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Overexpression of the *Neocallimastix frontalis* xylanase gene in the methylotrophic yeasts *Pichia pastoris* and *Pichia methanolica*

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Abstract

The xylanase gene from the rumen fungus *Neocallimastix frontalis* was expressed in *Pichia pastoris* and *Pichia methanolica*. Using a complex medium with medium replacement before induction and the maintenance of the methanol induction level at 0.5%, *P. pastoris* was able to produce about 5400 U/ml of xylanase after 10 days of induction. With *P. methanolica*, on the other hand, about 6200 U/ml of xylanase was reached after 10 days of induction using synthetic medium as first culture medium and then direct induction by continuous methanol feed at 1.8 ml l^{-1} h^{-1}. In general, the advantages of using *P. methanolica* to produce the xylanase included higher protein production, the lack of medium replacement, and an ease of scale up. However, because the *P. pastoris* culture supernatant contained fewer secreted non-target proteins compared to *P. methanolica*, xylanase purification would be easier with the *P. pastoris* system. In addition, experiments involving methanol pulses suggested that a relationship exists among base feeding, methanol consumption and xylanase activity.

Keywords: *Pichia pastoris*; *Pichia methanolica*; Ruminal fungi; Xylanase; High-level expression

1. Introduction

Expression of heterologous proteins is crucial for many biotechnology applications. Among the various available gene expression hosts, methylotrophic yeasts such as *Pichia pastoris* and *Pichia methanolica* are particularly attractive due to the availability of their strong and regulatory promoters that are involved in methanol metabolism [1,2]. Using the alcohol oxidase 1 gene (*AOX1*) promoter in *P. pastoris* or the alcohol utilizing gene (*AUG1*) promoter in *P. methanolica*, both of which are regulated by a carbon source-dependent repression/induction mechanism, expression of a target gene in methylotrophic yeast can be made highly inducible by methanol, but is repressed in the presence of other carbon sources [3,4]. *P. pastoris* and *P. methanolica* have similar features: (1) ease of genetic manipulation; (2) heterologous gene genetic stability after integration of an expression cassette into the host chromosome; (3) high cell density during fermentation is obtained easily; (4) accurate eukaryotic post-translational modification; and (5) extracellular protein secretion [5,6]. *P. methanolica*, however, has some distinct and superior characteristics in terms of carbon utilization and gene recombination. *P. methanolica* can efficiently shift its carbon source from dextrose to methanol and convert methanol into biomass [3]. Furthermore, *P. methanolica* predominately uses non-homologous recombination (>90%) to integrate expression cassettes, whereas *P. pastoris* usually uses homologous recombination [3,7].

β-1,4-Xylanases, which catalyze the hydrolysis of β-1,4-D-xylosidic linkages in xylans, have great commercial use in the food, animal feed, pulp and paper industries [8–10]. They are useful in bread making, clarification of beer and juices, and the conversion of xylan-containing lignocellulosic materials to oligoxyloses and xylose, which can then be converted to a variety of byproducts that have high aggregate value. Xylanases are also essential for improving the nutritional quality of animal feed. In addition, recent increasing concern about preserving the environment from industrial waste has raised interest in applying microbial xylanases to the pulp and paper industry. This has the aim of reducing or replacing the use of the harmful alkaline extraction hemicellulose process as well as the need for

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chlorine in the bleaching process. This needs to be done without affecting the cellulose fiber strength of the paper products and xylanases offer this possibility [8–10]. Rumen fungi are thought to be the most important contributors to fiber degradation in the rumen and thus they are known as a good source of potential xylanase [11]. Although several microbial xylanase genes have been cloned and expressed in heterologous expression systems such as *Escherichia coli* [12–15], *Kluyveromyces lactis*, *Penicillium roqueforti* [16], and *P. pastoris* [9,17], the expression levels have not been high enough for industrial applications.

In this study, the xylanase gene from the ruminant fungus *Neocallimastix frontalis*, *xyn11B*, was expressed in *P. pastoris* and *P. methanolica*. We have demonstrated that a high-level expression of the recombinant xylanase can be achieved in *P. pastoris* using high cell density culture and an induction strategy involving a constant methanol concentration. Furthermore, we have also demonstrated that *P. methanolica* has a similar high expression level obtained using an induction strategy of continuous methanol feeding at a fixed rate. In addition, the relationship between xylanase production and methanol induction together with the parameters of the fermentation were also investigated.

