J. Microbiol. Biotechnol. (2007), **17**(12), 1949–1954

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Variations in Protein Glycosylation in *Hansenula polymorpha* Depending on Cell Culture Stage

KIM, SO-YOUNG[1,2], JUNG-HOON SOHN[1], YU-RYANG PYUN[2], AND EUI-SUNG CHOI[1]*

[1]*Systems Microbiology Research Center, KRIBB, Daejeon 305-333, Korea*
[2]*Department of Biotechnology, Yonsei University, Seoul 120-749, Korea*

Received: March 12, 2007 Accepted: May 18, 2007

Abstract A simple way to prevent protein hyperglycosylation in *Hansenula polymorpha* was found. When glucose oxidase from *Aspergillus niger* and carboxymethyl cellulase from *Bacillus subtilis* were expressed under the control of an inducible methanol oxidase (*MOX*) promoter using methanol as a carbon source, hyperglycosylated forms occurred. In contrast, *MOX*-repressing carbon sources (*e.g.*, glucose, sorbitol, and glycerol) greatly reduced the extent of hyperglycosylation. Carbon source starvation of the cells also reduced the level of glycosylation, which was reversed to hyperglycosylation by the resumption of cell growth. It was concluded that the proteins expressed under actively growing conditions are produced as hyperglycosylated forms, whereas those under slow or nongrowing conditions are as short-glycosylated forms. The prevention of hyperglycosylation in the *Hansenula polymorpha* expression system constitutes an additional advantage over the traditional *Saccharomyces cerevisiae* system in recombinant production of glycosylated proteins.

Keywords: Glycosylation, *Hansenula polymorpha*, glucose oxidase, CMCase

The methylotrophic yeast *Hansenula polymorpha*, together with *Pichia apstoris*, has been recently studied as an efficient host for the production of foreign proteins, owing to its availability of strong regulated promoters such as methanol oxidase (*MOX*), and the ease of growth to high cell density [8, 9, 17, 22]. Although *H. polymorpha* has some superior features as an expression host compared with the traditional host *Saccharomyces cerevisiae*, little is known about the glycosylation of recombinant protein from this yeast. Many heterologous proteins are hyperglycosylated when produced in *S. cerevisiae* by the addition of long α-1,6-linked mannose chains with α-1,2- and α-1,3-side chains, which is different from mammalian glycosylation [19]. The hyperglycosylation significantly contributes to the high molecular mass and heterogeneity of most secretory proteins [4, 5, 16]. These types of glycosylations on the recombinant proteins could also be immunogenic, and thus the regulation of the glycosylation has been raised as an important issue in the biotechnology industry [10]. Although methylotrophic yeast showed a lesser extent of glycosylation on the foreign glycoproteins than those of *S. cerevisiae* [6, 11], yeast-derived recombinant glycoproteins are generally hyperglycosylated compared with those of mammals, which has been a major drawback of yeast as a host for the production of recombinant glycoproteins. To solve such problems in *H. polymorpha*, we previously cloned and characterized the *MNN9* of *H. polymorpha* for the development of a deletion strain, which might have a defect in the addition of a long mannose chain as found in *S. cerevisiae* [13, 24]. During the course of the work, we found a dramatic reduction of the extent of glycosylation of proteins by the addition of sorbitol used as a stabilizer for the Δmnn9 mutant. Further analyses with other carbon sources such as glucose and glycerol also showed apparent reductions of glycosylation on proteins in contrast to methanol as the carbon source. Finally, we found that the extent of glycosylation was greatly influenced by the cell culture stage in protein production.

*Corresponding author
Phone: 82-42-860-4453; Fax: 82-42-860-4489;
E-mail: choi4162@kribb.re.kr

Materials and Methods

Yeast Strains and Media

The *H. polymorpha* strain used in this study was DL1-L, a *LEU2* defective strain of DL1 (ATCC26012). All strains were routinely grown in YPD medium (1% yeast extract, 2% Bactopeptone, 2% glucose) or SD medium (0.67% yeast nitrogen base w/o amino acid, 2% glucose) with shaking at 37°C. To induce the expression of foreign proteins, the transformant was precultured overnight in

YPD medium, and transferred to YPM (1% yeast extract, 2% Bactopeptone, 2% methanol) or MM (0.67% yeast nitrogen base w/o amino acid, 2% methanol) media with or without 2% of an appropriate carbon source.

