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## **Bench-Scale Production of Heterologous Proteins from Extremophiles- Escherichia coli and Pichia pastoris based expression systems**

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**The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase from the bacterium *Rhodothermus marinus***

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**Abstract**

A thermostable glycoside hydrolase family 10 xylanase originating from *Rhodothermus marinus* was cloned and expressed in the methylotrophic yeast *Pichia pastoris* (SMD1168H). The DNA sequence from *Rmxyn 10A* encoding the xylanase catalytic module was PCR-amplified and cloned in frame with the *Saccharomyces cerevisiae*  $\alpha$ -factor secretion signal under the control of the alcohol oxidase (*AOX1*) promoter. Optimisation of enzyme production in batch fermentors with methanol as a sole carbon source, enabled secretion yields up to 3 grams xylanase per litre with a maximum activity of 3130 U/L to be achieved. N-terminal sequence analysis of the heterologous xylanase indicated that the secretion signal was correctly processed in *P. pastoris* and the molecular weight of 37 kDa was in agreement with the theoretically calculated molecular mass. Introduction of a heat-pretreatment step was however necessary in order to fold the heterologous xylanase to an active state, and at the conditions used this step yielded a 200-fold increase in xylanase activity. Thermostability of the produced xylanase was monitored by differential scanning calorimetry, and the transition temperature ( $T_m$ ) was 78 °C. *R. marinus* xylanase is the first reported thermostable gram-negative bacterial xylanase efficiently secreted by *P. pastoris*.

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**Keywords:** *Rhodothermus marinus*, bacterial xylanase, family 10 glycoside hydrolases, heterologous expression

## 1. Introduction

Xylanases (EC 3.2.1.8.) are glycoside hydrolases that catalyse hydrolysis of xylan, one of the main components of hemicellulose of wood and plant cell walls [1-3]. These enzymes are classified among the glycoside hydrolases (GH), a widespread group of enzymes that have the ability to hydrolyse the glycosidic bond between two carbohydrate moieties or between a carbohydrate and a non-carbohydrate moiety [4]. The catalytic module of xylanases are divided into two families of GH: 10 and 11 [5]. GH 10 enzymes have TIM-barrel folded catalytic module in the molecular weight range of 40-50 kDa [1,6,7], while xylanases of GH 11 are in principle consisting of two (antiparallel)  $\beta$ -pleated sheets [8] with molecular weights in the range 20-30 kDa [1,9-11].

Xylanases have proven useful when selective removal of hemicellulose is required for production of certain materials, or in processes where environmental concerns demand alternatives. Examples of such processes are found in food and feed industries and in pulp and paper industry [1,12-18]. To facilitate the application demand for xylanase, efficient heterologous expression systems capable of producing large amounts of secreted protein with an organism that can be grown to industrial scale is pivotal.

The *Pichia pastoris* expression systems are increasingly used for production of a variety of heterologous proteins due this methylotrophic yeast possessing a number of attributes which has rendered it as an attractive expression host [19]. Moreover, expression is under the control of the efficient and highly regulated promotor of the alcohol oxidase gene (*AOX 1*) and secretion of the protein of interest in to the cultivation medium is possible [20-22]. In addition *P. pastoris* is generally regarded as safe (GRAS), and has an added advantage for recombinant xylanase

production because this organism does not secrete any endogenous xylanase into the cultivation medium [23]. Despite this, only few reports on successful xylanase production in *Pichia* are thus far published [23-25]. The majority of those concerns GH11 xylanases of fungal origin, and include production of a thermostable xylanase from *Thermomyces* [26].

The presence of several xylanolytic enzymes has been reported in *Rhodothermus marinus* [27,28]. This thermophilic bacterium was first isolated from submarine hot springs in Iceland [29] and is phylogenetically (16S RNA sequence) closely related to the *Flexibacter-Cytophaga-Bacteroides* group [30]. The cloning and sequencing of the *xyn10A* gene coding for a thermostable multidomain xylanase from *R. marinus* has previously been reported [7]. This work reports the successful cloning and expression of the catalytic domain from the *xyn10A* gene of *R. marinus* in *P. pastoris*. To our knowledge this is the first thermostable GH10 xylanase, and also the first xylanase of Gram-negative bacterial origin to be successfully produced in *P. pastoris*.

## 2. Materials and Methods

### 2.1 Yeast strain, plasmid construction and cloning

*P. pastoris* expression vector pPICZ $\alpha$ B as well as yeast strain SMD1168H were from Invitrogen (San Diego, CA). Expand High Fidelity DNA polymerase was purchased from Roche Diagnostics (Mannheim, Germany) and restriction enzymes were from New England Biolabs Ltd. (Beverly, MA) and used according to manufacturers recommendation. Oligonucleotides were synthesised by MWG Biotech Scandinavia A/S (Denmark).

### 2.1.1 Construction of Yeast Expression Vector

A purified vector-construct comprising the gene encoding the full-length multidomain xylanase *xyn10A* cloned in pET22b [31] was used as a template in the PCR amplification of the sequence encoding the catalytic module of *xyn10A*. Forward (F) and reverse (R) primers were designed (see below, with the gene specific sequence in capital letters), introducing cleavage sites for *Pst*I and *Xba*I (underlined), respectively, and including a sequence coding for a hexa-histidine-tag and a stop codon in the reverse primer (figure 1):

(F)5'agctggccgactgcaggaCTTGCGGCTGATGTGGATAAG 3'

(R)5'agtgataattctagatcaatgatgatgatgatgGTTGGCGCTCAGGTACGAC  
TC 3'.

After DNA amplification the resulting PCR product was purified using the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, CA). The PCR product and plasmid pPICZ $\alpha$ B were cut with restriction enzymes *Pst*I and *Xba*I for 4.5 h and ligated using T4 DNA ligase (Invitrogen Life Technologies, Frederick, MD) at room temperature for 12 h followed by incubation at 4°C for 3 h. The resulting plasmid [pSRX1] was transformed into *Escherichia coli* NovaBlue (Novagen, Madison, WI) by electroporation (Gene Pulser II. Bio-Rad, Hercules, CA) and selected on LB plates (1% Tryptone, 1% NaCl, 5% Yeast extract, 1.2% Agar) containing 25  $\mu$ g/mL Zeocin. Transformants were selected and screened by direct PCR using the primers above. Positive transformants were grown in 100 mL liquid LB containing zeocin (25  $\mu$ g/mL) for 12 h and the recombinant plasmid [pSRX1] was isolated using a Qiagen column (Mini-Prep kit, Qiagen) and sequenced by dideoxy chain-termination method using ABI Prism BigDye Terminator Cycle Sequencing kit 3.0 (Applied Biosystems, Foster City, CA)

to confirm the *xyn10ACM* sequence, in frame with  $\alpha$ -factor secretion signal and thereby including the Kex2 cleavage site.

