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SHORT COMMUNICATION

Use of the glyceraldehyde-3-phosphate dehydrogenase promoter from a thermotolerant yeast, *Pichia thermomethanolica*, for heterologous gene expression, especially at elevated temperature

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Abstract The glyceraldehyde-3-phosphate dehydrogenase (*GAP*) gene from the thermotolerant yeast strain *Pichia thermomethanolica* BCC16875 was characterized. To investigate the efficiency of the *GAP* promoter for heterologous expression, especially at high temperature in various carbon sources, the promoter was employed for constitutive expression of a phytase reporter gene. The results showed that this promoter was able to drive efficient expression of phytase at 30 °C; the native promoter was highly robust compared with the heterologous *GAP* promoter from *Pichia pastoris*. More importantly, the *GAP* promoter was shown to be able to function at higher temperatures up to 42 °C, which could be useful for large-scale protein production to help reduce cooling costs in the fermenter. Expression in different carbon sources revealed that the *GAP* promoter was functional in glucose-, glycerol-, and methanol-containing media, with the highest level of expression in YPD medium. This strong promoter will help promote high expression of heterologous protein expression in *P. thermomethanolica*, especially in large-scale fermentation. In addition, a new tool for heterologous expression in yeast has been gained.

Keywords Thermotoloerant yeast · Glyceraldehyde-3-phosphate dehydrogenase · Heterologous gene expression · Carbon source

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Yeasts are efficient systems for heterologous expression of eukaryotic proteins (Böer et al. 2007). However, many common yeast strains, including *Saccharomyces cerevisiae* and *Pichia pastoris*, may not be optimal for large-scale production because they have optimal growth at relatively low temperatures (less than 37 °C). High temperature production has gained much attention because fermentation conducted at elevated temperatures will significantly reduce cooling costs and risk of contamination (Hensing et al. 1995). Thus, an alternative yeast strain that can grow well at high temperature is highly beneficial for many purposes. For example, ethanol production can be achieved by simultaneous saccharification and fermentation (SSF) done at a relatively high temperature (40 °C) with a thermotolerant yeast (Sandhu et al. 2012). Recently, a thermotolerant yeast strain isolated in Thailand named *Pichia thermomethanolica* (later renamed *Ogataea thermomethanolica*) was shown to grow at high temperature and to utilize methanol as the sole carbon source (Limtong et al. 2005, 2008). In addition, this strain has been used successfully as a host for expression of recombinant protein under *P. pastoris* promoters (Tanapongpipat et al. 2012). It could also be transformed with a relatively high efficiency (Tanapongpipat et al. 2012). This yeast strain, therefore, has high potential to be developed as a host for heterologous expression at moderately high temperature and the use of its

native promoter could further increase its effectiveness as a host.

In order to develop *P. thermomethanolica* as a host strain for heterologous protein expression especially at higher temperature than *P. pastoris* fermentation, a strong promoter (either constitutive or inducible) capable of functioning at elevated temperature is required. Currently, several inducible promoters have been isolated and utilized for protein expression in *P. pastoris* (Macauley-Patrick et al. 2005; Cai et al. 2013). However, inducible expression can pose problems with the requirement to change medium or the need to use hazardous substances that are toxic to environment. Therefore, efforts have been made to identify several constitutive promoters for use in constitutive expression. The glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter has been found to be a strong, constitutive promoter and is likely to be suitable for constitutive expression of heterologous proteins (Bitter and Egan 1984; Waterham et al. 1997; Heo et al. 2003). The robustness of the *GAP* promoter is also reflected by the fact that *GAP* mRNAs can represent up to 5 % of total mRNAs in eukaryotic cells (Van Bogaert et al. 2008). In this work, the *GAP* gene was isolated from *P. thermomethanolica* BCC16875 and its promoter was investigated for expression of heterologous protein. The ability of the promoter to function at normal temperature and high temperatures up to 42 °C was explored and compared with the *GAP* promoter from *P. pastoris*. Utilization of the *GAP* promoter to drive expression of a reporter gene in media containing fermentable and non-fermentable carbon sources was also investigated.

