Presence of protease activity was analysed semi-quantitatively from the xylanase producing cultivations, in 1.5 % agar (in 20 mM TRIS-HCl, pH 7.5) containing 0.4 % azocasein (Sigma) cast in a petri-dish. Small circle-shaped wells were cut in the gel and loaded with 100 µl samples or controls. The soluble and insoluble protein-fractions after sonication were loaded on the gel together with controls and incubated at 37 °C for approximately 12 h. As positive control, protease from *Bacillus thermoproteolyticus rokkeo* (Sigma) was used (25, 50 and 100 µL) in a

concentration of 25 µg/mL. Buffer (20 mM TRIS-HCl pH 7.5) was used as negative control.

After incubation, the gel was stained with Congo-Red at room temperature for 90 min, and destained in 1M NaCl until clearing zones appeared. If necessary the gel was counterstained using 10 % acetic acid to detect protease activity as clearing zones around the wells.

Endotoxin detection

For endotoxin analysis a commercial kit (Endpoint Chromogenic LAL Kit R160 (Charles River Laboratories, Wilmington, USA)) was used and gave endotoxin levels in endotoxin units (EU). Conversion of the EU to concentration of lipopolysaccharide (mg/L), was made using the conversion factor 0.1 ng / EU (Petsch *et al*, 2000).

Results

The target enzymes; xylanase and cyclodextrinase

Two model enzymes (*A/Cda13* and *RmXyn10A*) were chosen for production trials, both being glycoside hydrolases originating from bacteria isolated in Icelandic geothermal habitats. The genes encoding the respective enzyme were cloned in pET22b (Abou-Hachem *et al*, 2003a; Turner *et al*, 2005), a low copy number vector, with expression of the target gene under the control of the strong T7/lac promoter.

The cyclodextrinase gene is isolated from a Gram-positive *Anoxybacillus flavithermus* strain, and encodes an enzyme (*A/Cda13*) prone to aggregation, and hence a rather difficult target protein for production. This has led to selection of an *E. coli* host strain that allows tuning of the inducer concentration (the mutant strain Tuner(DE3)TM, with a deletion in *lacY1*) in order to overcome production of insoluble inactive protein (Turner *et al*,

2005). *A/Cda13* (EC 3.2.1.54,) consists of 586 amino acid residues, and is predicted to exhibit the 4-domain structure typical for this enzyme group (Turner *et al*, submitted manuscript) (**Fig. 2A**). The gene-construct is cloned to include an N-terminal pelB signal peptide, and an 8 residue C-terminal tag (Leu-Glu-His₆) encoded by the vector pET22b (totally 621 residues), resulting in a theoretically calculated molecular mass of the encoded protein of 73 kDa.

The xylanase (*RmXyn10A*) originates from the Gram-negative bacterium *Rhodothermus marinus*, and is a highly soluble protein (>30 g/L, data not shown). The catalytic module of this enzyme is previously shown to be produced in good yields using *E. coli* BL21(DE3) as a host (Nordberg Karlsson *et al*, 1999, Ramchuran *et al*, 2002), and results from batch cultivation of the full-length enzyme, also indicated relatively good production levels (Abou-Hachem *et al*, 2003a). *RmXyn10A* is a modular xylanase (EC 3.2.1.8, 962 residues, excluding the native 37 residue signal peptide) comprising 5 domains/modules, separated by short linker peptides (Nordberg Karlsson *et al*, 1997, Abou Hachem *et al*, 2003b) (**Fig. 2B**). The gene expressed in this work encodes a 970 residue enzyme (with a theoretically calculated molecular mass of 108 kDa) that starts with the sequence of CBM4-1, mutated in its first residue to include the start codon (G37M), and ends with the vector encoded C-terminal 8 residue insertion (Leu-Glu-His₆), to include a His-tag (Abou Hachem *et al*, 2003a).

Cyclodextrinase production & feeding strategies

The production of active soluble CDase was analysed using two feeding strategies in three cultivations: two SLFB and one TLFB-cultivation (**Table 2**). The first SLFB was run at a temperature of 37 °C

(SLFB₃₇), while in the second SLFB (SLFB₂₅), temperature was decreased to 25 °C during the last part of the feeding phase (30 min before induction) and kept at this temperature during the production phase in order to view the effect of the decreased temperature on the recombinant protein yield at substrate limited conditions (**Fig. 3**). In the third cultivation the TLFB-strategy was utilized, and in this case the temperature was down-regulated to 22 °C in the early feeding phase, and the probing feeding strategy with down-pulses was used, leading to a higher glucose concentration at the end of the cultivation (**Fig. 4**). The recombinant CDase production was in all cases initiated 2 h after the feed-start [at a cdw of 15.4 g/L (SLFB₃₇), 15.7 g/L (SLFB₂₅), and 16.3 g/L (TLFB)] by a single addition of the inducer IPTG, and the production phase lasted for 4 h (SLFB₃₇) or 6 h (SLFB₂₅, and TLFB). The IPTG-concentration used was based on an optimisation procedure in shake-flasks (using IPTG-concentrations ranging from 0.005 – 1 mM), in which cultivations at 37 and 25 °C, yielded optimal production of soluble active CDase at IPTG concentrations of 0.05 and 0.1 mM, respectively (data not shown). In the TLFB the inducer concentration was set to 0.1 mM. The maximum specific growth rate of the strain Tuner(DE3)TM at these temperatures in the mAT medium was 0.8 h⁻¹ (37 °C) and 0.3 h⁻¹ (25 °C). It was shown that the SLFB₂₅-strategy was not a good strategy for production of recombinant CDase: the specific activity was much lower, and the band observed on SDS-PAGE, much weaker than what was observed using the SLFB at higher temperature, or the TLFB-strategy (**Table 2, Fig. 5**). The SLFB₃₇ and TLFB-strategies resulted in more similar production values, although in the first case production appeared to be faster (likely as a consequence of the higher temperature).