### 2.1.2 Transformation, selection and analysis of *P. pastoris* clones

Plasmid [pSRX1] was linearized with *SacI*, followed by transformation by electroporation according to the Invitrogen manual with the following modifications. A 15  $\mu$ g amount of linearized DNA was transformed into 60  $\mu$ L electro-competent *P. pastoris* strain SMD1168H (protease deficient strain) using Gene Pulser II (Bio-Rad, Hercules, CA) with the following settings: T (2.5 kV), C (50  $\mu$ F), R (200  $\Omega$ ). Transformed colonies were selected on YPDS plates (1% Yeast extract, 2% peptone, 2% Glucose, 1 M Sorbitol, 1.2% Agar) containing 100  $\mu$ g/mL zeocin. Genomic DNA extraction of selected clones was conducted according to a procedure outlined in [32]. Screening for positive *xyn10ACM* inserts by PCR was carried out using total genomic DNA and the primers listed above, and one positive clone was selected for expression studies.

## 2.2 Expression optimisation

### 2.2.1 Shake flask experiments of *P. pastoris* expressing xylanase

In order to ascertain the optimum growth and xylanase expression media different media formulations were evaluated (Table 1). Mid-log cultures (1 mL), in 20% glycerol (stored at -80 °C), containing pSRX1, was used to inoculate the flasks containing 100 mL of the growth phase medium. The flasks were subsequently incubated at 28 °C at 200 rpm on a rotary shaker (Innova 4400, New Brunswick Scientific) to  $A_{600}$  of 4-5, harvested by centrifugation at 3000 rpm (Beckman Spinchron centrifuge) for 15 min and resuspended in 150 mL of the corresponding induction

phase medium and incubated as above. Methanol (0.05% v/v) was added every 24 h. Induction proceeded for 5 days and sample aliquots (1 mL) were taken, centrifuged at 13000 rpm (Biofuge A, Heraeus Sepatech) for 5 min, and both pellet and supernatants were stored (-20°C) until further analysis.

### 2.2.2 *Batch Induced Fermentations*

Batch fermentations were performed to evaluate protein expression in a more controlled environment with regard to pH and dissolved oxygen. The inoculum was prepared using 100 mL YPD media in a 1 L baffled Erlenmeyer flask. Mid-log cultures (1 mL), in 20% glycerol (stored at -80 °C), of the xylanase producing *P. pastoris* clone, was used to inoculate the flasks which were subsequently incubated at 30 °C at 200 rpm on a rotary, water bath shaker (Heto, Allerod, Denmark). After 20 h (late exponential phase of growth) 0.1% methanol was added to the flasks and the cells were grown for a further 12 h. Cells were then harvested by centrifugation (3000 rpm, 15 min) and the resulting pellet was resuspended in 150 mL of BMMY or YPTM medium to facilitate transfer into the fermentor. Cultivations were performed using a 3 L fermentor (Belach Biotek AB, Stockholm, Sweden) with a medium (BMMY or YPTM) volume of 2 L. Yeast extract, peptone and yeast nitrogen base were sterilised in the vessel at 121 °C for 45 min thereafter, sterile phosphate buffer or TSB, biotin and methanol were aseptically added (**Table 1**). Cultivation temperature was controlled at 28 °C and the pH was maintained at 6.0 by titration with 6.7 M aqueous ammonia and 0.5 M H<sub>2</sub>SO<sub>4</sub>. Aeration rate was set at 2 L/min. Dissolved oxygen concentration was measured using a polarographic

electrode calibrated to 100% at 800 rpm at 28 °C. The DO was automatically controlled at 35% saturation using stirrer speed.

**Table 1:** Media compositions for shake flasks and “Batch Induced” cultivations.

| Component           | Growth Media (mL/L) |     |      |      | Induction Media (mL/L) |     |                   |                   |
|---------------------|---------------------|-----|------|------|------------------------|-----|-------------------|-------------------|
|                     | MMG                 | BMG | BMGY | YPTG | MMM                    | BMM | BMMY <sup>d</sup> | YPTM <sup>d</sup> |
| Buffer <sup>a</sup> |                     | 100 | 100  |      |                        | 100 | 100               |                   |
| YNB <sup>b</sup>    | 100                 | 100 | 100  |      | 100                    | 100 | 100               |                   |
| Biotin (0.02%)      | 2                   | 2   | 2    | 2    | 2                      | 2   | 2                 | 2                 |
| 10% Yeast extract   |                     |     | 50   | 50   |                        |     | 50                | 50                |
| 20% Peptone         |                     |     | 50   | 100  |                        |     | 50                | 100               |
| 10% Glycerol        | 100                 | 100 | 100  | 100  |                        |     |                   |                   |
| 5% Methanol         |                     |     |      |      | 100                    | 100 | 100               | 100               |
| 2% TSB <sup>c</sup> |                     |     |      | 20   |                        |     |                   | 20                |
| H <sub>2</sub> O    | 798                 | 698 | 598  | 728  | 798                    | 698 | 598               | 728               |

<sup>a</sup>1 M potassium phosphate buffer, pH 6.5

<sup>b</sup>Yeast nitrogen base with ammonium sulphate and without amino acids (1.34%)

<sup>c</sup>Tryptone Soy Broth

<sup>d</sup>For “Batch Induced Cultivations” a final methanol concentration of 0.5% as well as 5 mL/L PTM<sub>i</sub> trace metal solution [51] was used and the added H<sub>2</sub>O volume was reduced by 5 mL.

## 2.3 Analytical Methods

### 2.3.1 Optical density

The OD was determined at 600 nm. Samples were appropriately diluted with 0.9% (w/v) NaCl at OD values exceeding 0.5.

### 2.3.2 Cell dry weight determination

Cell dry weight (CDW) was determined after centrifuging (1400 × g, 10 min, at room temperature) triplicate samples (3 mL) in pre-weighed glass tubes then dried overnight (105 °C) and subsequently weighed to determine CDW.