2. Materials and methods

2.1. Strains and media

E. coli strain DH5α (GIBCO-BRL Life Technologies, Grand Island, NY, USA) was used for plasmid manipulation and was grown in LB medium (Difco, Detroit, MI, USA). *P. pastoris* strain KM71 (*his4*, *arg4*, *aox1:: ARG4*, Mut^s) and *P. methanolica* strain PMAD11 (*ade2-11*, Mut$^+$) were purchased from Invitrogen (Carlsbad, CA, USA). The regeneration dextrose (RD) medium for *P. pastoris* regeneration, the minimal dextrose (MD) medium for *P. methanolica* regeneration, the buffered glycerol-complex medium (BMGY) for *P. pastoris* culture, the buffered dextrose-complex medium (BMDY) for *P. methanolica* culture, and the buffered methanol-complex medium (BMMY) for induction were prepared according to the manual of the Invitrogen's *Pichia* Expression kit. The modified media, mBMGHY and mBMMHY, were previously reported [18].

2.2. Plasmid construction

The *N. frontalis* xylanase gene was cloned using *xyn11B* cDNA as the template [15]. The primers 5′ ACGCGTCGACGAAATGCCCCTATCCTATCAACACTTC 3′ (xynFEcoRI) and 5′ ACGCGTCGACGCAACGGCCCGTGCCCCATTTCC 3′ (xynRNotI), was used to amplify *xyn11B* for *P. pastoris* transformation. The second of the above primers was replaced with xynRBamHI (5′ ACGCGTCGACGCAACGGCCCGTGCCCCATTTCC 3′) when used for the *P. methanolica* transformation. The amplified product with *Eco*RI and *Not* I sites was inserted into pPIC9K (Invitrogen), while the PCR product with *Eco*RI and *BamH*I sites was inserted into pMETαA (Invitrogen). The resulting plasmids were designated pPIC9Kxyn and pMETαAxyn, respectively.

2.3. Pichia transformation

Competent cell preparation and electroporation were performed according to the manual from the Invitrogen's *Pichia* Expression kit. For *P. pastoris*, pPIC9Kxyn was linearized with *Sal*I to favor integration at the *his4* locus and His$^+$ transformants were selected on RD medium plates. For *P. methanolica*, pMETαAxyn was linearized with *Asc*I and transformed into PMAD11 based on the methods of Raymond et al. [3]. The *P. methanolica* transformants were then screened on MD medium plates using adenine dropout by red and white selection.

2.3.1. Screening for xylanase activity

Both *P. pastoris* and *P. methanolica* candidate colonies were chosen, re-streaked on a regeneration plate at 30 °C for 2 days, and then inoculated in 5 ml of BMGY or BMDY medium, respectively. The overnight cultures were collected and resuspended in 1 ml BMMY medium. After 24 h of induction, the cultures were centrifuged at 1500 × *g* for 5 min at room temperature. The cell-free supernatant was used for the xylanase activity assay.

2.4. Enzyme assay

The xylanase activity was determined as described by Georis et al. [19] with some modifications. Oat spelts xylan (1%, w/v) was pre-warmed in citrate buffer (25 mM citrate acid and sodium citrate, pH 6) at 60 °C for 5 min; this was followed by the addition of enzyme solution to react for 5 min. Dinitrosalicylic acid reagent was added to terminate the reaction and color formation occurred after incubating the reaction mix for 5 min at 100 °C [20]. The amount of reducing sugar resulting from the enzymatic reaction was determined by measuring the absorbance at 540 nm using xylose as the standard. One unit (U) was defined as the amount of enzyme that released 1 μmol of reducing sugar per min. In order to measure the temperatures and pH stability of the enzyme, the xylanase was held at four different temperatures (25, 37, 50 or 70 °C) for 30 min or diluted in a series of buffers (25 mM citrate buffer (pH 3–6), 25 mM phosphate buffer (pH 6–8) or 25 mM Ammediol buffer (pH 8–10)) for 3 h then assayed for xylanase activity.