Table 2. Production-phase data from two substrate limited (SLFB at 25°C, with a 6 h production phase, and SLFB at 37 °C with a 4 h production phase) and one temperature limited fed-batch (TLFB) cultivation for production of cyclomaltoamylase (*A/Cda13*). The production phase was initialised by addition of IPTG 2 h after the feed start with the following inducer-concentrations: 0.05 mM IPTG, induced at a cdw of 15.4 g/L (Ec 0283, SLFB₃₇), 0.1 mM IPTG, induced at cdw 15.7 g/L (Ec0284, SLFB₂₅), and 0.1 mM IPTG, induced at cdw 16.3 g/L (Ec0307, TLFB).

	SLFB₃₇ (Ec0283)*	SLFB₂₅ (Ec0284)	TLFB (Ec0307)
Substrate consumption and biomass produced after induction			
Glucose consumed (g)	113	171	144
Biomass produced (g)	35	56	45
Recombinant enzyme production			
Total protein IC (g/L)	15	14	14
<i>A/Cda13</i> IC (g/L)	0.27	0.08	0.31
<i>A/Cda13</i> IC 4 h (U/L)	71 000	10 000	33 000
<i>A/Cda13</i> IC 6 h (U/L)	n.d.	21 000	81 000
Yield coefficients for the production phase			
Y _{X/S} (g/g)	0.29	0.33	0.31
Y _{P/S} (g/g)	0.0042	0.0008	0.0039
Y _{P/X} (g/g)	0.015	0.003	0.012
Volumetric	0.067	0.013	0.052
<i>CDa13</i> - productivity (g/Lh)			

* 4 h production phase, n.d. = not determined, IC = intracellular

Despite starting at 25 °C, in the TLFB, this process yielded significantly higher CDase activities than the SLFB₂₅-strategy. The activities were also steadily increasing during the production phase in the former under the cultivation conditions used (**Fig. 4**). Extracellular CDase was not detectable in any of the cultivations (**Fig. 5**). The SLFB₃₇ was, during the early production phase (in this case limited to 4 h), the most efficient CDase production strategy. A longer production phase under the SLFB-conditions (see below under xylanase production), was not judged to be

beneficial as cell lysis and loss of recombinant protein occurred at a later stage (>2 h into the post induction phase).

Xylanase production & feeding strategies

Xylanase production was analysed in one SLFB cultivation (run at 37 °C), and in two repeated cultivations using the pTLFB approach (**Table 3**, **Fig. 6**). In this case the enzyme to be produced was highly soluble, and inducer tuning not necessary, leading to use of the strain BL21(DE3), and the inducer concentration (1 mM IPTG) recommended for the vector in the manufacturers instruction. Induction of the target gene expression was started in the feed-phase at a cell mass of approximately 12 g cdw/L, and the production phase was in all cases allowed to proceed for 6 h. The maximum specific growth rate for BL21(DE3), corresponded well to that of strain Tuner(DE3)TM [0.8 h⁻¹ (37 °C)]. As expected, SDS-PAGE analysis showed that the produced xylanase was found in the soluble fraction, constituting about 10-35 % (dependent on the feeding approach and time in the production phase) of the soluble proteins (**Fig. 7**). In line with what was expected due to its high solubility, the level of insoluble xylanase found in the cell-debris was hardly detectable. Differences in production of the target enzyme were found by activity measurements, which showed that the pTLFB was the most beneficial strategy. The produced xylanase (intracellular) reached a slightly higher specific activity than in the SLFB cultivation, and especially kept a higher activity during a longer time interval (**Fig. 6**). Good reproducibility was also shown between the two pTLFB cultivations (**Table 3**). Although comparable levels were reached in the early production phase (0-2 h), xylanase produced using the SLFB strategy lost a significant amount of activity during the late production

phase. Using the pTLFB-strategy xylanase reached a maximum specific activity of 8000 U/g cdw (averaged from the two cultivations) 3-4 h after induction, with a final specific activity (after a 6 h production phase) approximately 10 % below this value, while the activity in U/mL was levelling out indicating a ceased production during the last 2 h. The maximum specific xylanase activity using the SLFB-strategy was 6900 U/g cdw, monitored already 2 h after induction, but the specific activity then decreased as much as 52 % after 6 h of production. A significant portion of the active xylanase was found in the medium in the late post induction phase in the SLFB (**Fig. 6A**) indicating cell-lysis (in line with the need to supply antifoam to the SLFB to avoid foaming in the late production phase). The total xylanase activity level in this sample (including the extracellular activity 840 U/g cdw), was only 74 % of the activity observed after 2 h production phase, showing a significant loss of recombinant protein during the late production phase. This trend, with decreasing specific activity during prolonged production, was also shown by Ramchuran *et al.* (2002) during production of the xylanase catalytic module using the same feeding-strategy.

As a consequence of cell-lysis, total protein concentration in the medium was higher in the SLFB, than in the two pTLFBs (**Table 3**). The majority of the extracellular protein contribution was however not due to recombinant protein, as the medium fraction of the pTLFB cultivations showed marginal xylanase activity (corresponding to approximately 0.1 g/L, **Table 3**). A probable cause is instead protein leakage from the periplasm, including for example the ampicillin-degrading enzyme β -lactamase, which is frequently found in the medium fraction after induction using this host-vector system.