Plasmid Construction

General DNA manipulations were performed as described by Sambrook *et al.* [20]. Plasmid pDLMOX-HIR [11], which contains the *MOX* promoter, HARS36 for autonomous replication [21], and *HLEU2* gene [1], was used as a backbone plasmid for construction of the glucose oxidase (GOD) expression plasmid. The 1.8 kb glucose oxidase gene and the α-amylase signal sequence were obtained by polymerase chain reaction (PCR) from the pGAL-GO2 plasmid containing the GOD gene of *Aspergillus niger* [12] and α-amylase secretion signal sequence of *Aspergillus oryzae* [23], using primer pairs GODN (5'-GCTCTTC-TAGCAATGGCATTGAAGCCAG-3') and GODC (5'-CTCGAGAAGCTTCTAGATCACTGCATGGAAGCATAA-3'), and amy1 (5'- CGAATTCAAAAATGGTCGCGTGGT-GGTGGTCTC-3') and amy2 (5'-TGGCGCCGGCCAAA-GCAGGTGCCGCGAC-3'), respectively. The amplified GOD gene and α-amylase signal sequence were subcloned into the pBluescript KS (+) (Stratagene) vector to construct pBKS-GOD and pBKS-AMY, respectively. pDLMOX-GOD (Fig. 1) was constructed by subcloning the HindIII/NaeI fragment of the α-amylase signal sequence from pBKS-AMY and the 1.8 kb SapI/HindIII fragment of the GOD gene from pBKS-GOD into the HindIII site of pDLMOX-HIR. To construct the carboxymethyl cellulase (CMCase) expression vector, pLDKCMC8 (Fig. 1), the 1.4 kb CMCase gene from the pBS1 plasmid containing the CMCase gene of *Bacillus subtilis* [18] was PCR-amplified and subcloned into the XhoI site of plasmid pSM1 [2], to be placed under the control of the *MOX* promoter and killer toxin signal sequence.

Analysis of the Glycosylation Pattern of GOD and CMCase

H. polymorpha transformants possessing pDLMOX-GOD or pLDK-CMC8 were cultured in an appropriate medium for 24 h and the culture supernatant was analyzed by Western blot using the polyclonal antibodies against GOD and CMCase, respectively. Glucose oxidase antibody was purchased from Accurate Biochemicals, and CMCase antibody was kindly given by J. S. Ahn (KRIBB, Korea).

Activity staining of the electrophoresis gels and halo plates indicated that the extent of glycosylation of the reporter enzymes seemingly did not create much difference in their enzyme activities (data not shown).

RESULTS

Reduced Extent of Protein Glycosylation by Sorbitol in *H. polymorpha*

A foreign glycoprotein, glucose oxidase (GOD) from *A. niger*, was selected and expressed in the yeast *H. polymorpha* to analyze the pattern of glycosylation. GOD is a dimeric flavoprotein converting β-D-glucose to glucono-δ-lactone and hydrogen peroxide. Heterologous expression of GOD in *S. cerevisiae* and *H. polymorpha* has previously been described [7, 12]. In both cases, GOD was hyperglycosylated even though the extent of glycosylation was different. When GOD was produced in *H. polymorpha* under the condition of a fully induced *MOX* promoter and exponential cell growth, the GOD thus produced was more heterogeneous in size and had a higher molecular weight compared with that from *S. cerevisiae* [7]. In our study, however, it was accidentally found that a mere addition of sorbitol to the normal growth and induction medium, YPM, greatly reduced the extent of glycosylation of GOD expressed using a similar expression mode in *H. polymorpha*. The GOD gene was fused to the *A. oryzae* α-amylase signal sequence and expressed under the control of the methanol oxidase (*MOX*) promoter (Fig. 1). A transformant was cultivated in the YPM media with or without 2% sorbitol for 24 h. The culture supernatant and cell-free extract were then analyzed for the extent of glycosylation on GOD by Western blot using anti-GOD antibody (Fig. 2A). The GOD expressed in the YPM medium with sorbitol showed