### 2.3.3 *Enzyme activities*

Prior to measuring heterologous xylanase activity, samples were heat treated at 70°C (30 min) cooled on ice (10 min) and centrifuged (13000 rpm, 5 min). The supernatants were then transferred to new eppendorf tubes for activity determinations.

Qualitative xylanase activity was determined by a halo plate assay containing 2% (w/v) agar and 0.1% (w/v) azo-xylan (Megazyme, Ireland). Samples (80 µL) were loaded into wells and the plate was incubated at 65°C for 40 min. Thereafter, stained in 1% (w/v) Congo Red solution and destained with 1 M NaCl.

Quantitative xylanase activity [extra-cellular] was determined using the DNS (3,5-dinitrosalicylic acid) method [33]. Xylanase activity was determined under the conditions described by [34]. Xylose was used as standard (2-10 µmol/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. 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.

Alcohol oxidase was extracted from 1 mL pellet samples taken during the fermentation by treatment with Yeast Protein Extraction Reagent (Y-PER) according to manufacturers instructions (Pierce, Rockford, IL). Activity was determined using a Sigma quality control test protocol (Enzymatic Assay of Alcohol Oxidase EC 1.1.3.13, Sigma) with the following modifications. Reagent F was replaced by the supernatant obtained after the Y-PER treatment mentioned above. Supernatant samples were diluted in Reagent A and analysis was performed in triplicate.

Alcohol oxidase activity was expressed in U/mL (One unit corresponds to the amount of enzyme which oxidises 1.0  $\mu$ mol methanol to formaldehyde per minute at pH 7.5 at 25°C).

#### 2.3.4 *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. Measured contribution of the peptide rich medium components to total protein concentration was 2.3 g/L and was subtracted from the values obtained.

#### 2.3.5 *Deglycosylation, Removal of His-tag and Deglutathionylation*

A deglycosylation reaction was carried out on the produced xylanase using Endoglycosidase H and PNGase F according to the manufacturers instructions (New England Biolabs Ltd, Beverly, MA) however, the incubation time was extended to 12 h. Samples were analysed by SDS-PAGE and activity was determined by the agarose over-layer gel method listed below.

The His-tag was removed by treatment with carboxypeptidase-A type II from bovine pancreas (Sigma), which causes the successive cleavage of amino acids from the C-terminus. Two sets of reaction mixtures were set-up consisting of the supernatant samples (before heat treatment) containing the heterologous xylanase (protein concentration 2 mg/mL) and carboxypeptidase-A (50:1, 25:1, 10:1 and 5:1) in 0.05M Tris-HCl pH 7.5. One sample set, was incubated at room temperature while the other set was incubated at 37°C for 1.5 h. For the deglutathionylation study, the reaction mixture was set-up as follows: 10  $\mu$ L of supernatant sample (before heat treatment containing 2 mg/mL Protein) + 10  $\mu$ L (1 M Tris-HCl pH 9) +

10  $\mu$ L (1 M DTT) + 70  $\mu$ L (H<sub>2</sub>O). This was incubated at room temperature for 2 h after which dialysis was performed in 0.02 M Tris-HCl pH7.5 + 0.01 M DTT buffer for 24 h. A second dialysis was then performed for 24 h in 0.02 M Tris-HCl pH 7.5 buffer without DTT. After completion of the carboxypeptidase and deglutathionylation reactions, quantitative heterologous xylanase activity was measured using the DNS-method mentioned above to determine the influence on enzyme activity.

As a positive control, a portion of the same sample was heat-treated according to the procedure listed above, and included in the activity measurement.

### 2.3.6 *Electrophoresis and N-terminal sequencing*

Protein production was analysed by the sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) according to [35] and stained with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany). Enzyme activity was detected by an over-layer agarose 1% (w/v) gel, containing 0.05% (w/v) birch xylan (Carl Roth, Germany). The SDS-PAGE gel was washed with 20 mM Tris-HCl pH 7.5 containing 2.5% Triton X-100 (Merck) for 30 min. Thereafter, the gel was washed with 20 mM Tris-HCl pH 7.5 covered with the overlayer gel, and incubated for 30 min at 65°C. The agarose gel was stained in 1% (w/v) Congo Red solution and destained with 1 M NaCl. Densitometric measurements were performed using a Gel Doc 2000 system (Bio-Rad).

Protein samples for N-terminal sequencing were separated electrophoretically as above, and subsequently transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA) by semi-dry electroblotting using Sartoblot IIS electroblotting unit (Sartorius, Göttingen, Germany). The membrane was stained with Coomassie Brilliant

Blue and the protein bands were sequenced by Edman degradation at a commercial laboratory (Karolinska Institutet, Stockholm, Sweden).

### 2.3.8 *Differential scanning calorimetry (DSC)*

DSC measurements were performed on a differential scanning calorimeter (VP-DSC, MicroCal Inc., Northampton, MA) with a cell volume of 0.5 mL. A scanning rate of 1°C min<sup>-1</sup> was used and the temperature range was set between 25 to 120°C. Prior to the measurements the samples were degassed for 15 min at room temperature. Baseline scans, collected with buffer in the reference and sample cells, were subtracted from sample scans. Protein concentrations were 0.5 mg/mL, and samples were dialysed in 20 mM Tris-HCl pH 7.5 prior to scanning. The reversibility of thermal transitions was judged from the reproducibility of the calorimetric traces upon immediate cooling and rescanning.

## 3. Results

### 3.1 *Construction of expression vector, selection of clones and expression in shake flasks*

The PCR-amplified DNA sequence encoding the catalytic domain of Xyn10A from *Rhodothermus marinus* was cloned in frame with the secretion signal (*S. cerevisiae*  $\alpha$ -factor) into the expression vector pPICZ $\alpha$ B under the control of the *AOX1* promoter (figure 1). The resulting expression plasmid was transformed into *P. pastoris* (SMD1168H) by electroporation and spreading 200  $\mu$ L of the transformed cells onto YPDS/zeocin-plates, resulted in approximately 300-350 transformants per plate after a 57 h incubation at 30°C. Total genomic DNA extraction of 5 randomly selected colonies (from the original transformants), followed by screening for the *xyn10A* insert (encoding Xyn 10ACM) by PCR resulted in

4 positive clones of which one was selected for expression studies in shake flasks.