Table 3. Production-phase data (after a 6 h production unless otherwise stated) from substrate limited (SLFB) and probed temperature limited fed-batch (pTLFB) cultivations for production of the xylanase (Xyn10A). The production phase was initialised by addition of the inducer IPTG (1mM), 2 h after the feed start, at a cdw of 11.2 (Ec0320), 12.2 (Ec0321), and 12.6 (Ec0322) g/L and allowed to proceed for 6 h.

	pTLFB (Ec0320)	pTLFB (Ec0321)	pTLFB (Av ± SD)	SLFB (Ec0322)
Substrate consumption and biomass produced after induction				
Glucose consumed (g)	133	160	147 ± 19	183
Biomass produced (g)	62	56	59 ± 4	42
Recombinant enzyme production				
Total protein IC (g/L)	16	14	15 ± 1	13
Total protein EC (g/L)*	4.6	4.9	4.7 ± 0.2	6.0
Xyn10A IC (g/L)	4.4	5.1	4.7 ± 0.5	1.6
Xyn10A EC (g/L)	0.13	0.08	0.10 ± 0.03	0.51
Xyn10A IC 2 h (U/L)	144000	157000	150000 ± 9000	160000
Xyn10A IC (U/L)	243000	282000	263000 ± 27000	90000
Xyn10A EC (U/L)	7000	5 000	6000 ± 2000	28000
Yield coefficients for the production phase				
Y _{X/S} (g/g)	0.47	0.35	0.41 ± 0.08	0.23
Y _{P/S} (g/g)	0.067	0.064	0.066 ± 0.002	0.022
Y _{P/X} (g/g)	0.14	0.18	0.16 ± 0.03	0.10
Volumetric Xyn10A- productivity (g/Lh)	0.75	0.86	0.81 ± 0.08	0.35

* The medium- contribution (0.91 g/L) is withdrawn from all estimated values, IC = intracellular, EC = extracellular.

Protease analysis

Presence of protease activity was analysed at selected time-intervals during the production phase, to evaluate if presence of host cell proteases could be related to the feeding strategy or the extent of recombinant protein production. A novel semi-quantitative protease analysis was made

with agar-gels containing azocasein (a red-colored protease substrate) shown for the first time in this paper. It was shown that protease activity was present during the post-induction phase in all cultivations, the size of the clearing zones however indicated a higher level at the end of SLFB-cultivations run at 37°C (**Table 4**). This result indicated that the feeding strategy with lower glucose levels available led to higher metabolic stress when the gene encoding the recombinant protein was well expressed.

Table 4. Semiquantitative protease-activity in the soluble fraction detected as clearing zones using samples applied to wells in azo-casein plates. + = detectable clearing zone, ++ = significant clearing zone (0.5-1.5 mm), +++ = large clearing zone (> 2 mm)

Cultivation		Post induction time			
		0h	2h	4h	6h
pTLFB	Ec0320	-	-	+	++
pTLFB	Ec0321	-	-	+/-	+
SLFB	Ec0322	-	+	++	+++
SLFB	Ec0283	-	-	++	ND
SLFB ₂₅	Ec0284	-	ND	+	+
TLFB	Ec0307	-	ND	+	+

- = not detectable, nd = not determined

Released endotoxin

The amount of endotoxin released to the cultivation medium was measured at induction, and in the final sample. Higher amounts of endotoxin were released in the final samples, compared to at induction, but when relating to the biomass produced in the respective cultivation no significant differences could be observed between the two related *E. coli*

strains or between the feed strategies (**Table 5**). The values obtained in the final samples using TLFB-strategies were slightly higher than the corresponding from SLFB-cultivations, but due to the relatively large between-batch variation, the difference between the strategies was too small to be significant.

Table 5. Released endotoxin in the medium at induction and in the final sample of the fed-batch cultivations

Feed strategy/ Enzyme	Cult. code	Cdw (g/L) induction (final)	Endotoxin EU/L (EU/g cdw)		Endotoxin (mg/L)	
			induction	final	induction	final
SLFB ₃₇ / <i>AjCda13</i>	Ec0283	15 (34)	0.50×10^{10} (3×10^8)	$0.9 \times 10^{10*}$ ($3 \times 10^{8*}$)	500	900*
SLFB ₃₇ / <i>RmXyn10A</i>	Ec0322	13 (34)	0.12×10^{10} (1×10^8)	1.4×10^{10} (4×10^8)	120	1 400
SLFB ₂₅ / <i>AjCda13</i>	Ec0284	16 (43)	0.20×10^{10} (1×10^8)	0.7×10^{10} (2×10^8)	200	700
TLFB/ <i>AjCda13</i>	Ec0307	16 (42)	0.20×10^{10} (1×10^8)	2.1×10^{10} (5×10^8)	200	2100
pTLFB/ <i>RmXyn10A</i>	Ec0320/ Ec0321	11 / 12 (42 / 39)	$0.09 \times 10^{10\#}$ ($0.8 \times 10^{8\#}$)	$2.5 \times 10^{10\#}$ ($6 \times 10^{8\#}$)	90 ± 10 [#]	2400 ± 700 [#]

* 4 h production phase, #average from two cultivations

Discussion

In this investigation different feed control strategies were used to produce two types of recombinant glycoside hydrolases (a CDase and a xylanase) of thermophilic origin in *E. coli*. The CDase (GH13) was previously shown to be an aggregation-prone protein, difficult to produce in active soluble form (Turner *et al*, 2005) while the xylanase (GH10) was highly soluble, and previously shown to be well-produced and purified from *E. coli* (Abou-Hachem *et al*, 2003a). In addition, release of endotoxins (which are putative health hazards during *E. coli* cultivations) was monitored during the recombinant protein production phases of all cultivations, to complement the data from a previous investigation (Svensson *et al*, in press) that indicated different release levels as a consequence of feeding strategy in uninduced cultures.