Pichia thermomethanolica BCC16875 was obtained from the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Thailand. *P. thermomethanolica* BCC16875 was grown in YPD (1 % yeast extract, 2 % peptone, and 2 % dextrose), YPG (1 % yeast extract, 2 % peptone, and 2 % glycerol), or YPM (1 % yeast extract, 2 % peptone, and 2 % methanol) medium. To obtain partial sequence of the *GAP* gene from *P. thermomethanolica* BCC16875, the conserved regions of GAP protein sequences from various yeast species (*Hansenula polymorpha*, *Ogataea parapolymorpha*, *Pichia minuta*, *Pichia methanolica*, *Pichia stipitis*, *Pichia pastoris*, *Pichia ciferrii*, and *Pichia guilliermondii*) were aligned with the ClustalW program (Larkin et al. 2007). The resulting sequence alignment was used to design degenerate primers GAP_degenF1 (5′-GCNTAYATGTTYAARTAYGAYWSNACNCAYGG-3′) and GAP_degenR2 (5′-CCRTCNGGNCCRTCNACNGTYTTYTGNGTiGC-3′). PCR was done with *P. thermomethanolica* BCC16875 genomic DNA as a template. PCR conditions were initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min with a final incubation at 72 °C for 10 min. The PCR product was subsequently subjected to DNA sequencing (BioDesign, Klong Luang Pathumthani, Thailand).

The partial sequence of the *GAP* coding region obtained by degenerate-primed PCR was used to design primers for genome walking in order to obtain the upstream and downstream regions of the *GAP* gene. Two primers, GAPwalkingR1 (5′-CAAGTACTTGCCCTCGGAGGTGACAGTTCC-3′) and GAPwalkingR2 (5′-CTCCGGTGGAGTCCAAGACGTAGTCAACG-3′), were used for PCR walking in the upstream direction. To obtain downstream *GAP* sequence, GAPwalk_downF1 (5′-GGTAAGGAGGGCGTTGACTACGTCTTGGAC-3′) and GAPwalk_downF2 (5′-GTTAACCACGAGGAGTACAAGCCAGAGATC-3′) primers were used. The resulting PCR products were then cloned into a plasmid vector and sequenced. The nucleotide sequence for the *GAP* gene, including its promoter and 3′ untranslated regions, from *P. thermomethanolica* BCC16875 was deposited in GenBank database under accession number JX392982. The transcription start site within the putative promoter region was predicted using the promoter prediction tool from Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html) (Reese 2001). MatInspector 8.0 (Cartharius et al. 2005) was used to predict transcription factor binding sites.

To clone the putative *GAP* promoter, named PthGAP, from *P. thermomethanolica* BCC16875, approximately 610 bp of *GAP* upstream region was amplified by PCR containing GAP_thermomet_promoterF1 (5′-GCATGGATCCGATTTACTGAGGAGAAGCTATAAAT-3′) and GAP_thermomet_promoterR1 (5′-GCGGTTCGAATATATTATCTTGTTGTTATAAGCAATTG-3′) primers. The resulting PCR product was digested with *Bam*HI and *Bst*BI and used to replace the *Bgl*II–*Bst*BI fragment of the pGAPZαA-HIS plasmid, which was previously constructed by inserting *P. pastoris HIS4* gene into the *Bam*HI site of the pGAPZαA vector (Invitrogen, Carlsbad, CA). The resulting construct was named pPthGAPZα. To clone rPhyA170 phytase gene under the control of the PthGAP promoter, the phytase gene from the pPICZαA-rPhyA170 plasmid (Promdonkoy et al. 2009) was cloned into *Eco*RI–*Xba*I site of the pPthGAPZα construct. The resulting plasmid was named pPthGAPZα-PHY and the correct construct was verified by sequencing. The phytase gene was also ligated with the pGAPZαA-HIS vector to generate pGAPZα-PHY construct. The construct was then transformed into *P. thermomethanolica* BCC16875 according to description in Tanapongpipat et al. (2012).

To express rPhyA170 at various temperatures, the recombinant yeast was incubated in 5 mL YPD medium at 30 °C overnight with vigorous shaking. The overnight culture was diluted into 50 mL YPD to make a final concentration of 0.2 OD_{600} and further incubated at 30, 37, 40, or 42 °C. Culture supernatant was then collected every 24 h for 3 days. To analyze phytase expression in media containing different carbon sources, cells from overnight culture were collected by centrifugation, washed twice with dH_2O, and cultured in

50 mL YPD, YPG, or YPM medium. Cultures were incubated at 30 °C and culture supernatant was collected every 24 h for 3 days. Samples equivalent of 0.5 OD_{600} were subjected to SDS-PAGE analysis. Phytase activity was determined as described by Promdonkoy et al. (2009) and shown as average values from three independent experiments. The recombinant phytase rPhyA170 was deglycosylated using PNGaseF according to the manufacturer's instructions (New England Biolabs, Ipswich, MA).