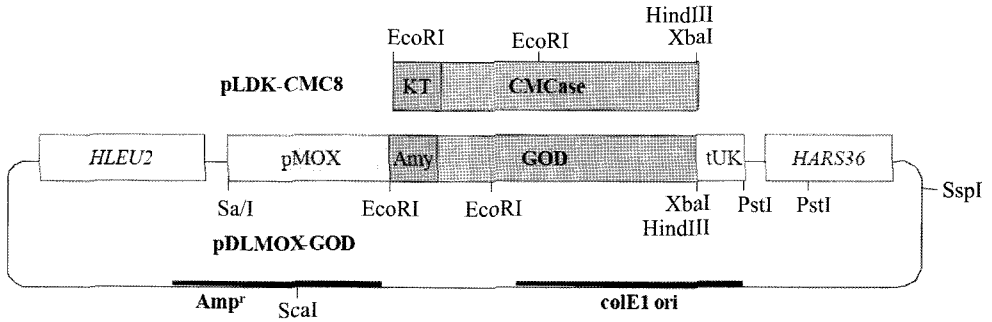

Fig. 1. Expression plasmids, pDLMOX-GOD and pLDK-CMC8, used in this study.
Abbreviations: pMOX, *MOX* promoter; Amy, α-amylase signal sequence; KT, killer toxin signal sequence; tUK, unknown terminator [9].

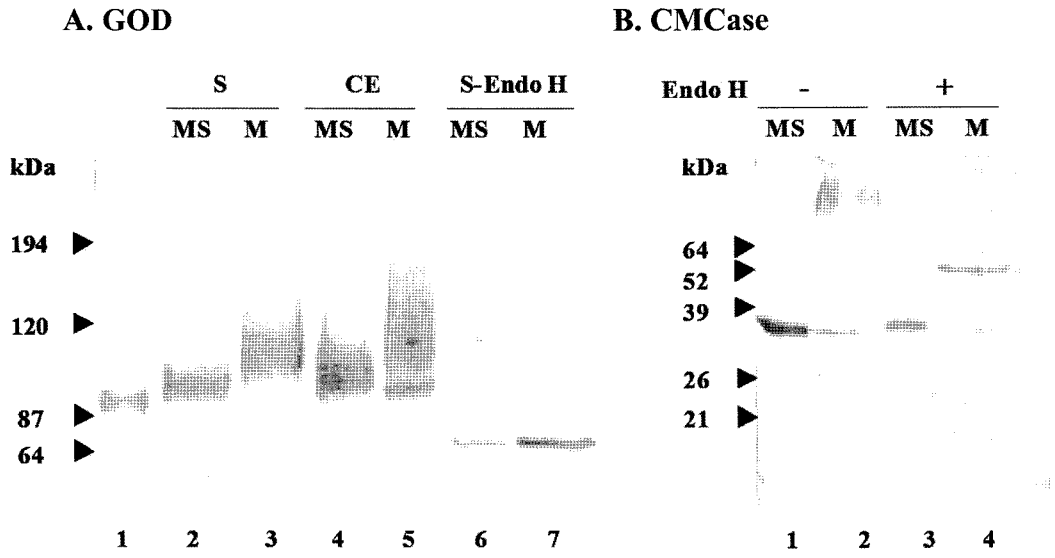

Fig. 2. Glycosylation patterns of GOD and CMCase expressed in *H. polymorpha*. **A.** Expression of GOD.
Standard GOD from *A. niger* was used as control (lane 1). The culture supernatant (S: lanes 2, 3, 6, 7) and total cell extract (CE: lanes 4, 5) were prepared from a transformant grown for 24 h in YP media containing 2% methanol and 2% sorbitol (MS: lanes 2, 4, 6) or YP media containing 2% methanol (M: lanes 3, 5, 7). The culture supernatant of each sample was incubated with Endo H (lanes 6, 7) under the condition suggested by the supplier (NEB). **B.** Expression of CMCase. The culture supernatant was prepared from a transformant grown for 24 h in YP media containing 2% methanol and 2% sorbitol (MS: lanes 1, 3) or YP media containing 2% methanol (M: lanes 2, 4). Each sample was incubated with Endo H (lanes 3, 4).

a discrete band (lane 2), slightly bigger than the core-glycosylated authentic GOD from *A. niger* (lane 1). In contrast, the protein from the medium without sorbitol showed a diffuse hyperglycosylated band (lane 3). Endo-H-treated GOD from either case showed no difference in size (lanes 6 and 7), indicating that the difference is caused by the amount of mannose residue. The GOD present in the intracellular fraction was also affected by sorbitol in a similar fashion (lanes 4 and 5). These results clearly indicate that the extent of glycosylation of secreted GOD was greatly influenced by sorbitol.