During the production of the CDase, the SLFB-strategy at 37 °C led to a relatively fast production of enzyme, with activity levels after 4 h that were comparable to those obtained after 6 h with the TLFB-strategy. Due to the previous difficulties to produce this enzyme in active soluble form, the inducer levels had been tuned in shake-flasks (Turner *et al*, 2005), prior to the cultivation in the stirred tank reactor in order to optimise the amount IPTG applied. This tuning was found to be cultivation temperature dependent (data not shown) requiring higher inducer amounts for maximised recombinant enzyme activity at lower temperatures, and hence it was difficult to compare expression levels in cultivations run at fixed temperatures (like SLFB) with the TLFB, in which the cultivation temperature varied.

It was also found that a decrease of the cultivation temperature (from 37 to 25 °C), using the SLFB-strategy was not beneficial for the production

of CDase. The CDase activity was significantly lower at 25 °C, despite using a higher amount of the inducer at the apparent optimum concentration (0.1 instead of 0.05 mM), an unexpected result, as inclusion body formation is generally reported to decrease (and the soluble protein fraction to increase) with decreasing cultivation temperature (Chalmers *et al*, 1990).

During xylanase production, no major differences in activity were found using SLFB or pTLFB control strategies during the early production phase (< 2h). However, use of the SLFB-strategy resulted in cell lysis in the later stage of the cultivation, visible when a 6 h production phase was used. A corresponding cell-lysis was not noticed using the TLFB-strategies (where instead production ceased in the late post-induction phase) which lead us to speculate that the cells may be more sensitive to shear forces if grown under a prolonged time in medium with limited amounts of the carbon source glucose available. It was also found that although initially fast, the xylanase activity ceased and even decreased during the late production phase (> 2 h) using the SLFB-strategy. Similar results have previously been obtained by Ramchuran *et al* (2002) using another construct encoding the same xylanase catalytic module, and as in this work, using glucose as the sole carbon source in SLFBs fed with minimal medium. In that case visible levels of protease activity were found using the detection method C-PAGE. Protease activity (determined by an azo-casein plate method) was in this work visible in all the cultivations (independent of feeding strategy), but the level of activity appeared higher in the SLFB-cultivations. However, as reported in Ramchuran *et al* (2002), incubation with protease containing extracts did not reduce the activity of previously produced xylanase, and it was therefore speculated that aggregation might play a role in the reduction of the overall xylanase activity. In the current

investigation, both the soluble and insoluble protein fractions have been analysed by SDS-PAGE, and as no proteins were accumulated in the insoluble fraction, aggregation could be out-ruled. In addition, a previous concern was the effect of the export to the periplasm (Ramchuran *et al*, 2002), but in the current investigation the same trend is seen using intracellularly produced xylanase. Misincorporation of amino acids upon depletion of available amino acid pools as suggested by Tsai *et al*, (1988), is however still a valid explanation that could make the produced polypeptide more prone to protease degradation in the later stages of the production phase, as compared to a purified enzyme and may hence explain the decreased activity levels observed during the late production phase of the SLFB cultivation. In addition, down-regulation of expression as a consequence of sigma-factor competition (Schweder *et al*, 2002) may explain the ceased production observed in all cases (at production phase > 4 h), using both SLFB and TLFB strategies.

E. coli can release endotoxin (pyrogen), in the form of LPS from the outer cell wall (Raetz, 1993), which is highly undesirable, as these endotoxins causes fever if it is introduced in the bloodstream of humans or animals (according to U.S. Food and Drug administration guideline, the upper limit of the pyrogen level is 5.0 EU / kg bodyweight). Authors have previously reported extracted endotoxin levels of approximately 50×10^8 EU/g lyophilised cells using *E. coli* strains JM83 and BMS67C12 grown in LB (Somerville *et al*, 1996), and the endotoxin level released to the whole broth using *E. coli* strain TG1 producing recombinant protein, grown in a fed-batch process on defined medium with glucose as the carbon source using an exponential feed ($\mu_{\text{set}}=0.12 \text{ h}^{-1}$), was approximately 0.1×10^{10} EU/L (or 0.1×10^8 EU/g cdw) (Garke *et al*, 2000, Seeger *et al*, 1995)