2.5. Pichia fermentation

The *P. pastoris* fermentation process was divided into three phases, namely glycerol batch fermentation, glycerol-fed batch fermentation and methanol induction. The *P. pastoris* transformant with the highest xylanase activity was cultured in BMGY at 30 °C until the OD_{600} reached 20, after which 10 ml of the seed culture was added into a 5 l jar fermenter (B. Braun Biotech International GmbH, Melsungen, Germany) containing 2 l of mBMGY supplemented with 4% (v/v) glycerol. The batch and fed-batch fermentation of *P. pastoris* have been described elsewhere [18]. Cells were then collected by centrifugation and resuspended in 2 l of fresh mBMMY containing 0.5% (v/v) methanol and returned to the fermenter for xylanase production. The methanol concentration was maintained at 0.5% using a MC-168 controller (PTI Instruments Inc., Lincoln, NE, USA). The air input rate was 3 VVM and the agitation rate was 800 rpm. Samples were removed periodically throughout this phase to be assayed for xylanase activity, for electrophoretic protein analyses and to measure the viable cell count.

The fermentation of *P. methanolica* was similar to that of *P. pastoris* except that the medium and the induction were altered. The carbon source in batch culture was dextrose rather than glycerol. After dextrose depletion, methanol containing 12 ml/l of PTM_1 trace salts (Invitrogen) was fed continuously at the rate of 1.8 ml/(l h).

2.6. SDS-PAGE, glycoprotein staining and Western blot analysis

SDS-PAGE was performed using 13% (w/v) polyacrylamide gels with a Mini-Protein III system (Bio-Rad, Hercules, CA, USA). Proteins were visualized in the gel by Coomassie blue staining. For Western blotting, the proteins were transferred to a PVDF membrane by semi-dry transfer (Genemaster Biotechnology, Taipei, Taiwan). The membrane was incubated with a polyclonal mouse anti-XYN serum at $1:10^5$ dilution (Blossom Biotechnologies, Taipei, Taiwan). Immunoreactive protein was visualized using an alkaline phosphatase-linked goat anti-mouse IgG at 1:5000 dilution together with its chromogenic substrate (PerkinElmer, Wellesley, MA, USA).

3. Results

3.1. Expression of recombinant xylanase in P. pastoris

The nucleotide sequence encoding the entire *N. frontalis xyn11B* without its original signal sequence was fused in frame

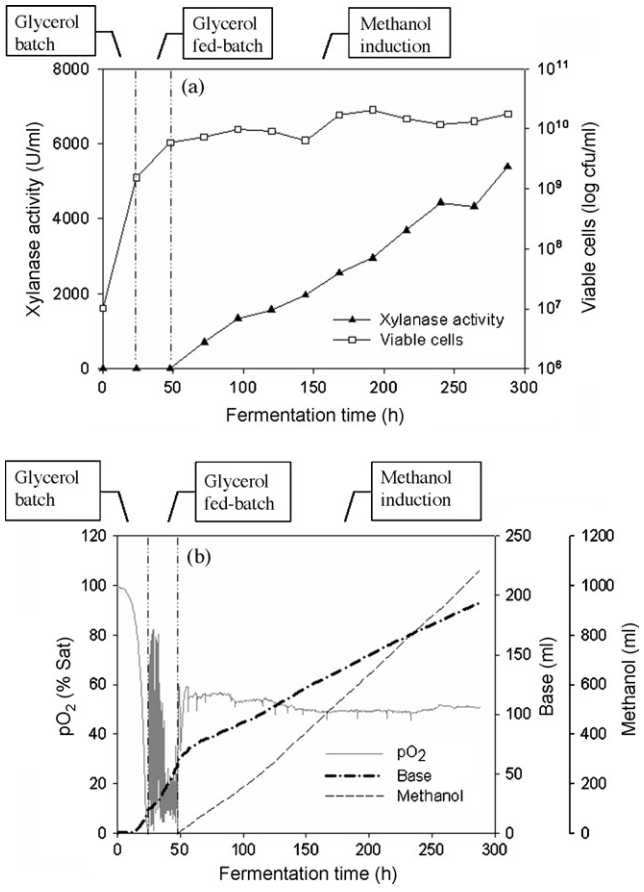

Fig. 1. Viable cell density, xylanase activity and fermentation parameters in high cell density cultures of the *P. pastoris* Mut^s YS27. Induction of the culture was initiated at 48 h, and the methanol concentration was maintained at 0.5% (v/v) using an on-line methanol controller. (a) Xylanase activity (▲) and viable cell density (□). (b) Fermentation parameters: dissolved oxygen concentration (pO_2) (gray line), total amount of 10% ammonia added (dash-dot line), and total amount of 100% methanol added (dash line).