Partial *GAP* gene sequence from *P. thermomethanolica* BCC16875 was obtained by PCR with degenerate primers. The 5′ and 3′ flanking sequences were obtained by genome walking to finally obtain 2,136 bp genomic sequence revealing a putative open reading frame of 1,008 bp and 5′ and 3′ flanking regions of 769 and 359 bp, respectively (Fig. 1 and data not shown). Sequence analysis showed that its deduced ORF of 335 amino acids is similar to GAP proteins from other yeasts, especially *H. polyporpha* and *O. parapolymorpha* with 93 % and 92 % identity, respectively (supplementary Figure 1), indicating close phylogeny among these three organisms. The predicted GAP protein contained amino acids that are thought to be important and highly conserved among GAP proteins from yeast species (Supplementary Figure 1). For example, the N-terminal 1–147 amino acids contain the putative conserved NAD^+-binding domain. Conserved amino acids Cys-151, His-178, Lys-185 thought to involved in catalysis are also present along with the deduced active site at $_{149}$ASCTTNCL$_{156}$. Possible polyadenylation signals were also found downstream of the stop codon.

ClustalW analysis showed that the 769 bp region upstream of the coding region contained only moderate similarity to the *GAP* promoter of other yeasts. This upstream region, especially 200 bp immediately preceding the start codon, was more similar to *H. polymorpha GAP* than *P. pastoris* or *S. cerevisiae GAP* (data not shown). This 769-bp upstream region contained a putative transcription start site, a TATA-like sequence and two CAAT-like sequences, implying its function as a promoter. Moreover, a Kozak translation initiation sequence is also present (Fig. 1). This putative *GAP* promoter, named PthGAP, is also likely to contain several regulatory elements. For example, putative GRF1 (General Regulatory Factor1)-binding site and a GPE (GRF1 site Potentiator Element) (Bitter et al. 1991), which are important for *GAP* promoter activation in *S. cerevisiae*, are present (Bitter and Egan 1984). As in the case in *S. cerevisiae*, these DNA elements are likely to be important for activation of *GAP* promoter function in *P. thermomethanolica* BCC16875, although more direct evidence is required to test this hypothesis. Three putative carbon source-responsive elements (CSRE), with the consensus sequence CCRTYSRNCCG in *S. cerevisiae*, are also present in the PthGAP promoter. These elements are normally present in many genes involved in gluconeogenesis (Bitter et al. 1991) and are activated when glucose concentration is low. Thus, the role of CSREs in the *GAP* promoter is consistent with involvement of the *GAP* gene in gluconeogenesis. Furthermore, a putative heat shock responsive element (HSE), with a perfect match to consensus sequence CNNGAANNTTCNNG, could also be identified. This HSE may be important for the function of the identified *GAP* promoter at high temperature.

Since the *GAP* promoter is a strong promoter, it is most likely an efficient promoter to be utilized for expression of heterologous gene expression (Delroisse et al. 2005; Wang et al. 2012). To utilize the identified *GAP* promoter for efficient heterologous expression, the 610 bp sequence immediately upstream of the *GAP* start codon (containing putative transcription start site, TATA box and CAAT box) from *P. thermomethanolica* BCC16875 was used to test the ability to function as a promoter. This upstream region was placed upstream of the rPhyA170 phytase reporter gene and the resulting pPthGAPZα-PHY construct was then transformed into *P. thermomethanolica* BCC16875. As seen in Fig. 2a, when the strain harboring pPthGAPZα-PHY was grown at 30 °C in YPD medium, protein bands with molecular weight higher than 86 kDa could be detected by SDS-PAGE analysis, whereas no protein band could be detected in the strain harboring pPthGAPZα vector. This indicated that the PthGAP promoter can be utilized effectively for constitutive expression of heterologous protein. The recombinant protein bands detected were similar to those observed in Tanapongpipat et al. (2012) when expressed by the *P. pastoris GAP* promoter and therefore should represent rPhyA170 with a heterogeneous glycosylation pattern. After treatment with PNGaseF to eliminate the *N*-linked glycan moiety, the expressed rPhyA170 became more homogenous and exhibited a mobility of 51 kDa on SDS-PAGE as expected (Fig. 2b). After 1 day of cultivation, the strain harboring the pPthGAPZα-PHY construct exhibited phytase activity more than twice as much as that exhibited by the strain with pGAPZα-PHY construct containing the *GAP* promoter from *P. pastoris* (Fig. 2C). This phenomenon was subsequently more pronounced after 2 days. After 2 days, the culture reached saturation and the level of expression did not increase further.