corresponding to 100 mg endotoxin/L. The *E. coli* strains BL21(DE3), and Tuner(DE3), used in this investigation were found to release endotoxin in the range $0.7\text{-}2.5 \times 10^{10}$ EU/L (or $2\text{-}6 \times 10^8$ EU/g cdw) at the end of the production phase, which is one order of magnitude higher than reported for strain TG1, but in the same range as those reported by Svensson *et al* (in press), using uninduced cultures of *E. coli* strains W3110, and BL21(DE3) grown applying the SLFB-technique (0.85×10^{10} EU/L or 850 mg/L). In the work of Svensson *et al* (in press) uninduced cultures of the same strain released 1-2 orders of magnitude lower levels of endotoxin (20 mg/L) if the TLFB-technique was applied. No significant differences in endotoxin release could however be found between the feeding strategies in the induced cultures of the current investigation (**Table 5**), contradicting the results from the uninduced cultures by Svensson *et al*, (in press). The lack of difference between feeding strategies in the current investigation could be due to the presence of antibiotics as a selection pressure to keep the vector, which previously has been shown to promote endotoxin release in enteric bacteria (Prins *et al*, 1994), and could have increased the endotoxin release in the TLFB-cultures (as seen at the point of induction where batch variation appeared larger than variation between strategies). Another reason for the higher level of released endotoxin could be the induction of recombinant protein, which increased the metabolic stress on the cells and led to overall (slightly) higher release of endotoxin at the end of the cultivations than reported by Svensson *et al* (in press), especially using TLFB-strategies. The metabolic stress could also be monitored by the presence of clearly detectable protease activity in all cultures (although to different degrees) at the end of the production phase (**Table 4**). In accordance with the previous investigations (Svensson *et al*, in press; Han *et*

al., 2003) was the fact that release of endotoxin increased dramatically towards the end of the cultivation (comparing the level at induction with the level released at the end of the production phase).

In conclusion our results show that in terms of production of active recombinant enzyme, the type of control-strategy applied (SLFB, TLFB or pTLFB) appeared to be of minor importance for the activity obtained in the initial production phase (0 - 2 h), but as a general conclusion the TLFB and pTLFB-strategies appeared to be beneficial when a longer production-phase (> 3 h) was applied. The released endotoxin levels monitored in this investigation could not be concluded to vary significantly as a consequence of the feeding strategy applied. Higher amount of endotoxin were however always released per produced cell mass at the end of the production-phase compared to at the time of induction.

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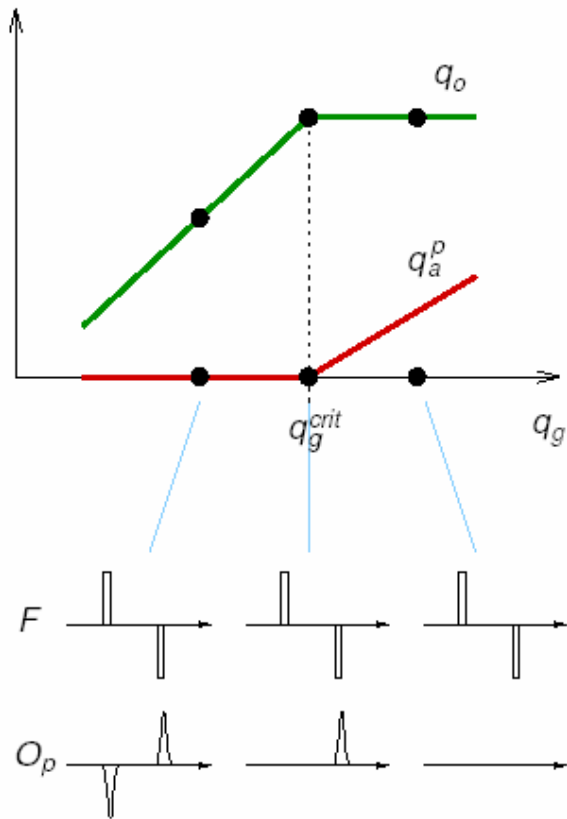


Figure 1. Schematic diagram showing the relations between specific glucose uptake, q_g , specific oxygen uptake, q_o , and specific acetate production, q_a^p . Below: The cellular response (O_p measured by the dissolved oxygen sensor) to up and down pulses in the feed rate (F), without acetate formation (left), at q_g^{crit} , the onset of acetate formation (middle), and above the onset of acetate formation (right).

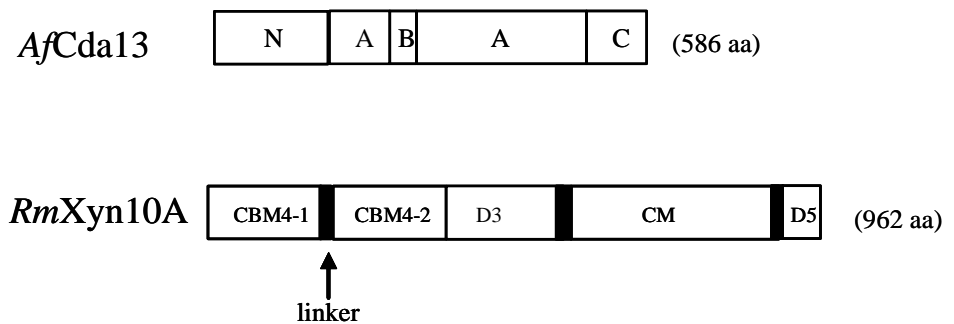


Figure 2. Domain organisation of *AfCda13* and *RmXyn10A*.

AfCda13 consists of 4 domains linked together via loop-regions: an N-terminal domain (N, 118 residues), a catalytic module (GH13, 375 residues) composed of two domains [A ((β/α)₈-barrel) and B protruding from this], and finally domain C (C, 80 residues). In *RmXyn10A*, two N-terminal tandemly repeated family 4 carbohydrate binding modules (CBM4-1 and 4-2, each comprising 165 residues), are followed by a domain of unknown function (D3, 189 residues), a catalytic module belonging to GH family 10 (320 residues) and finally a small domain (D5, 84 residues) with a likely function in cell-attachment.