This phenomenon seemed to be a quite unusual case found in protein glycosylation in that the extent of protein glycosylation is regulated by a media component. To verify whether this phenomenon is restricted to a protein GOD, we tested another foreign glycoprotein, CMCase from *B. subtilis* [18]. Expression of CMCase in *S. cerevisiae* showed that the CMCase excreted into the culture supernatant was mostly hyperglycosylated [15]. CMCase expression was carried out using the same conditions used for GOD expression to test the sorbitol effect on the glycosylation of CMCase in *H. polymorpha*. The glycosylation patterns of CMCase in *H. polymorpha* were checked by Western blot using anti-CMCase antibody (Fig. 2B). As expected, the CMCase produced in YPM medium with sorbitol migrated as a discrete 35 kDa CMCase band, whereas that produced in the same medium without sorbitol appeared as the hyperglycosylated forms. The molecular mass of 35 kDa observed for the less-glycosylated form is smaller than the molecular mass of 52 kDa calculated from the nucleotide sequence. This discrepancy in the molecular masses can be explained by the fact that the molecular mass of the native CMCase from *B. subtilis* or the recombinant CMCase from *E. coli* is 35 kDa, resulting from either processing or degradation of the primary translation product [18]. This posttranslational modification was not observed in *S. cerevisiae*, most probably because of the protection of CMCase by hyperglycosylation. Endo-H-treated CMCase from *S. cerevisiae* was found mostly as a 52 kDa protein [15]. Interestingly, CMCase from *H. polymorpha* expressed with the medium containing sorbitol was detected as a 35 kDa protein with or without treatment of Endo H, suggesting the occurrence of its posttranslational processing being owing to the absence of hyperglycosylation, as found in *B. subtilis* and *E. coli*. In contrast, Endo-H-treated CMCase expressed under the condition without sorbitol appeared as a 52 kDa protein, as found in hyperglycosylated CMCase from *S. cerevisiae*. The result indicated that sorbitol could play an important role in reduction of the extent of glycosylation of CMCase as well as the GOD expressed in *H. polymorpha*. Therefore, it appeared that the presence of sorbitol in the growth medium might be generally effective in the reduction of hyperglycosylation of proteins expressed in *H. polymorpha*.

Influence in the Extent of Glycosylation by Cell Growth Stage

Because this sorbitol effect was minimal in *S. cerevisiae* (data not shown), we initially assumed that sorbitol functions as a stabilizer for the cells growing in methanol, which may be related to the reduced level of glycosylation

by an unknown mechanism. Thus, we tested other known stabilizers for cell integrity, potassium phosphate and NaCl, for possible influence on the level of glycosylation. Addition of these stabilizers into the medium, however, resulted in hyperglycosylated forms of proteins and showed no difference with those obtained from the medium without sorbitol. Next, it was noted that the effect of sorbitol could be different for *H. polymorpha* and *S. cerevisiae* because of the differences in sorbitol metabolism in these two organisms. Unlike *S. cerevisiae*, *H. polymorpha* could utilize sorbitol as a sole carbon source [3]. Sorbitol represses the transcription from the *MOX* promoter, as in the case of other repressing carbon sources such as glucose and glycerol (Sohn *et al.*, unpublished results). Therefore, in *H. polymorpha*, the expression of GOD or CMCase, which is controlled by the *MOX* promoter, can be repressed in the medium containing both methanol and sorbitol until this repression is derepressed by depletion of sorbitol and induction by methanol occurs. In the medium without sorbitol, on the other hand, the expression of GOD or CMCase would be induced by methanol continuously from the beginning of cell culture. This difference appeared to influence greatly the extent of protein glycosylation. If this is the case, other repressing carbon sources for the *MOX* promoter might also give the same effect on the extent of protein glycosylation. Cells were cultivated in YPM medium with one of the two repressing carbon sources, glucose or glycerol, or a nonrepressing carbon source, xylose (Sohn *et al.*, unpublished results), and the glycosylation patterns of GOD were analyzed. As shown in Fig. 3, the extent of glycosylation was reduced in the cases of the repressing carbon sources relative to that of methanol, even though the extent was slightly different. In contrast, no change was found in a nonrepressing carbon source, xylose. The result indicated that the effect should not be specific for sorbitol, but rather, general for repressing carbon sources for the *MOX* promoter.