The most promising condition for efficient secretion of heterologous thermostable xylanase was initially assessed in shake flask cultivations employing four different media formulations (Table 1). Absence of endogenous xylanases was also confirmed using plasmid-free *P. pastoris* strain SMD1168H, in the same media.

In all cases, xylanase activity could only be detected after the supernatant samples had undergone a heat pre-treatment step (70°C, 30 min followed by cooling on ice, 10 min). No expression of xylanase was observed during the pre-induction phase when glycerol was used as the sole carbon source (figure 2). Also, during the post-induction phase employing methanol as the sole carbon source the secretion of xylanase was undetected in minimal media (MM). However, moderate amounts of xylanase activity were observed when a buffered minimal media was employed (BMM). Furthermore, additions of peptide-rich supplements [such as yeast extract (BMMY) or tryptone soy broth and peptone (YPTM)] to the expression media, resulted in efficient secretion of thermostable xylanase when methanol was used a sole carbon source.

### 3.2 *Expression optimisation in Batch Induced Fermentations*

Xylanase expression was optimised in a stirred tank bio-reactor which provided a more stable environment in terms of controlled pH and increased aeration as compared to shake flasks. Results obtained from the media formulations in shake flask experiments suggested that BMMY and YPTM would be ideal media for evaluation of xylanase activity.

A series of “batch induced cultivations” were hence performed using two alternative strategies in the two above mentioned media. In the first

strategy identical 100 mL pre-induction cultures or inocula, were grown in shake flasks with glycerol as a sole carbon source. These cells were grown to late exponential phase (approximately 20 h) before transfer into the fermentor, in which the sole carbon source was methanol. However, a lag phase of approximately 18-23 h was then observed in the bioreactor and heterologous xylanase was first detected 20 h after the start of the cultivation (data not shown).

An alternate strategy for the pre-induction phase or inoculum preparation was then investigated with the focus geared not only towards the establishment of a suitable cell density but also towards transcription of the *AOX1*-promoter controlled genes (endogenous alcohol oxidase and heterologous xylanase). This involved the addition of methanol during the late exponential phase of growth in the shake-flask and cultivation for a further 12 h. Using this strategy, no apparent lag phase was observed upon inoculation into the fermentor (in which methanol was the sole carbon source) indicated by an immediate decrease in the dissolved oxygen (DO) concentration. A DO concentration of 35% was attained 15 h after inoculum transfer and was maintained due to a concomitant increase in the stirrer speed until the end of the cultivation (figure 3A). Also, the total cultivation time was reduced to approximately 41 h as compared to 65 h (data not shown) when the previous inoculum strategy stated above was used. No significant difference in the growth rates or cell dry weight titers (13.3 g/L and 12.5 g/L, figure 3B) was noted when media formulations YPTM and BMMY was employed respectively. Thus, based on this finding and the similar xylanase production profiles for the two media in shake flasks, samples were selected from the cultivation in YPTM medium and were analysed for alcohol oxidase and heterologous xylanase production.

Alcohol oxidase activity was detected throughout the cultivation, but a decrease in approximately 50% was observed during the latter stages (35 - 41 h) of the cultivation (figure 3). A plausible explanation could be low residual methanol concentrations at this stage due to its uptake as a carbon source during growth and metabolism hence, decreasing the *AOX 1* transcription rate. The *AOX 1* gene is under the control of both a general repression/derepression mechanism and a methanol specific induction mechanism. Furthermore, in carbon-starved *P. pastoris* cultivations the *AOX 1* transcription and protein levels reach only 2% of those observed in methanol-grown cells [36].

In terms of heterologous thermostable xylanase production, supernatant samples (heat-pre-treated & cooled) were evaluated by SDS-PAGE and over-layer gels for the presence of extracellular enzyme activity. Judging from the gel presented in figure 4A, a single band of (approximately) 37 kDa corresponding well to the theoretically calculated molecular mass of the cloned secreted xylanase (37.4 kDa, after cleavage at the Kex2-site) was visible 4 hours after inoculation. Also, from densitometric analysis an incremental increase in band intensity proportional to an increase in cultivation time was observed. The 37 kDa band was confirmed as heterologous thermostable xylanase by the over-layer gel (figure 4B), which showed visible zones of clearing indicative of xylan degradation (after incubation at 65 °C) at a position corresponding to the band of the expected molecular mass. Also, an increase in xylanase activity was visible as the cultivation proceeded corresponding to the increase in band intensity observed by densitometry (figure 4A), after which it was estimated that heterologous xylanase constituted approximately 70 % of the total extracellular protein at the end of the cultivation. It should be noted that samples run without heat pre-treatment

and cooling prior to SDS-PAGE separation showed a similar banding pattern of the 37 kDa protein, although in this case no activity could be observed by the overlayer technique. N-terminal sequencing of the 5 first amino acids of the protein-band (Gly-Leu-Ala-Ala-Asp) observed both before and after the heat pre-treatment procedure proved that this band was indeed the heterologous xylanase. It also showed that in both cases the secretion-tag was removed at the Kex2-site, and that the protein was further processed by the *STE13*-gene product, to remove the Glu-Ala repeats, leaving a single Gly-residue introduced in the cloning-design before the start of the Xyn10ACM-sequence as the first identifiable residue (figure 1). Using Gly as the first residue, the theoretically calculated molecular weight was 37.4 kDa.

### 3.3 *Quantification of xylanase activity*

Initial experiments to quantify the activity of the produced heterologous xylanase before heat pre-treatment using the DNS method showed a maximum extracellular enzyme activity of only approximately 3 U/g extracellular protein. Variations in the incubation time (up to 35 h) and/or temperature (in a range 50-95 °C) during the activity assay only resulted in insignificant variations (data not shown). This showed that neither a fixed high temperature nor prolonged incubations at a fixed temperature in the assay affected activity, and indicated the protein to be produced in an inactive form.

However, with the introduction of the heat pre-treatment and cooling step (70°C, 30 min + 0°C, 10 min) of the culture supernatants prior to enzyme activity analysis (65°C for 5 min) the heterologous xylanase regained activity, indicating refolding into an active conformation. The



enzyme activity profiles showed an approximately 200-fold increase in enzyme activity after heat pre-treatment of supernatant samples from the cultivation employing the two media formulations BMMY and YPTM, respectively. Further evaluation of enzyme production profiles between the two media formulations showed the maximum xylanase activity (maximum 3130 U/L) to be 11% lower when BMMY was used as an induction media. The highest specific xylanase activity achieved was 1050 U/g extracellular protein (figure 5), obtained after 38 h cultivation in YPTM, at an estimated heterologous xylanase concentration of 2.6 g/L. In the final sample, obtained after 40 h, and at a cell dry weight of 13.3 g/L the estimated extracellular xylanase concentration had increased to 3.1 g/L, but resulted in a specific activity of 1000 U/g (likely as a consequence of small differences in the yield from the heat-treatment).