with the *Saccharomyces cerevisiae* α-factor secretion signal into the expression vector pPIC9K, and the resulting plasmid was used for transformation of *P. pastoris*. Sixty-seven transformants with high growth rates were picked and inoculated in 5-ml cultures to measure xylanase activity. The clone expressing the highest level of xylanase activity, designated YS27, was used for the remainder of the study. Representative results of xylanase expression in *P. pastoris* and its fermentation parameters are shown in Fig. 1. The fed-batch was initiated after the initial glycerol had been exhausted, as evidenced by an abrupt increase in dissolved oxygen. The viable cell density had reached 5.9×10^9 cfu/ml at the end of the glycerol-fed batch phase. After fed-batch cultivation and medium replacement, the methanol concentration was maintained at 0.5% using a methanol concentration controller. The cell density did not change significantly during the induction phase. Xylanase activity was first detected after 24 h of induction and increased with induction time, reaching about 5400 U/ml after 10 days. The amount of methanol used for feeding increased in proportion with the xylanase activity. The amount of base added to maintain the pH at 5.0 reflected glycerol utilization in the batch phase. In the induction phase, the base feeding paralleled both methanol feeding and xylanase production.

3.2. Expression of recombinant xylanase in *P. methanolica*

N. frontalis xyn11B lacking its signal sequence but with the *S. cerevisiae* α-factor secretion signal cloned in the expression vector pMETαA was used for transformation of *P. methanolica*. Twenty-three *P. methanolica* transformants were picked and screened for xylanase activity. The clone expressing the highest level of activity, designated M6-16, was used for the remainder of the study. Due to its ability to efficiently shift from using dextrose as a carbon source to methanol and its ability to convert methanol into biomass, the fermentation of *P. methanolica* was divided into batch and methanol-fed batch phases. Preliminary study had indicated that no medium replacement at the end of the growth phase followed by continuous methanol feeding at a rate of $1.8\,ml\,l^{-1}\,h^{-1}$ comprised the best induction strategy for *P. methanolica* (data not shown). Fig. 2 shows representative results of xylanase expression in *P. methanolica* and its fermentation parameters. The viable cell density reached

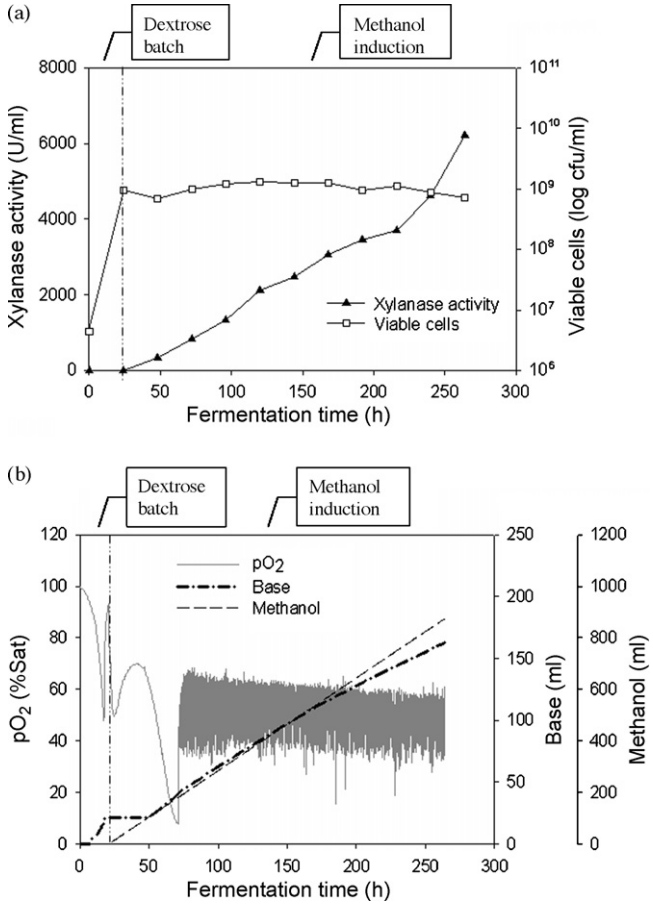

Fig. 2. Viable cell density, xylanase activity and fermentation parameters in fed-batch cultures of the *P. methanolica* Mut$^+$ M6-16. The culture was induced at 24 h utilizing a continuous methanol feeding of $1.8\,ml\,l^{-1}\,h^{-1}$. (a) Xylanase activity (▲) and viable cell density (□). (b) Fermentation parameters: pO_2 concentration (gray line), total amount of 10% ammonia added (dash-dot line), and total amount of 100% methanol added (dash line).