To analyze further the correlation between the extent of glycosylation and the repressing carbon sources, the effect of the repressing carbon source on the extent of

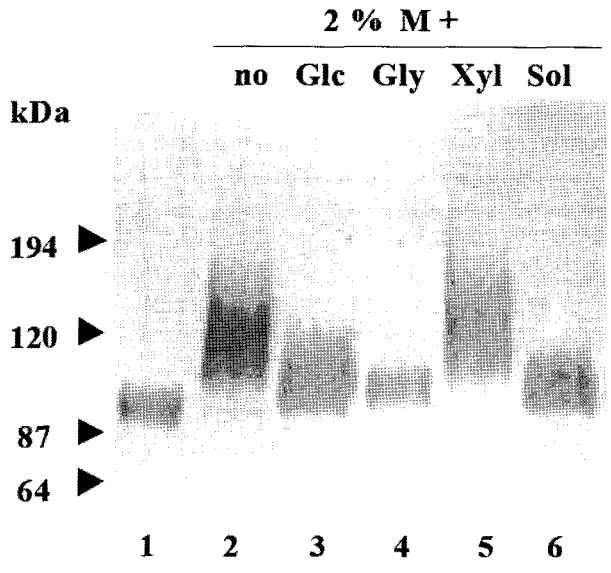

Fig. 3. Effect of carbon sources on the extent of glycosylation.
Standard GOD from *A. niger* was used as control (lane 1). The culture supernatant was prepared from a transformant grown for 24 h in YPM medium with or without an appropriate repressing carbon source. Lane 2: YPM; lane 3: YPM plus 2% glucose; lane 4: YPM plus 2% glycerol; lane 5: YPM plus 2% xylose; lane 6: YPM plus 2% sorbitol.

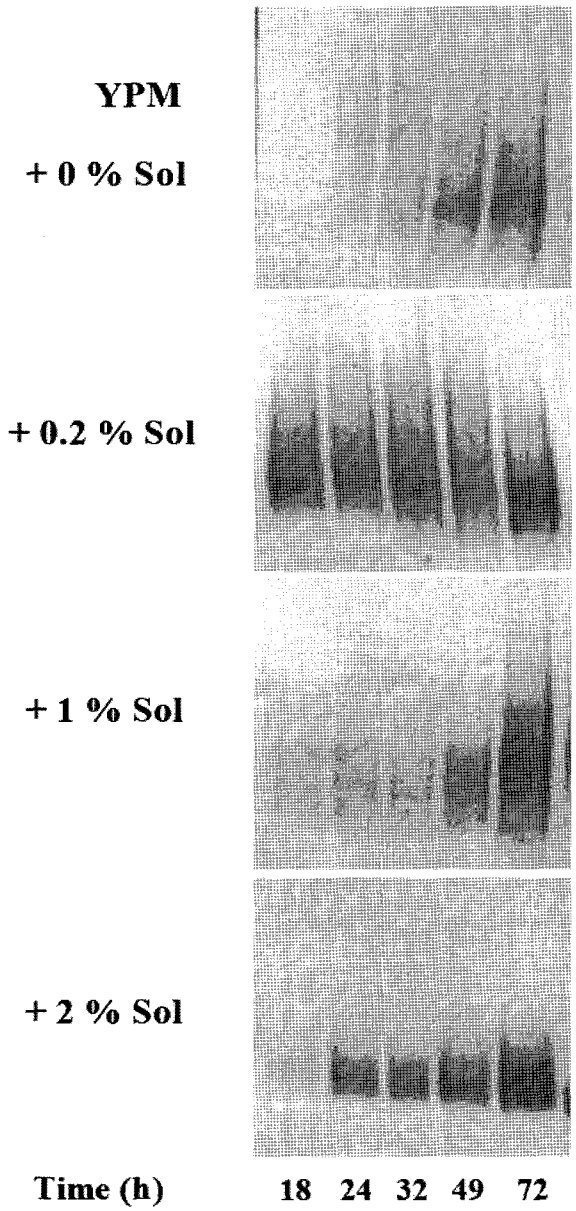

Fig. 4. Effect of different concentrations of sorbitol on the extent of glycosylation.
Cells were cultivated in YPM medium with the indicated concentration of sorbitol, and culture supernatants were taken with time intervals.