#### *Characterisation of the produced xylanase*

Analysis of the sequence, revealed two putative glycosylation sites for N-glycosylation (residue combination Asn-Xaa-Ser/Thr). Deglycosylation reactions using EndoH or PNGaseF, did after prolonged incubations (only partially visible after PNGase treatment), resulted in an apparent down-shift in molecular weight of approximately 4.2 kDa (estimated from SDS-PAGE, indicating N-glycosylation), but this was not accompanied with any change in activity, and did not reactivate the xylanase (figure 6B). Moreover, removal of the His-tag with carboxypeptidase A did not yield active xylanase, excluding deactivation caused by His-tag interaction with the catalytic domain of the xylanase. Previous reports have shown that S-glutathionylation can occur at Cys-residues in proteins produced in *P. pastoris* [37-38]. As Cys-residues are present in the sequence of the catalytic module of Xyn10A, a

deglutathionylation reaction was also tried, but failed to transform the xylanase to an active state.

Xylanase activity could only be detected in heat pre-treated samples, confirming the heating/cooling treatment as a prerequisite for attaining active xylanase. In order to access possible differences between the inactive and reactivated enzyme, both native-PAGE and SDS-PAGE gels combined with activity overlay gels were run. As a positive activity-control another construct of the catalytic module of the xylanase was used (Xyn10A $\Delta$ NC  $M_r = 39.5$  kDa), previously produced in active form in *E. coli* [39]. It was observed that the migration patterns differed between the heat-treated and non-heat-treated *P. pastoris* produced xylanase on native PAGE (figure 6A). Using SDS-PAGE on the other hand (figure 6B), no apparent difference in migration pattern between heat treated (lane 6 & 7) and non heat treated (lane 1) samples could be seen, confirming that no change in the apparent molecular mass of the polypeptide had occurred during this treatment. Due to the migration being mainly dependent on native charge using native PAGE, while it is size dependent on SDS-PAGE (the technique often used to monitor mobility changes due to glycosylation), a plausible explanation for the mobility difference would be a change in conformation due to heat-treatment.

To further investigate the thermostability of the produced xylanase, in order to rationalise the effect of heat pre-treatment, the thermal unfolding of the polypeptide was monitored using differential scanning calorimetry (DSC). The unfolding curve obtained showed that the unfolding process for the produced xylanase had started at 70°C (the temperature of the heat pre-treatment), but had not reached the transition point, as the transition temperature ( $T_m$ ) was observed to be 78°C.

Although this is a thermostable polypeptide, the  $T_m$  was significantly lower (10.8°C lower) as compared to the most similar construct of the xylanase catalytic module expressed using an *E. coli* host-vector system (differing only in 3 amino-acid residues, the N-terminal, and with two residues inserted preceding the His-tag [31]). This indicates that removal of these residues, could have removed a stabilising interaction, or that the stability of the heterologous protein is affected by glycosylation. More importantly the results show that the produced xylanase requires only partial unfolding (heat treatment at 70°C) in order to correctly refold (while cooling on ice) to an active conformation.

#### 4. Discussion

This study is the first to report the use of the methylotrophic yeast *P. pastoris* for extra-cellular production of thermostable xylanase originating from a bacterial source. The catalytic module of the bacterial xylanase (Xyn10A), produced in this study, originates from the extreme thermophile *Rhodothermus marinus*, and its cloning and characterisation was first described using an *E. coli* expression system [7]. Although, since then both bacterial and fungal xylanases have been expressed in different eukaryotic hosts [23,40-42], this is also one of the first studies to describe the production of a GH 10 enzyme in *P. pastoris*.

*R. marinus* is an aerobic, heterologous, G-negative bacterium growing optimally at 65 °C, which in the presence of xylan produces a number of glycan-hydrolysing activities. Xylanase activity has been reported to be produced to extracellular levels of 1000-2000 U/L in batch cultivations [27,43], and involving at least two enzymes [44]. The maximum cell densities obtained when growing this organism, under conditions

inducing xylanase activity are reported to be 4.2 g/L [43] which makes recombinant production of a selected enzyme in a host capable of acquiring high cell densities a tempting alternative. Other difficulties with the native organism related to purification and enzyme characterisation is the presence of more than one xylanase activity, and the fact that the majority of the xylanase activity in *R. marinus* is cell-attached [44]. Advantages using *P. pastoris* as a host for recombinant production of this enzyme are thus the possibility to grow the organism to high cell densities, the lack of endogenous xylanase, and the possibility to efficiently secrete the enzyme [21,23].

The xylanase from *R. marinus* has previously been analysed on the sequence level, after cloning in *E. coli* [7], and a number of different constructs of the enzyme, have also been successfully produced in this microorganism [31,39,45]. A major drawback using *E. coli* as a host is however its lack of secretion mechanism, and therefore the methylotrophic yeast *P. pastoris* was selected as an alternative host, being an organism capable of efficient secretion, and with commercial cloning vectors available.

In order to avoid limitations during the expression of the foreign protein which has been reported to decrease the yield (caused by choice of/or change in cultivation conditions and medium composition during growth [22]), the induction phase for xylanase expression was carried out in a bioreactor containing peptide rich media components and methanol as the sole carbon source. This strategy yielded 3 g/L of extracellular xylanase (judged from SDS-PAGE and total protein estimation) with a maximum activity of 3130 U/ L during 41 h of induction. The volumetric productivity obtained ( $76 \text{ UL}^{-1}\text{h}^{-1}$ , without further optimisation of the “batch induced” process or reactivation conditions) is 1.6 times higher than

what was obtained in batch cultivations of *R. marinus* (47 UL<sup>-1</sup>h<sup>-1</sup>, including both xylanase activities) in our laboratory [27]. Although, the xylanase activity was considerably lower than previous production results using *E. coli* as an expression host (intracellular production in fed-batch cultures yielding 7,000 - 21,000 U/g cell dry weight) [39] it should be noted that *P. pastoris* cultivations can reach cell densities >130 gram cell dry weight using fed-batch fermentation processes which in turn would yield a significantly higher yield of recombinant protein. Furthermore, extracellular production of the xylanase using the *P. pastoris* expression system has a significant impact on lowering production costs due to product accumulation in the fermentation broth.