9.3×10^8 cfu/ml at the end of the batch phase and did not change significantly during the methanol feeding phase. Xylanase activity was detected immediately after induction and continued to increase throughout the methanol induction phase. After induction for 10 days, xylanase activity had reached about 6200 U/ml. The dissolved oxygen concentration curve was similar to that of *P. pastoris* during the batch phase but consistently varied between 70 and 30% once methanol induction was initiated and continued within these parameters until the end of the experiment. With *P. methanolica*, the amount of base feeding also served as a good indicator of growth and induction of protein expression.

3.3. Effect of methanol pulses on recombinant xylanase expression during P. methanolica induction

The above results suggested that there was a proportional relationship between methanol feeding and base feeding as well as between base feeding and xylanase activity production. To verify this phenomenon, pulses of methanol were added to the media after 24-h periods of no methanol being fed. *P. methanolica* was cultured and induced as described above. After 72 h of induction, the methanol feed was stopped for 24 h, then resumed for another 24 h; this was repeated four times. The total volume of methanol used in pulse-chase experiment was about 600 ml. When methanol feeding was stopped, the dissolved oxygen immediately reached saturation levels. This was accompanied by an arrest in the increase in xylanase activity and in base feeding (Fig. 3). After methanol feeding was resumed, the level of dissolved oxygen dropped tremendously and base feeding and the total xylanase activity of the culture again began to increase.

3.4. Enzyme characteristics of the recombinant xylanases from P. pastoris and P. methanolica

To further assess the expression of xylanase, SDS-PAGE analysis of the supernatants of the *P. pastoris* and *P. methanolica* cultures was performed. The predicted molecular weight of the recombinant protein is 36.74 kDa. Two major bands were observed at 27.9 and 31.8 kDa in the induced *P. pastoris* culture medium. In contrast, two bands at 19 and 27.9 kDa and a smear running from 30 kDa to more than 94 kDa were present in the *P. methanolica* culture medium. The intensity of these bands from both strains increased with induction time, indicating recombinant xylanase secretion was proportional to the level of methanol used for induction. Comparison of the Coomassie-blue stained SDS-PAGE gels and Western blots using anti-XYN indicated that most of the protein in the culture supernatants of induced *P. pastoris* and *P. methanolica* was xylanase (Fig. 4). In order to find the effects of different glycosylation level on two recombinant xylanases, thermostability and enzyme pH stability tests were performed. For the xylanase thermostability assay, the xylanase activity of enzyme stored at 25 °C was defined as the 100% and the xylanase activity after the other treatments at different temperatures was compared to this to obtain a relative activity. The xylanase from *P. pastoris* retained up to 90% of its activity at