glycosylation was investigated with varying concentrations of sorbitol. Cells were cultivated in YPM media with different concentrations of added sorbitol. Culture supernatants were taken with time intervals and analyzed for patterns of protein glycosylation (Fig. 4). The extent of glycosylation in GOD was apparently increased by the decrease of sorbitol concentration. Specifically, severe hyperglycosylation was found when GOD expression was induced by depletion of sorbitol during the exponential growth phase (up to 32 h). Fast depletion of sorbitol during the exponential phase induces the diauxic shift to use methanol as a second carbon source. Thus, the expression of GOD was induced by methanol at the exponential growth phase and resulted in hyperglycosylation of GOD. In contrast, prolonged repression of the *MOX* promoter with a high concentration of sorbitol to the stationary phase resulted in the reduced glycosylation. In order for the hyperglycosylation to occur, it seemed to be necessary to have a condition of exponential cell growth using methanol as a carbon source in addition to the promoter induction by methanol. However, GOD expression with a constitutive *GAPDH* promoter also produced the hyperglycosylated form using any carbon sources in the exponential growth phase (data not shown). Taken together, these suggested that GOD produced from the fast growing cells in the exponential phase is exclusively the hyper-forms, whereas GOD from slow or no-growing cells in the stationary phase is of short-form, independent of the carbon sources used.

The carbon source starvation experiment clearly showed the effect of cell growth stage on the extent of protein glycosylation (Fig. 5A). Cells of stationary phase producing short-glycosylated forms of GOD (YPDM) were transferred to the minimal medium without (M) and with a carbon source, methanol (MM). M medium could not support cell growth but could derepress the *MOX* promoter. GOD produced without cell growth remained as a short-glycosylated form. On the other hand, cell growth resumed in the MM medium and, at the same time, methanol induced the GOD expression from the *MOX* promoter. Thus, GOD expression under active cell growth resulted in the increased extent of glycosylation. Furthermore, cells producing hyperglycosylated GOD from methanol-containing medium (YPM) were also transferred to the fresh MM and M (Fig. 5B). Whereas resumption of cell growth in MM showed no change in the extent of glycosylation, GOD expression by derepression of the *MOX* promoter without cell growth in M medium dramatically reduced the extent of glycosylation.

DISCUSSION

From the results described above, it was concluded that one of the major factors determining the extent of glycosylation in *H. polymorpha* is the cell growth stage and it may be possible to avoid the hyperglycosylation of heterologous glycoproteins produced in *H. polymorpha* by a simple adjustment of growth condition. Little is known about the regulation of protein glycosylation. Kukuruzinska and Lennon-Hopkins [14] recently reported that the mRNA concentration of the *ALG* gene family, related with the first *N*-glycosylation pathway, is regulated depending on the cell growth stage in *S. cerevisiae*, even though the mannosylation pattern of the protein was not affected. We also made the same situation for *S. cerevisiae* and another