Since *P. thermomethanolica* is considered thermotolerant, the pPthGAPZα-PHY-containing strain was also grown at 37, 40, and 42 °C to investigate the ability of the *P. thermomethanolica GAP* promoter to function at a temperature higher than 30 °C. The results in Fig. 2c showed that the recombinant phytase could be produced at all temperatures tested, indicating that the *GAP* promoter could drive expression of the rPhyA170 gene effectively at least up to 42 °C. The ability of the PthGAP promoter to drive heterologous gene expression at moderately high temperature up to 42 °C is a considerable advantage over *P. pastoris*, especially for large-scale fermentation because cooling costs can be greatly reduced. The recombinant strain harboring pPthGAPZα-PHY

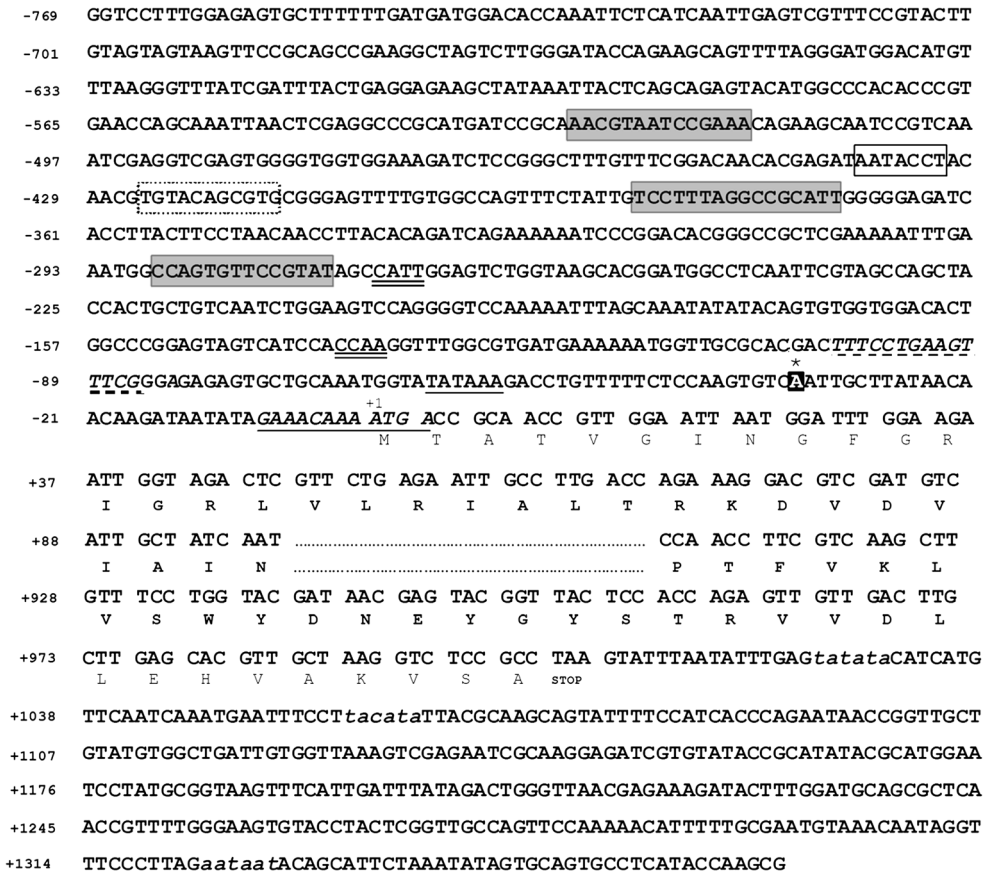

Fig. 1 *Pichia thermomethanolica* BCC16875 *GAP* 5′ and 3′ flanking regions. The nucleotide sequence of glyceraldehyde-3-phosphate dehydrogenase (*GAP*) 5′ and 3′ flanking regions is shown. *Black shading/asterisk* Putative transcription start site, *bold letters underlined* putative TATA box, *bold letters double underlined* two possible CAAT boxes, *underlined italic letters* potential Kozak sequence (including the start codon), *dotted rectangular box* putative GRF1 binding site, *closed box* putative GPE element, shaded boxes three possible carbon source responsive elements (CSREs), *underlined by dashed line* putative heat shock element, *italic lower case letters* putative polyadenylation signals in the 3′ flanking region. Several amino acids at the start and the end of the coding region are shown as *one-letter code* below the nucleotide sequence (the majority of the coding region is omitted for clarity). The number *+1* indicates the adenine residue of the ATG start codon