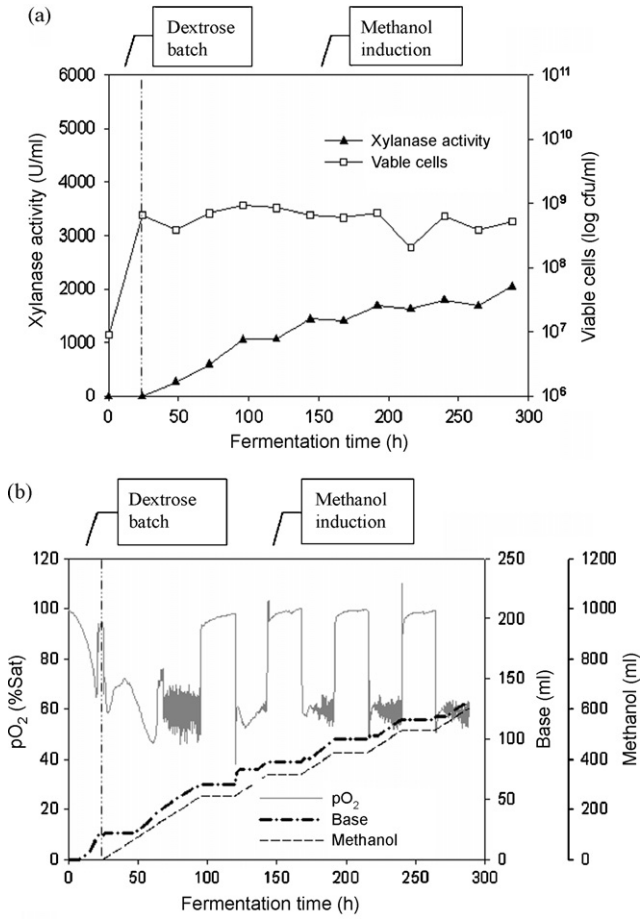

Fig. 3. Effect of pulses of methanol on induction of xylanase and base feeding in a *P. methanolica* culture. The culture was induced at 24 h utilizing a continuous methanol feeding of 1.8 ml l^{-1} h^{-1}. After 72 h of induction, the methanol feeding was stopped for 24 h, then resumed for another 24 h. Four sequential pulses/chases were performed. (a) Xylanase activity (▲) and viable cells (□). (b) Fermentation parameters: pO_2 concentration (gray line), total amount of 10% ammonia added (dash-dot line), and total amount of 100% methanol added (dash line).

37 °C for 0.5 h, and 70% activity at 50 °C for 0.5 h; however, the activity had dropped below 50% activity after treatment at 70 °C got 0.5 h. The xylanase from *P. metahnolica*, retained 96% activity at 37 °C for 0.5 h, and retained 80% activity after treatment at 50 °C for 0.5 h (Fig. 5A). For the pH stability assay, the xylanase activity of enzyme incubated with 25 mM citrate buffer pH 6.0 was defined as 100% and the xylanase activity after treatments at other pH levels were compared to this as a relative activity. Recombinant xylanases from *P. pastoris* and *P. metahnolica* retained 50 and 75% activity across a pH range from 3 to 10 (Fig. 5B and C).

4. Discussion

In this study, we demonstrated that both the *P. pastoris* and *P. methanolica* are useful for the heterologous expression of a ruminant xylanase gene. Although both of these methylotrophic yeast utilize the alcohol oxidase promoter, there are important technical aspects that distinguish the two species. Firstly,

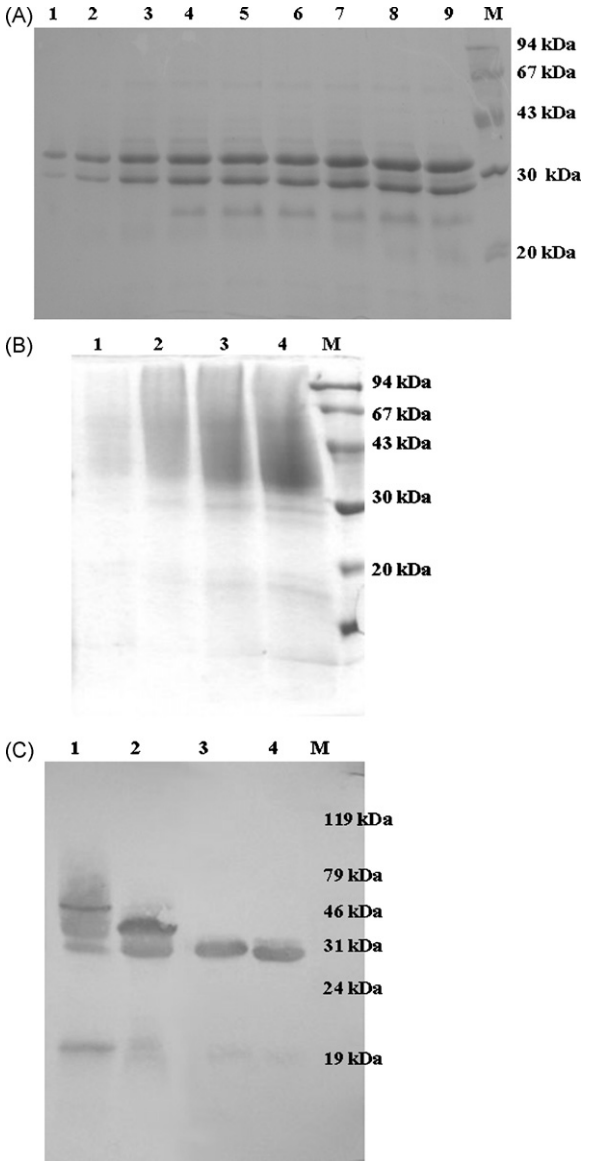

Fig. 4. SDS-PAGE and Western blot analyses of the recombinant xylanases from *P. pastoris* and *P. methanolica*. (A) *P. pastoris* culture supernatant at different induction time. Lanes 1–9: 7.5 μl of culture supernatant collected every day at days 2–10 were loaded. Lane M, protein marker. (B) *P. methanolica* culture supernatant at different induction time. Lanes 1–4: 7.5 μl of culture supernatant collected after 1, 4, 7, and 10 days of induction were loaded. Lane M, protein marker. (C) Western blot analysis of recombinant xylanases. Lane 1, culture supernatant of *P. methanolica* expressing xylanase; lane 2, culture supernatant of *P. pastoris* expressing xylanase; lane 3, affinity-purified his-tagged xylanase from a cell extract of *E. coli* expressing xylanase; lane 4, cell extract of *E. coli* expressing xylanase [15]. Lane M, prestained protein marker.