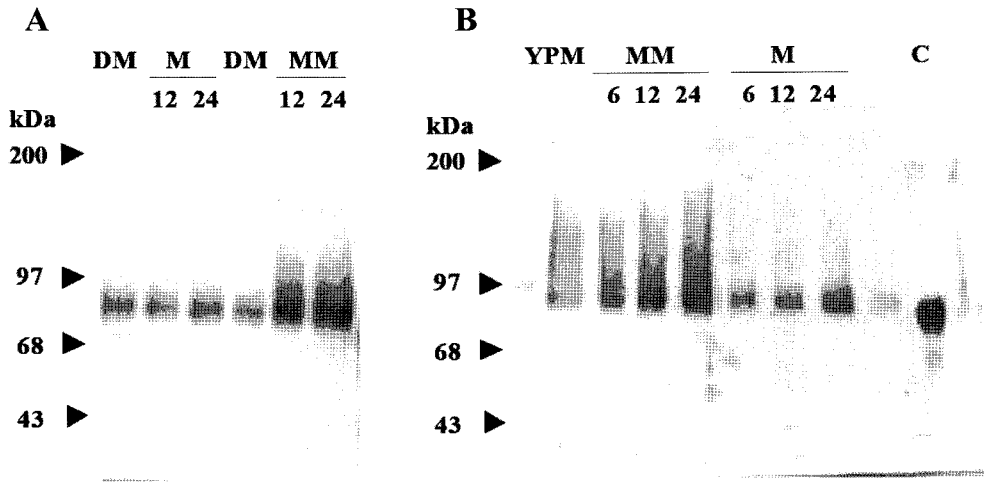

Fig. 5. Effect of cell culture condition on the extent of glycosylation.
A. Alteration of short forms of glycosylation to hyper-forms by resumption of cell growth. A transformant grown for 24 h in YPDM medium (DM) was transferred into fresh minimal medium without carbon source (M) or with 2% methanol (MM). Culture supernatant was taken 12 and 24 h after medium change. **B.** Alteration of hyper-forms of glycosylation to short-forms. A transformant grown for 24 h in YPM medium (YPM) was transferred into fresh minimal medium with 2% methanol (MM) or without carbon source (M). Culture supernatant was taken 6, 12, and 24 h after medium change. Standard GOD from *A. niger* was used as control (C).

methylotrophic yeast, *Pichia pastoris*, as in *H. polymorpha* using GOD expression under the control of the *GAL* promoter in *S. cerevisiae* and the *AOX* promoter in *P. pastoris* and tested the effect of growth condition on the extent of glycosylation. In these two species, however, such a remarkable change of glycosylation pattern observed in *H. polymorpha* was not found either in the cells from induction medium containing a repressing carbon source for each promoter or in stationary phase cells (data not shown). Therefore, the phenomenon observed in this study appears to be quite specific in *H. polymorpha*. The yeast *H. polymorpha* may have a unique regulation pathway responsible for the different extent of glycosylation depending on cell growth conditions. Further mutational approaches and analyses of glycosylation patterns of proteins expressed in various conditions would provide new clues for the extraordinary regulation mechanisms of glycosylation in *H. polymorpha* and may help obtain recombinant proteins with glycan structures more suitable for biotechnological purposes.