A drawback using the current production system was the necessity to include a heat-treatment step in order to fold the enzyme into an active conformation. Heat precipitation is however routinely used to remove cell debris and cell particulate during the purification of heterologous proteins of thermophilic origin produced in mesophilic hosts, and is considered advantageous being a relatively simple purification step [14,46]. In this case it is also necessary, in order to gain an active form of the produced xylanase. The current results indicate an approximately 200-fold increase in xylanase activity after the heat pre-treatment step during which unfolding and refolding of the enzyme confers xylan degradation ability. However, the specific activity obtained after the currently used, unoptimised treatment is significantly lower (1 U/mg) than what can be obtained after extensive purification of native xylanase from *R. marinus* (30 U/mg, ref-Leifs thesis), or after purification of *E. coli* produced enzyme (190 U/mg, extremophiles), pointing out that a large part of the produced enzyme is not refolded, and shows the need for optimisation of this step in order to make the production system more competitive.

*P. pastoris* is capable of adding *N*- and *O*-linked carbohydrate moieties to secreted proteins [20]. *N*-glycosylation occurs at the amide nitrogen of Asn-residues, when found in the consensus Asn-Xaa-Ser/Thr, and the *N*-linked oligosaccharides formed in *P. pastoris* are generally in the range (Man<sub>8-14</sub>GlcNAc<sub>2</sub>) which is shorter than those of *S. cerevisiae* [47]. Despite this shorter length, it is still possible to determine *N*-glycosylation by catalysing hydrolysis of linkages in the *N*-linked oligosaccharide (by Endo H or PNGase F), followed by separation on SDS-PAGE in order to demonstrate shifts in mobility. Two putative sites for *N*-glycosylation were found in the Xyn10ACM-sequence, and a mobility shift was observed after incubation with EndoH (catalysing GlcNAc $\beta$ -1,4GlcNAc hydrolysis), but was not clearly observable after the PNGase treatment (catalysing hydrolysis of GlcNAc $\beta$ -Asn linkage). This indicates glycosylation, but still leaves some doubt on the specificity of the reaction as the mobility shift, and the apparent molecular mass after the treatment (33 kDa) was lower than the theoretically calculated. The data collected from SDS-PAGE (after heat treatment, compared to after deglycosylation), however speak against *N*-glycosylation as a reason for the need to reactivate the enzyme as Endo H and PNGase treatment did not affect activity. Moreover, mobility on SDS-PAGE did not change after the reactivating heat-treatment, indicating that the molecular mass is unchanged.

Evidence for *O*-glycosylation of recombinant proteins expressed in *Pichia* is limited, but has been reported to occur on Ser and Thr hydroxy-groups [47]. The *O*-linked saccharides are generally short (<5 residues of  $\alpha$ -1,2-linked mannose units), and their presence not normally visible on SDS-PAGE, which makes their extent in the recombinant protein more difficult to analyse. Glycosylation has not been verified in *R. marinus*, and the xylanase was expressed in active form in *E. coli* (which is incapable of

glycosylating exported proteins). Therefore *O*-glycosylation could be the reason for the necessity to reactivate the enzyme. This could also lead to the apparent lower thermostability, seen as the lower melting temperature ( $T_m$ ) observed on DSC-thermograms, compared to the  $T_m$  of the same module purified from an *E. coli* based expression system [31].

It was also found that the *Pichia* produced xylanase failed to purify by immobilized metal-ion affinity chromatography (IMAC), which could be an indication of interaction between the enzyme and the C-terminal His-tag, possibly leading to a conformational change, and also resulting in a shift in melting temperature. In the *E. coli* produced protein, the C-terminus of the catalytic module is linked via the two residues (Leu-Glu) to the His-tag, while in the *Pichia* produced module these two residues are left out. Destabilising interactions between the His-tag and the protein can at this stage not be ruled out as the reason for the lowered thermostability, and reports show that a single amino acid or substitutions can dramatically influence the denaturation pattern of a protein [48]. However, treatment with carboxypeptidase showed that interaction with the His-tag could be excluded as a reason for the need to reactivate the enzyme.

In conclusion, this report shows the first successful expression of thermostable GH10 xylanase from a Gram-negative bacterium (*R. marinus*) using the *P. pastoris* host vector system. Although optimisation of the refolding process seems necessary, the system is promising for development of high cell density fed-batch fermentations of thermostable xylanase of bacterial origin for applied purposes. The produced xylanase displays a thermostability sufficient for use in applications such as bleaching processes, which so far have been dominated by fungal xylanases used e.g. in the treatment of sugar cane bagasse pulp [49] and bagasse soda pulp [50]. Using *P. pastoris* in a controlled environment with regards to pH,

aeration and carbon feed rate will ensure increased extracellular heterologous protein production levels for trials and use in large scale processes.

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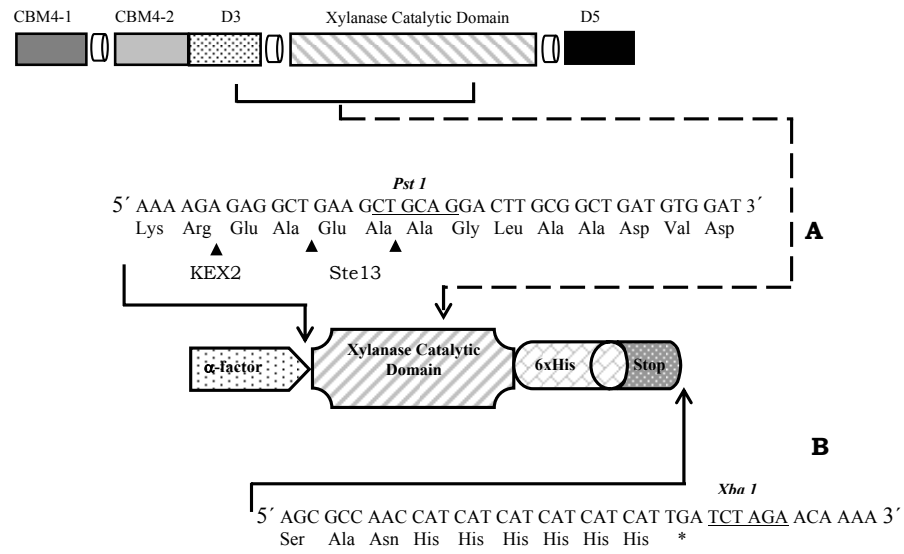