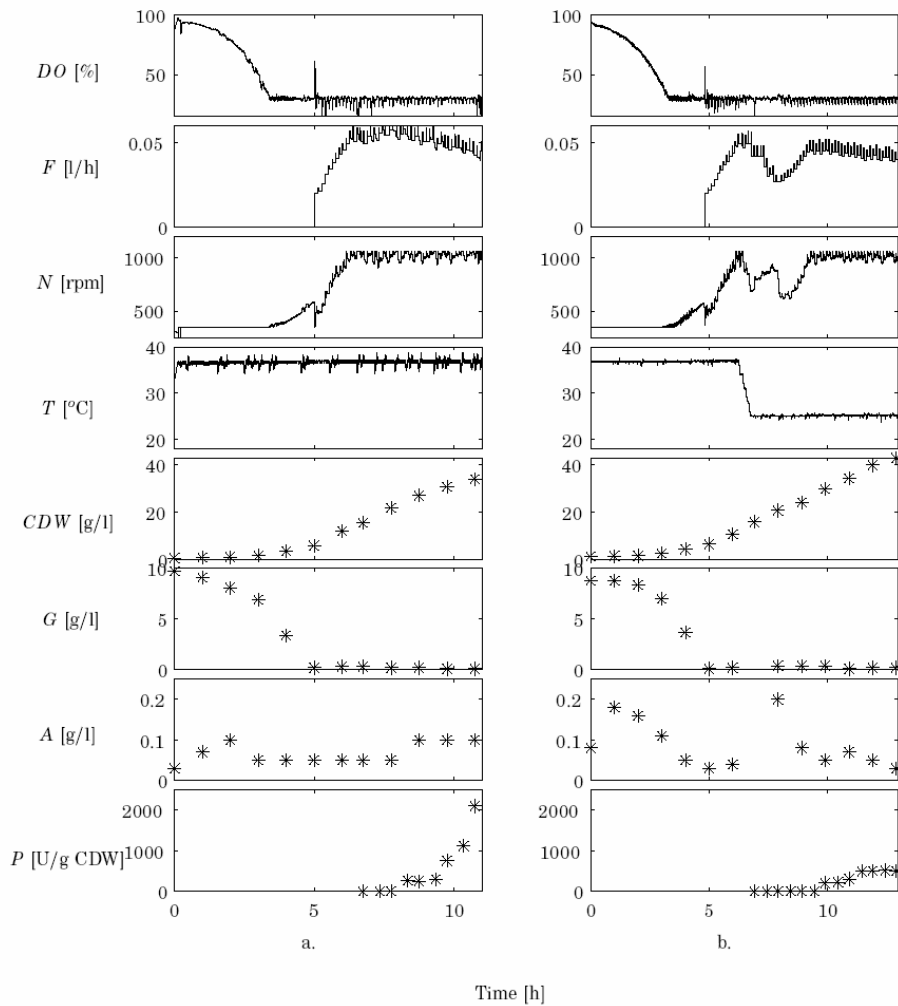


Figure 3. Substrate Limited Fed Batch cultivations (SLFB), with adjustments of the feed-rate by *up-pulses* with production phase temperature at 37 °C (a) and 25 °C (b) for CDase production. It can be noted that during the temperature-decrease to 25 °C, this cultivation is not substrate-limited (as shown by the acetic acid data (A)). The logged parameters are displayed as follows: response in dissolved oxygen (DO), feed profile (F), stirrer speed (N) and temperature (T). Variables measured off-line are biomass (displayed as cell dry weight (CDW)), glucose (G), acetate (A) and intracellular activity (P) of the recombinant CDase.

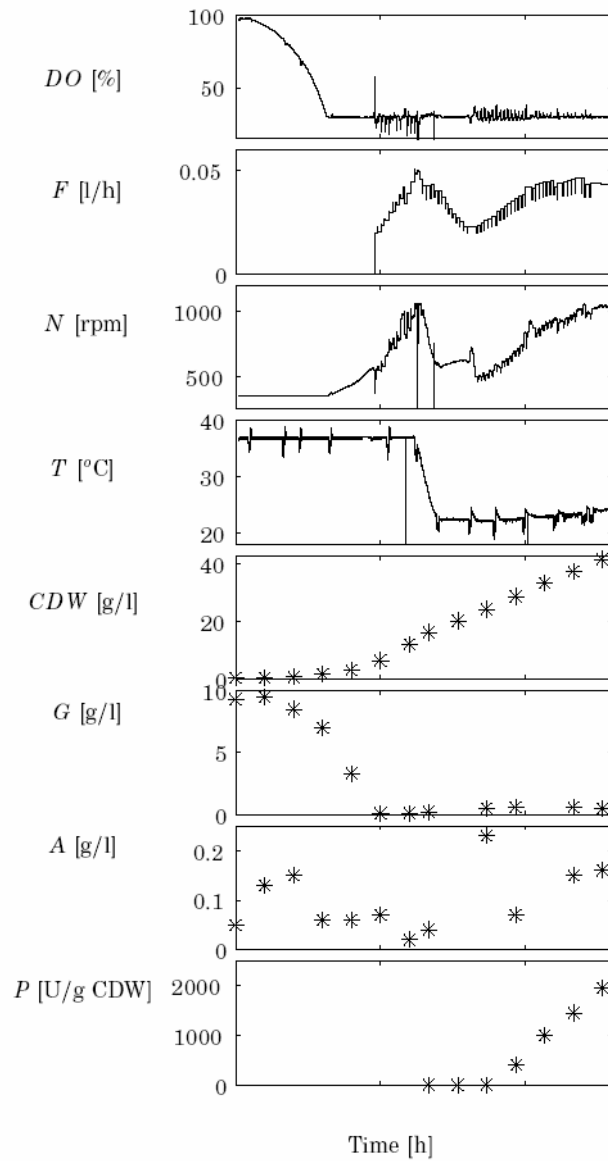


Figure 4. Temperature limited fed-batch cultivation (TLFB) with production of CDase, using adjustments of the feed rate after monitoring the response to *down pulses* in the feed rate. The logged parameters are displayed as follows: response in dissolved oxygen (DO), feed profile (F), stirrer speed (N) and temperature (T). Variables measured off-line are biomass (displayed as cell dry weight (CDW)), glucose (G), acetate (A) and intracellular activity (P) of the recombinant CDase.