REFERENCES

1. Agaphonov, M. O., M. Beburov, M. D. Ter-Avanesyan, and V. N. Smirnov. 1995. A disruption-replacement approach for the targeted integration of foreign genes in *Hansenula polymorpha*. *Yeast* **11:** 1241–1247.
2. Agaphonov, M. O., A. I. Poznyakovski, A. I. Bogdanova, and M. D. Ter-Avanesyan. 1994. Isolation and characterization of the LEU2 gene of *Hansenula polymorpha*. *Yeast* **10:** 509–513.
3. Barnett, J. A., R. W. Payne, and D. Yarrow. 1990. *Yeasts: Characteristics and Identification*, 2nd Edn., Cambridge University Press, Melbourne, Sydney.
4. Boer, P. and E. P. Steyn-Parve. 1966. Isolation and purification of an acid phosphatase from Baker's yeast (*Saccharomyces cerevisiae*). *Biochim. Biophys. Acta* **128:** 400–402.
5. Gascon, S. and J. O. Lampen. 1968. Purification of the internal invertase of yeast. *J. Biol. Chem.* **243:** 1567–1572.
6. Grinna, L. S. and J. F. Tschopp. 1989. Size distribution and general structural features of *N*-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. *Yeast* **5:** 107–115.
7. Hodgkins, M., D. Mead, D. J. Ballance, A. Goodey, and P. Sudbery. 1993. Expression of the glucose oxidase gene from *Aspergillus niger* in *Hansenula polymorpha* and its use as a reporter gene to isolate regulatory mutations. *Yeast* **9:** 625–635.
8. Hollenberg, C. P. and G. Gellissen. 1997. Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.* **8:** 554–560.
9. Hong, I.-P., S. Anderson, and S.-G Choi. 2006. Evaluation of a new episomal vector based on the GAP promoter for structural genomics in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **16:** 1362–1368.
10. Jenkins, N., R. B. Parekh, and D. C. James. 1996. Getting the glycosylation right: Implications for the biotechnology industry. *Nat. Biotechnol.* **14:** 975–981.
11. Kang, H. A., J. H. Sohn, E. S. Choi, B. H. Chung, M. H. Yu, and S. K. Rhee. 1998. Glycosylation of human alpha 1-antitrypsin in *Saccharomyces cerevisiae* and methylotrophic yeasts. *Yeast* **14:** 371–381.
12. Kapat, A., J. K. Jung, and Y. H. Park. 2001. Enhancement of glucose production in batch cultivation of recombinant *Saccharomyces cerevisiae*: Optimization of oxygen transfer condition. *J. Appl. Microbiol.* **90:** 216–222.
13. Kim, S. Y., J. H. Sohn, H. A. Kang, S. K. Yoo, Y. R. Pyun, and E. S. Choi. 2001. Cloning and characterization of the *Hansenula polymorpha* homologue of the *Saccharomyces cerevisiae* MNN9 gene. *Yeast* **18:** 455–461.
14. Kukuruzinska, M. A. and K. Lennon-Hopkins. 1999. ALG gene expression and cell cycle progression. *Biochim. Biophys. Acta* **1426:** 359–372.
15. Lee, J. W., D. O. Kang, B. Y. Kim, W. K. Oh, T. I. Mheen, Y. R. Pyun, and J. S. Ahn. 2000. Mutagenesis of the glucoamylase signal peptide of *Saccharomyces diastaticus* and functional analysis in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **193:** 7–11.
16. MacKay, V. L., S. K. Welch, M. Y. Insley, T. R. Manney, J. Holly, G. C. Saari, and M. L. Parker. 1988. The *Saccharomyces cerevisiae* BAR1 gene encodes an exported protein with homology to pepsin. *Proc. Natl. Acad. Sci. USA* **85:** 55–59.
17. Oh, K. S., O. S. Kwon, Y. W. Oh, M. J. Sohn, S. G. Jung, Y. K. Kim, M. G. Kim, S. K. Rhee, G. Gellissen, and H. A. Kang. 2004. Fabrication of a partial genomic microarray of the methylotrophic yeast *Hansenula polymorpha*: Optimization and evaluation of transcript profiling. *J. Microbiol. Biotechnol.* **14:** 1239–1248.
18. Park, S. H., H. K. Kim, and M. Y. Pack. 1991. Characterization and structure of the cellulase gene of *Bacillus subtilis* BSE616. *Agric. Biol. Chem.* **55:** 441–448.
19. Romanos, M. A., C. A. Scorer, and J. J. Clare. 1992. Foreign gene expression in yeast: A review. *Yeast* **8:** 423–488.
20. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratoty Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Sohn, J. H., E. S. Choi, C. H. Kim, M. O. Agaphonov, M. D. Ter-Avanesyan, J. S. Rhee, and S. K. Rhee. 1996. A novel autonomously replicating sequence (ARS) for multiple integration in the yeast *Hansenula polymorpha* DL-1. *J. Bacteriol.* **178:** 4420–4428.
22. Veale, R. A., M. L. Giuseppin, H. M. van Eijk, P. E. Sudbery, and C. T. Verrips. 1992. Development of a strain of *Hansenula polymorpha* for the efficient expression of guar alpha-galactosidase [published erratum appears in *Yeast* 1992 Sep;**8**(9):813]. *Yeast* **8:** 361–372.
23. Wirsel, S., A. Lachmund, G. Wildhardt, and E. Ruttkowski. 1989. Three alpha-amylase genes of *Aspergillus oryzae* exhibit identical intron-exon organization. *Mol. Microbiol.* **3:** 3–14.
24. Yip, C. L., S. K. Welch, F. Klebl, T. Gilbert, P. Seidel, F. J. Grant, P. J. O'Hara, and V. L. MacKay. 1994. Cloning and analysis of the *Saccharomyces cerevisiae* MNN9 and MNN1 genes required for complex glycosylation of secreted proteins. *Proc. Natl. Acad. Sci. USA* **91:** 2723–2727.