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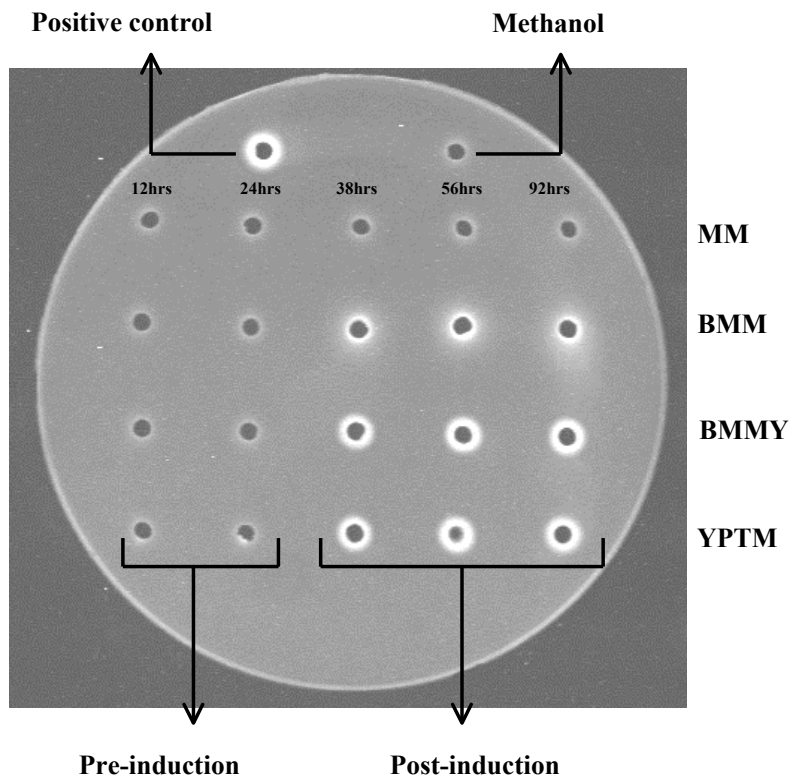
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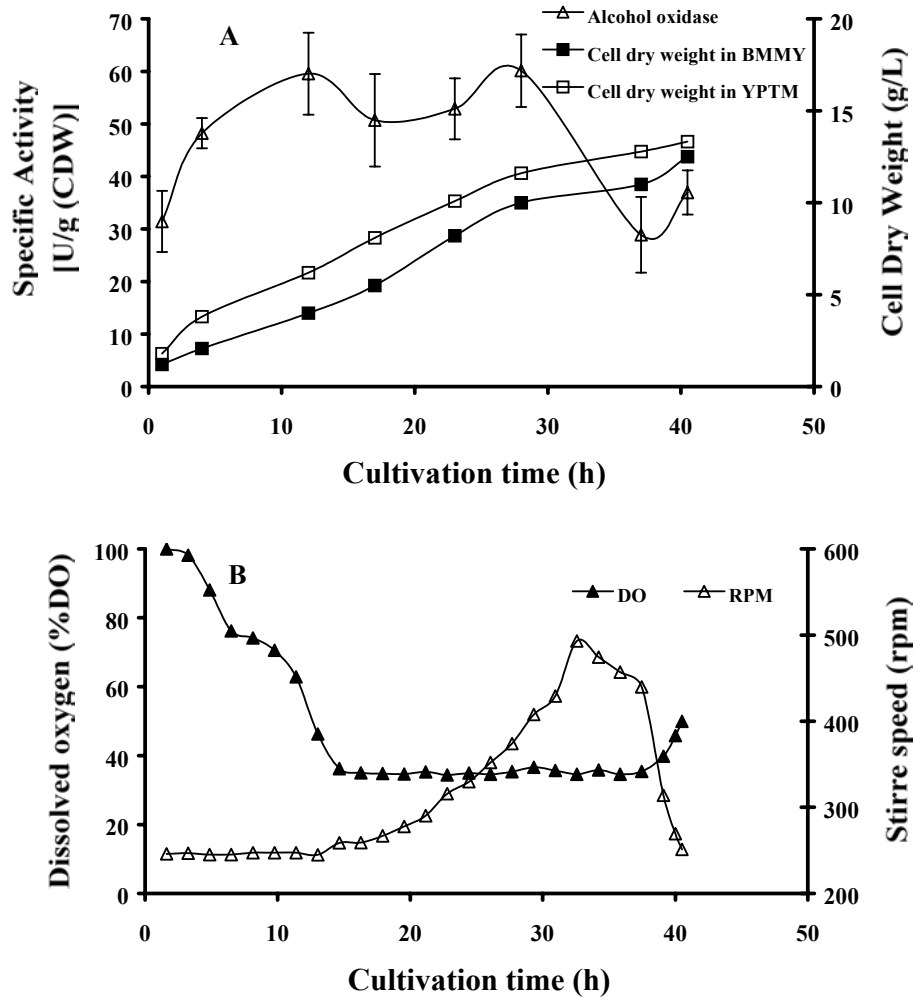
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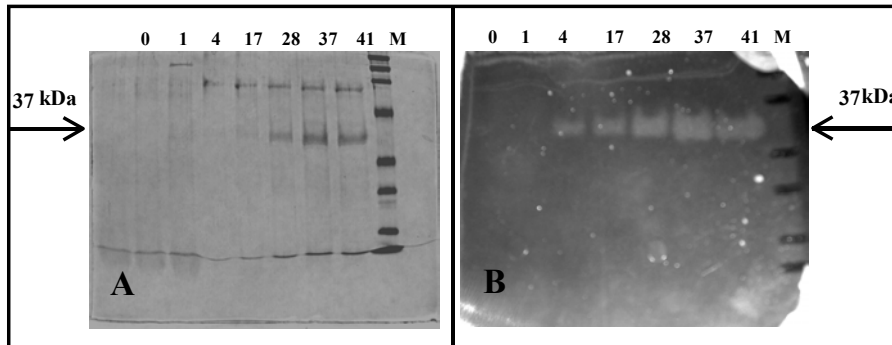
**Figure 1.** Schematic representation of the full-length xylanase (Xyn 10A, 107.6kDa) from *R. marinus* with two carbohydrate modules (CBM4-1 and CBM4-2, 165 residues each); domain three (D3, 189 residues); xylanase catalytic module (Xyn10ACM, 320 residues) and the fifth domain (D5, 84 residues). A linker is present between (CBM4-1 and CBM4-2), (D3 and Xyn10ACM) and (Xyn10ACM and D5). Also, the plasmid construction for the expression of the catalytic domain of Xyn10A (Xyn10ACM) in *P. pastoris*, using the expression vector pPICZ $\alpha$ B is shown. **(A)** 5' region of the recombinant pPICZ $\alpha$ B/pSRX1-construct displaying the last five amino acids of the *S. cerevisiae*  $\alpha$ -factor secretion signal and the dipeptide AlaGly ahead of the first six amino acids of the xylanase catalytic domain. The *KEX2* and *Ste13* protease cleavage sites are essential to ensure proteolytic processing of the secretion amino acids of the xylanase catalytic module followed by a six-histidine tag and a stop codon.