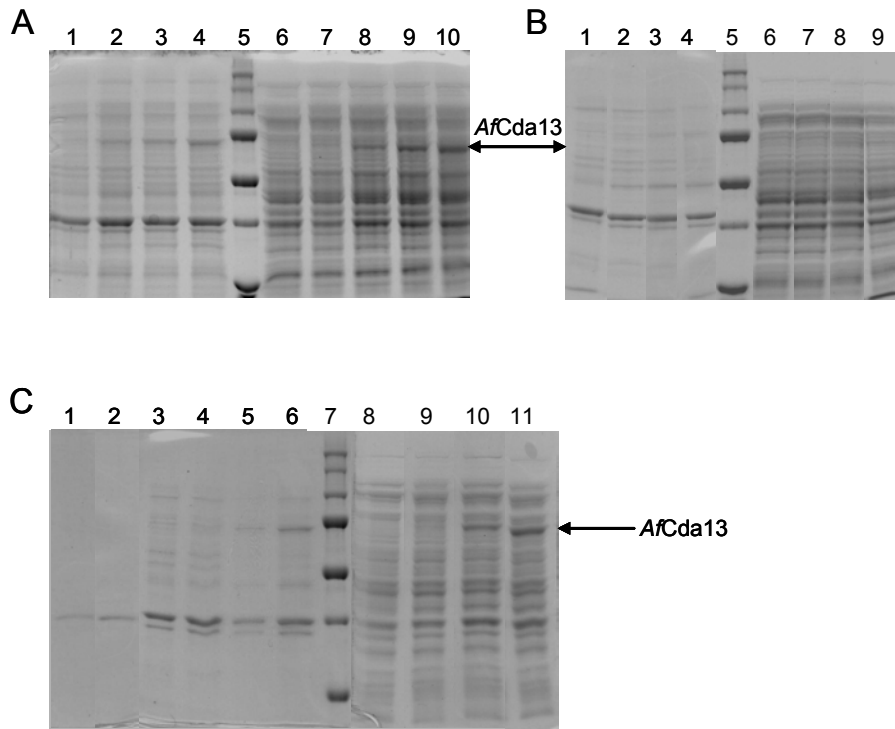


Figure 5. SDS-PAGE showing the production of *AfCda13* (indicated by an arrow) in SLFB cultivations at 37 °C **A**, 25 °C **B** and in an TLFB cultivaton **C**. **a** Lanes 1-4 insoluble protein 0, 2, 3 and 4 h post induction, lane 5 standard Precision Plus (BioRad), lanes 6-10 soluble protein 0, 1, 2, 3 and 4 h post induction. **b** Lanes 1-4 insoluble protein 0, 2, 4 and 6 h post induction, lane 5 standard Precision Plus, lanes 6-9 soluble protein 0, 2, 4 and 6 h post induction. **c** lanes 1-2 medium fraction 0 and 6 h post induction, lanes 3-6 insoluble protein 0, 2, 4 and 6 h post induction, lane 7 standard Precision Plus, lanes 8-11 soluble protein 0, 2, 4 and 6 h post induction.

