J. Microbiol. Biotechnol. (2009), **19**(9), 966–971
doi: 10.4014/jmb.0901.018
First published online 29 April 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Optimization of the Functional Expression of *Coprinus cinereus* Peroxidase in *Pichia pastoris* by Varying the Host and Promoter

Kim, Su-Jin[1], Jeong-Ah Lee[1], Yong-Hwan Kim[2]*, and Bong-Keun Song[1]*

[1]*Korea Research Institute of Chemical Technology, 100 Jang-dong, Yuseong-gu, Daejeon 305-343, Korea*
[2]*Department of Chemical Engineering, Kwangwoon University, 447-1, Seoul 139-701, Korea*

Received: January 8, 2009 / Revised: February 20, 2009 / Accepted: March 12, 2009

Peroxidase from *Coprinus cinereus* (CiP) has attracted attention for its high specific activity and broad substrate spectrum compared with other peroxidases. In this study, the functional expression of this peroxidase was successfully achieved in the methylotrophic yeast *Pichia pastoris*. The expression level of CiP was increased by varying the microbial hosts and the expression promoters. Since a signal sequence, such as the alpha mating factor of *Saccharomyces cerevisiae*, was placed preceding the cDNA of the CiP coding gene, expressed recombinant CiP (rCiP) was secreted into the culture broth. The Mut$^+$ *Pichia pastoris* host showed a 3-fold higher peroxidase activity, as well as 2-fold higher growth rate, compared with the Mut^s *Pichia pastoris* host. Furthermore, the *AOX1* promoter facilitated a 5-fold higher expression of rCiP than did the *GAP* promoter.

Keywords: *Coprinus cinereus* peroxidase, promoter, *Pichia pastoris*, heterologous expression, recombinant peroxidase

Peroxidases (E.C. 1.11.1.7) are enzymes that use peroxide to oxidize a broad range of compounds, including phenols, aromatic amines, and reduced inorganic compounds, and have active sites that usually contain protoporphyrin IX (heme) and coordinated ferric ions. Occurring in a wide variety of organisms, including plants, animals, and microorganisms with specificities and biological functions that depend on the source, these enzymes have attracted attention for their various potential industrial applications [6, 12, 19, 27].

Horseradish peroxidase (HRP) is well known for its high catalytic activity and broad specificity for electron donors. Since *Coprinus cinereus* peroxidase (CiP), a fungal peroxidase found in the culture filtrate of an ink cap basidiomycete, was reported to be similar to HRP, CiP has also been receiving attention [16, 20] and, essentially identical to *Arthromyces ramosus* (a hyphomycete) peroxidase (ARP), exhibits a substrate-specific activity and broad substrate specificity similar to HRP. Notably, CiP consists of a single species of enzyme, whereas HRP consists of at least 12 isozymes possessing different catalytic properties.

Industrial applications of a fungal peroxidase require an efficient and economical production system. To date, *Saccharomyces cerevisiae* [21], *Aspergillus oryzae* [17], and *Aspergillus awamori* [15] have been examined as heterologous expression systems for CiP/ARP production.

The methylotrophic yeast *Pichia pastoris* is well known for being effective in producing recombinant genetic material when facilitated by including a gene coding for a foreign protein behind the promoter of the *AOX1* gene normally needed for methanol utilization [18, 24, 26], such that high quantities of foreign protein (10–100 times more than *S. cerevisiae*) can be expressed [2, 3, 8, 9]. As *P. pastoris* has properties similar to *S. cerevisiae*, a yeast widely used in genetic engineering and industry, the technologies of molecular biology, genetics, and biochemistry developed for *S. cerevisiae* can be applied to *P. pastoris*. Using *P. pastoris* as a host, the desired foreign protein can be expressed at the inside or outside the cell, and because of the very limited amounts of protein that *P. pastoris* secretes, most of the secreted protein can be the desired foreign protein. In addition, different from the glycoproteins of *S. cerevisiae*, *P. pastoris* has specific posttranslational modification pathways that result in secreted glycoproteins similar to those of higher eucaryotes. By benefit of these differences, problems that arise with the use of *S. cerevisiae* can be solved or avoided [7, 22].