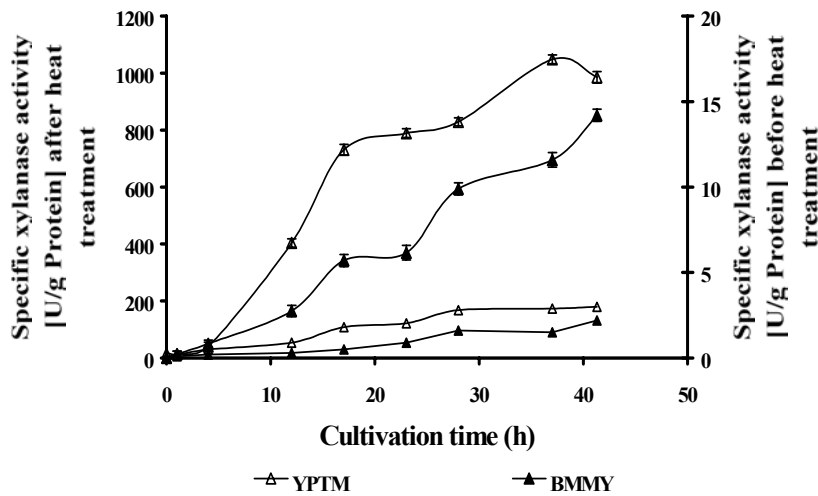
**Figure 2.** Qualitative xylanase production, measured by a halo-plate assay using the substrate azo-xylan and supernatant samples from shake flask experiments with varying medium composition (Table 1). Methanol (0.5%) was used as negative control and purified Xyn10A $\Delta$ NC produced in *E. coli* was used a positive control.



**Figure 3.** Cultivation parameters **(A)** On-line data capture of dissolved oxygen concentration (DO) and stirrer speed from a representative batch induced cultivation in YPTM. **(B)** Off-line analysis of cell dry weight and alcohol oxidase activity per gram cell dry weight.

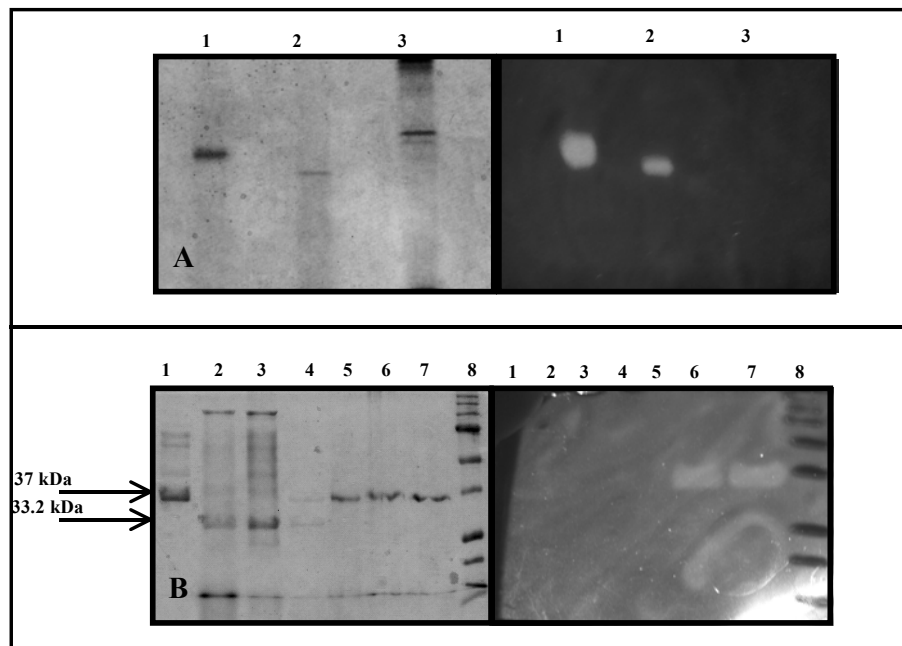


**Figure 4.** (A) SDS-PAGE gel of heat-treated samples from a batch induced cultivation in YPTM medium. (B) Corresponding agarose over-layer gel containing birch xylan as the substrate. [Lanes 0-41] corresponds to cultivation time in hours; [Lane M] molecular mass standard (Biorad wide range: 198, 113, 96.4, 52.9, 35.9, 28.5 & 18.5 kDa).



**Figure 5.** Specific xylanase activity before and after heat treatment from representative batch induced cultivations in YPTM and BMMY medium.





**Figure 6. (A)** Native PAGE (left) with corresponding activity overlay gel (right). [Lanes 1] Positive control (xylanase previously produced in *E. coli*); [Lanes 2] heat-treated supernatant sample ; [Lanes 3] supernatant sample with no heat treatment. **(B)** SDS-PAGE and corresponding activity overlay gel. [Lanes 1] supernatant sample with no heat treatment, [Lanes 2-3] sample after deglycosylation with Endo H for 15 hours and 1 hour respectively; [Lanes 4-5] sample after deglycosylation with PGNase F for 15 hours and 1 hour respectively; [Lanes 6-7] supernatant sample after heat treatment; [Lane 8] molecular mass standard (Biorad Precision Plus: 250, 150, 100, 75, 50, 37, 25, 20 & 15 kDa)