displayed higher levels of expressed phytase than the recombinant strain harboring pGAPZα-PHY at all temperatures tested, demonstrating the higher efficiency of the native *GAP* promoter from *P. thermomethanolica* BCC16875 than the promoter from *P. pastoris* at various temperatures.

To investigate the effect of different carbon sources on expression of the recombinant phytase from the *GAP* promoter, the strain harboring the pPthGAPZα-PHY construct was cultured in media containing glucose, glycerol, or methanol. It was found that PthGAP promoter was functional in media containing glucose, glycerol, or methanol which is useful for heterologous gene expression in different carbon sources. The steady-state levels of rPhyA170 were found to be the highest in glucose-containing medium and lowest in the methanol-containing medium (Fig. 2d, Supplementary Figure 2). After 2 days of cultivation, approximately 45 % and 11 % of phytase activity was detected from YPG and YPM media, respectively, compared with the activity detected from YPD medium. The high level of rPhyA170 expression in glucose-containing medium is similar to the report by Waterham et al. (1997) with the *GAP* promoter from *P. pastoris* and β-lactamase reporter gene. In contrast, Heo et al. (2003) found that the *GAP* promoter from *H. polymorpha* gave higher expression of the reporter gene in glycerol than in glucose. Expression by the PthGAP promoter is low in methanol, which is consistent with results in *P. pastoris* and *H. polymorpha* (Waterham et al. 1997; Heo et al. 2003). This may be due to the down-regulation of the *GAP* gene in the presence of methanol similar to *H. polymorpha* (van Zutphen et al. 2010) and rat retinal tissue (Chen et al. 2012). In addition, another factor that most likely contributes to the low expression of the reporter gene is the slow growth of *P. thermomethanolica* in YPM medium which may be caused by differential regulation of methanol metabolism similar to *P. pastoris* (Kumar and Rangarajan 2012). Finally, some regulatory element(s) may be responsible for different rates of transcription in fermentable and non-fermentable carbon sources, similar to the *GAP* promoter from *S. cerevisiae* (Kuroda et al. 1994). Deletion mapping of the PthGAP promoter may help identify regulatory elements controlling promoter activity in fermentable and non-fermentable carbon sources.

In conclusion, the *GAP* promoter was isolated successfully from *P. thermomethanolica* BCC16875 and utilized to drive efficient expression of a reporter gene at various temperatures up to 42 °C, which is advantageous for large-scale industrial fermentation. This ability makes *P. thermomethanolica* BCC16875 a possibly superior host compared with *P.*