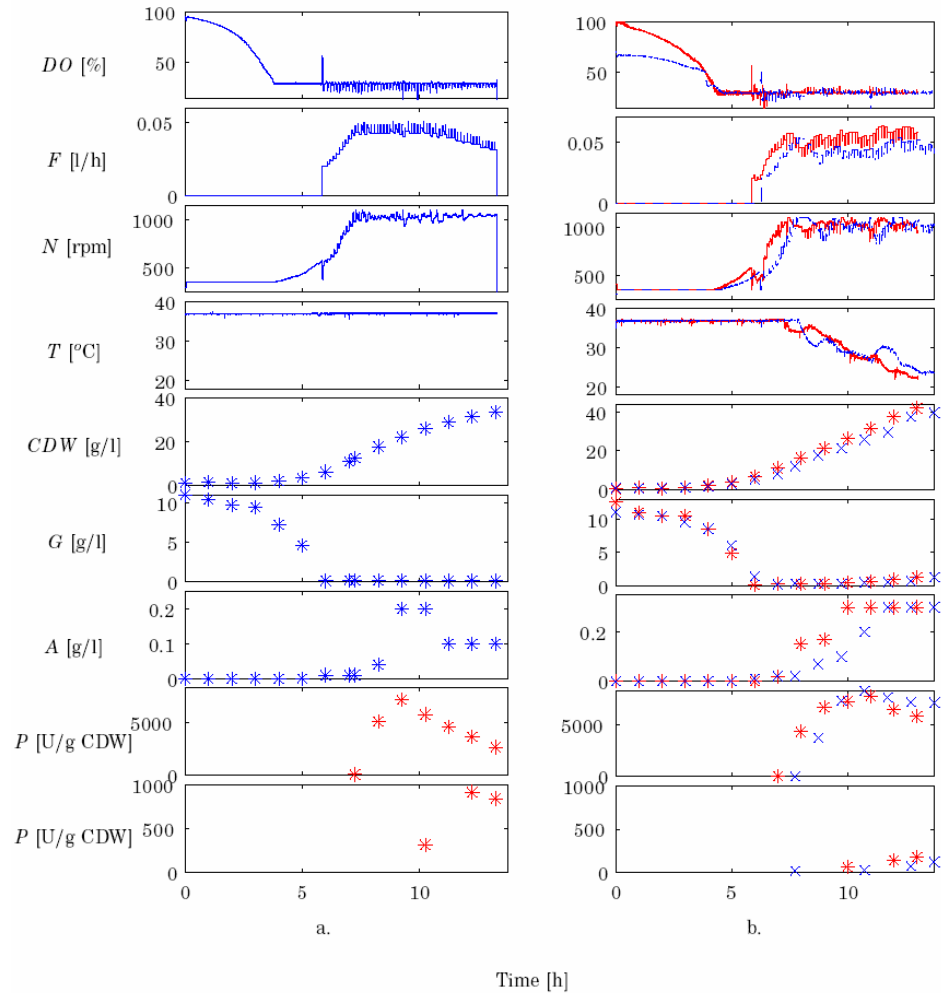


Figure 6. Substrate limited Fed Batch (SLFB, a) and probed Temperature Limited Fed Batch (pTLFB, b) producing xylanase. In the (SLFB), the feed-rate is adjusted after evaluating the response to *up-pulses* in the feed-rate. In the pTLFB, the feed rate is adjusted by *up-pulses* in the feed-rate until maximum stirrer speed is reached, and thereafter (during the temperature-limited phase) the feed-rate is adjusted after *down-pulses* in the feed-rate. The logged parameters are displayed as follows: response in dissolved oxygen (DO), feed profile (F), stirrer speed (N) and temperature (T). Variables measured off-line are biomass (displayed as cell dry weight [CDW]), glucose (G), acetate (A), intracellular (P above) and extracellular activity (P below) of the recombinant xylanase.

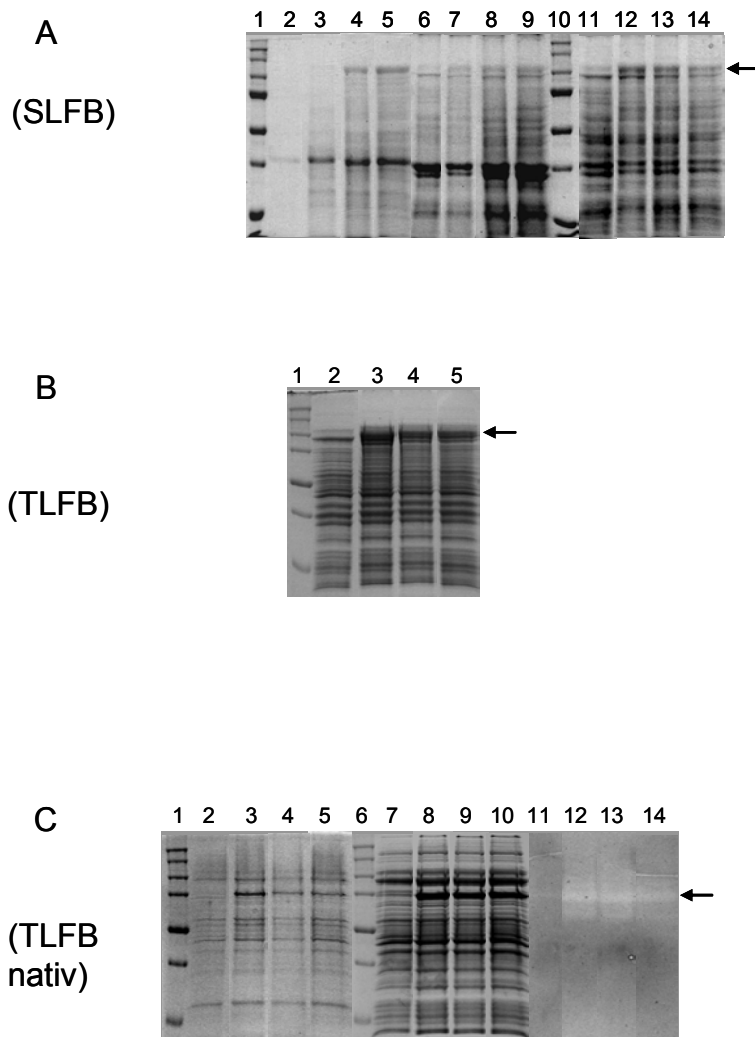


Figure 7. SDS-PAGE and activity staining showing the production of *RmXyn10A* (indicated by an arrow) in an SLFB cultivation **a** and a pTLFB cultivation (**b** samples boiled, **c** samples unboiled). **a** Lanes 1, 10 standard Precision Plus (BioRad), lanes 2-5 medium fraction 0, 2, 4 and 6 h, lanes 6-9 insoluble protein 0, 2, 4 and 6 h post induction, lanes 11-14 soluble protein 0, 2, 4 and 6 h post induction. **b** Lane 1 standard Precision Plus, lanes 2-5 soluble protein. **c** Lanes 1, 6 standard Precision Plus, lanes 2-5 insoluble protein 0, 2, 4 and 6 h post induction, lanes 7-10 soluble protein 0, 2, 4 and 6 h post induction, lanes 11-14 zymogram soluble protein 0, 2, 4 and 6 h post induction.