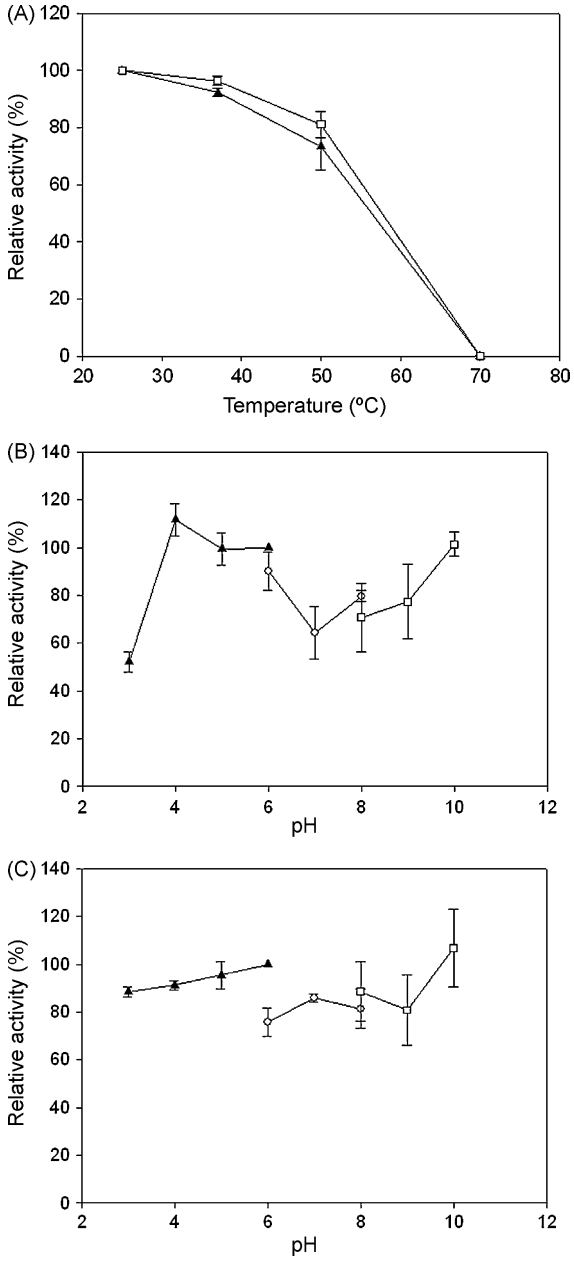

Fig. 5. Thermostability and pH stability of the recombinant xylanase from *P. pastoris* and *P. methanolica* cultures. (A) Thermostability of the recombinant xylanases. The xylanase activity from *P. pastoris* (▲) and *P. methanolica* (□) at 25 °C was set as 100% = 4036 and 6181 U ml^{-1}, respectively. The pH stability of the recombinant xylanases from (B) *P. pastoris* and (C) *P. methanolica* in 25 mM citrate buffer (▲), 25 mM phosphate buffer (◯) and 25 mM Ammediol buffer (□). The xylanase activity from *P. pastoris* and *P. methanolica* in 25 mM citrate buffer at pH 6.0 was set as 100% = 4616 and 5563 U ml^{-1}, respectively.

medium replacement is required with the *P. pastoris* culture before methanol induction, whereas direct methanol induction can be applied to *P. methanolica* cultures. This difference can be attributed to the host regulatory machinery of the *P. pastoris AOX* genes. *P. pastoris* is regulated according to a classical repression/induction mechanism that requires the absence of glucose or glycerol as well as the presence of methanol for the full induction of *AOX* promoter driven expression [21]. Thus, the induced protein expression level is much higher if the medium is replaced before induction [18]. In contrast, *P. methanolica*, which has the ability to more rapidly shift its carbon utilization from dextrose to methanol, needs no medium replacement before induction.