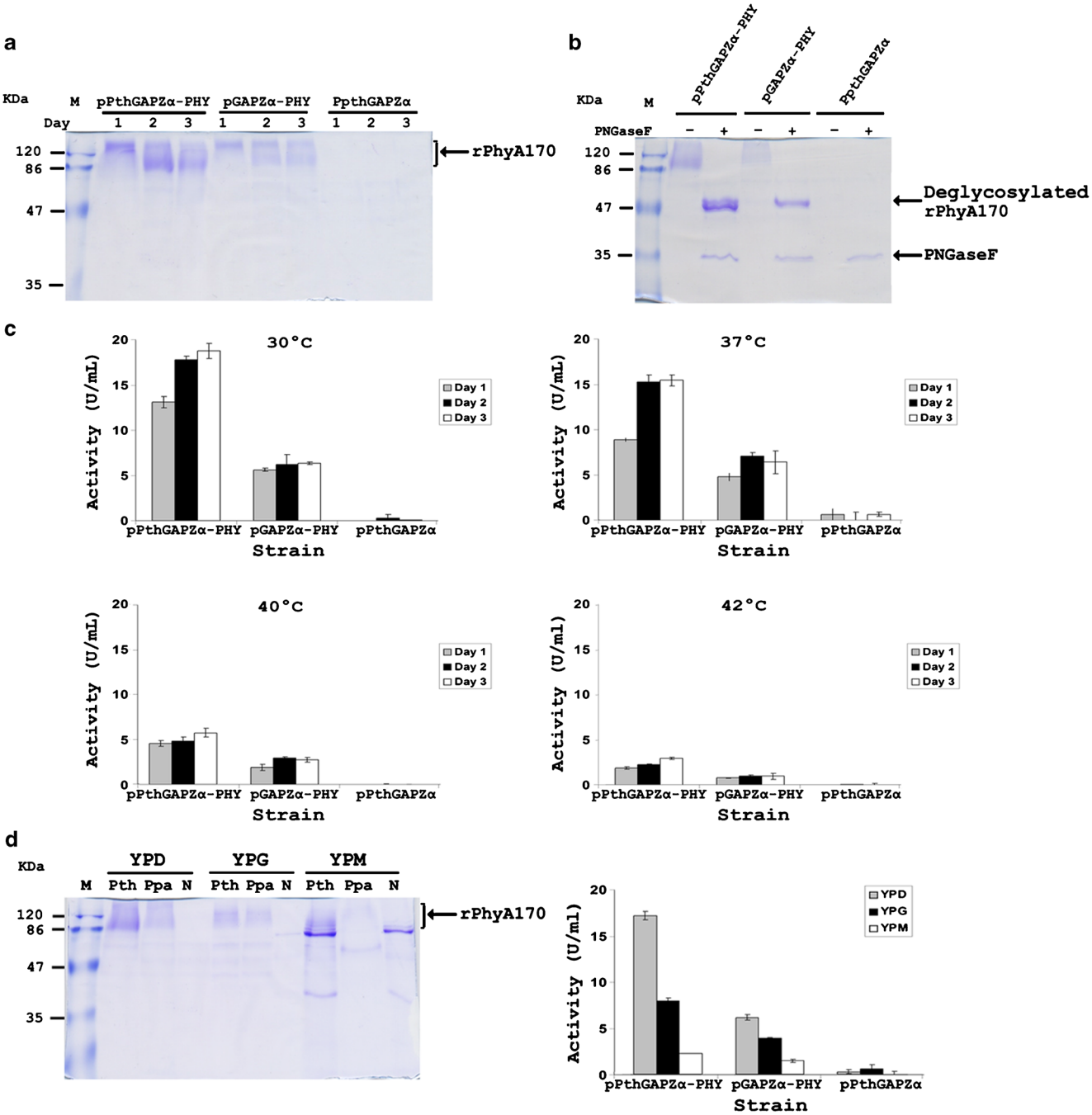

Fig. 2 **a**–**d** Expression of phytase by the *GAP* promoter at various temperatures and in medium containing glucose, glycerol, or methanol. **a** SDS-PAGE analysis showing recombinant phytase expressed by the *GAP* promoter at 30 °C. Culture supernatant from cells harboring pPthGAPZα-PHY, pGAPZα-PHY, and pPthGAPZα constructs grown in YPD was collected every 24 h for 3 days. After subjecting to SDS-PAGE, the gels were stained with Coomassie Brilliant Blue R-250. Lane *M* Protein marker, *arrow* expressed rPhyA170. **b** SDS-PAGE analysis of deglycosylated rPhyA170 after treatment (+) or not (−) with PNGaseF. Positions of the deglycosylated phytase and PNGaseF are indicated by *arrows*. **c** Phytase activity from cells harboring pPthGAPZα-PHY, pGAPZα-PHY, and pPthGAPZα constructs grown at 30, 37, 40, and 42 °C. Activities shown are average values from three independent experiments. *Error bars* Standard deviation. **d** SDS-PAGE analysis and phytase activity showing recombinant phytase expressed by the *GAP* promoter in medium containing glucose (YPD), glycerol (YPG), or methanol (YPM). Culture supernatant from cells harboring pPthGAPZα-PHY, pGAPZα-PHY, and pPthGAPZα grown in indicated medium was collected after 48 h. Lanes: *Pth* pPthGAPZα-PHY strain, *Ppa* pGAPZα-PHY strain, *N* pPthGAPZα strain, *M* protein marker. Activities shown are average values from three independent experiments. *Error bars* Standard deviation

pastoris for heterologous gene expression. In addition, the promoter can also be used for heterologous protein expression in both fermentable and non-fermentable carbon sources, thus providing a new tool for yeast expression systems.

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