Secondly, we found that using a high cell density culture and keeping a constant 0.5% methanol concentration gave a high level of xylanase expression with *P. pastoris*, whereas different culturing parameters were required for *P. methanolica* involving the use of a continuous methanol feed at a fixed rate of 1.8 ml l^{-1} h^{-1} in order to produce high xylanase activity.

This is because *AUG1* promoter expression predominates at a low-methanol concentration [22] and therefore a low methanol feeding rate strategy allowed a high level of recombinant protein expression in *P. methanolica*. Although the amount of methanol fed was similar for both *P. pastoris* and *P. methanolica*, the volume of methanol utilization on a per cell basis for the Mut^+ strain of *P. methanolica* was higher than that with the Mut^s strain of *P. pastoris*.

Thirdly, the dissolved oxygen concentration curve remained stable during the methanol induction in *P. pastoris*, but there were rapid and dramatic shifts observed with *P. methanolica*. This can be explained by the different methanol utilization phenotypes of *P. pastoris* and *P. methanolica*. The methanol utilization slow (Mut^s) phenotype of *P. pastoris* leads to slow methanol metabolism and oxygen consumption and this consequently creates a smooth dissolved oxygen concentration curve. In fast methanol utilization (Mut^+) of *P. methanolica*, the fast methanol utilization and oxygen consumption depletes methanol quickly; hence, when methanol is limited, the dissolved oxygen concentration spikes accordingly.

Lastly, the presumed extent of glycosylation of the recombinant protein produced by the two yeast species was different. The SDS-PAGE and Western blot analyses of the xylanase expressed by *P. methanolica* showed a smear from 30 kDa up, and a similar observation was also reported by Raymond [23]. No such smear was found in the supernatant of induced cultures of *P. pastoris*. Thus, the glycosylation level was higher in *P. methanolica* than that in *P. pastoris* [1]. This glycosylation probably also caused the difference in the specific enzyme activity and thermostability between the *P. pastoris* and *P. methanolica*-secreted xylanases. The results from the silver nitrate-periodic acid glycoprotein staining showed that both recombinant xylanases produced by *P. pastoris* and *P. methanolica* were glycoproteins. By *N*-glycosylation and *O*-glycosylation prediction, *xyn11B* has no N-linked glycosylation but many O-link sites. Since the *O*-glycosidase is only able to degrade the core structure of the O-link glycoprotein, the recombinant xylanase from *P. methanolica*, which might have more complex O-link glycosylation, would seem to need more enzyme to remove all the glycosylation modification. We also treated the recombinant xylanase from *P. pastoris* and *P. methanolica* with the N-link glycosyl hydrolase enzyme endo Hf, but there was no apparent band shift found (data not shown). Further biochemical analysis should be required to define the presence of *O*-glycans on the recombinant xylanases produced by both yeasts.

For some time, dissolved oxygen has been used as an indicator with large-scale fermentations. Although the concentration of dissolved oxygen in the medium immediately reflects changes in cell growth and carbon source utilization, it does not directly reflect the production of recombinant protein. In the methanol pulse experiment, we not only found a relationship between methanol feeding, the dissolved oxygen concentration and xylanase activity during fermentation, but we also found a relationship between base feeding and methanol metabolism. Methanol is oxidized by alcohol oxidase to generate formaldehyde, which then enters the dissimilatory pathway and produces formic acid [1,2], therefore the amount of base needed to maintain the pH of the culture at 5.0 during fermentation reflects methanol metabolism, the activation of the alcohol oxidase promoters, and consequently the expression of recombinant xylanase. In our previous study, this phenomenon was also observed when phytase and laccase production using *P. pastoris* and *P. methanolica* were investigated (data not shown). Based on these observations, we have demonstrated that methanol metabolism and recombinant protein production can be best monitored on-line by detecting the amount of base feeding.

5. Conclusion

In our study, we have shown that both *P. pastoris* and *P. methanolica* can be used to overexpress the xylanase gene from the rumen fungus *N. frontalis*. Using fermenter cultures, 5400 and 6200 U/ml of xylanase activity were produced by *P. pastoris* and *P. methanolica*, respectively. The *P. pastoris* culture supernatant contained lower levels of secreted non-recombinant proteins than the *P. methanolica* culture and hence use of *P. pastoris* ought to result in easier enzyme purification. However, *P. methanolica* might be preferred for large-scale culture because of the simpler culture procedure and induction strategy. Lastly, we also found the level of base feeding to be a useful indicator of recombinant protein production for both *P. pastoris* and *P. methanolica*.

Acknowledgment

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