P. pastoris can use methanol as a carbon source and regulates the transcription and synthesis of alcohol oxidase with the *AOX* promoter, *AOX1*. The expression of the *AOX1* promoter is similar to the control pathway of *GAL1* of *S. cerevisiae*, but differs from *GAL1* in that *AOX1* cannot function with, and is repressed by, glucose in the medium,

*Corresponding author
Phone: +82-2-940-5675; Fax: +82-2-941-1785;
E-mail: metalkim@kw.ac.kr, bksong@krict.re.kr

being only induced by methanol. Thus, using *P. pastoris*, glycerol is commonly used for high-density cultures and methanol for induction of a desired protein [14].

There are three types of available *P. pastoris* host strains that vary with regard to their methanol utilization abilities: the wild type or methanol utilization plus (Mut^+) phenotype; those resulting from deletions in the *AOX1* gene, methanol utilization slow (Mut^s); or both *AOX* gene and methanol utilization minus (Mut^-). An important advantage of Mut^s strains is that the culture is not as sensitive to residual methanol in the cultivation media relative to Mut^+ strains, facilitating the process of scale-up [25]. Unfortunately, the lower maximum specific growth rate of the wild-type Mut^s strain, compared with wild-type Mut^+ strains, limits the productivity of the Mut^s utilizing process.

In this paper, the degree of rCiP host expression was compared with various strains of *P. pastoris* Mut^+ and Mut^s, and through comparison of the expression of rCiP under the control of *AOX1* and *GAP* promoter, optimal conditions for CiP production were suggested.

MATERIALS AND METHODS

Materials

All chemicals were of reagent-grade purity. Classical peroxidase substrate ABTS [2,2'-azinobis (ethylbenzthiazoline-6-sulfonate)] and H_2O_2 were obtained from Sigma (U.S.A.), restriction enzymes and ligase were purchased from New England BioLabs (U.S.A.), EX*Taq* was from Takara Bio Inc. (Japan), and *E. coli* HIT DH5α Competent Cells were from RBC (Taiwan). *P. pastoris* strain X-33 (wild-type) and plasmid pPICZaA were obtained from Invitrogen (U.S.A.), and the yeast transformation kit (MicroPulser) and the plasmid miniprep kit were purchased from Bio-Rad (U.S.A.) and Takara Bio Inc. (Japan), respectively. Plasmid propagation was carried out in *E. coli* DH5α cells using low-salt LB medium (Zeocin 25 μg/ml).

Vector Construction for CiP Expression in *P. pastoris*

The peroxidase cDNA from *C. cinereus* was isolated from the previously constructed vector pPICZαA-rCiP [13]. The EcoRI-NotI DNA fragment containing the rCiP (1,095 bp) was purified and cloned into the pPICZαA and pGAPZαA (Fig. 1). Table 1 shows the yeast strains and plasmids used in this study.

P. pastoris Transformation and Selection

The pPICZαA-rCiP and pGAPZαA-rCiP were linearized with SacI and BlnI, respectively, and used to transform competent X33 (Mut^+, His^+) or KM71H (Mut^s, His^+) cells by electroporation according to accompanying instructions (Invitrogen, U.S.A.), after which the linearized vectors were integrated into the *Pichia* genome *via* homologous recombination. The resulting transformed cells were plated onto YPDS medium (1% yeast extract, 2% peptone, 2% glucose, and 1 M sorbitol) supplemented with 100 μg/ml Zeocin. For the CiP construct, typically 4–6 transformants were chosen and purified as isolated single colonies on new YPDS plates (supplemented with Zeocin) and then screened to identify clones with the highest CiP activities.

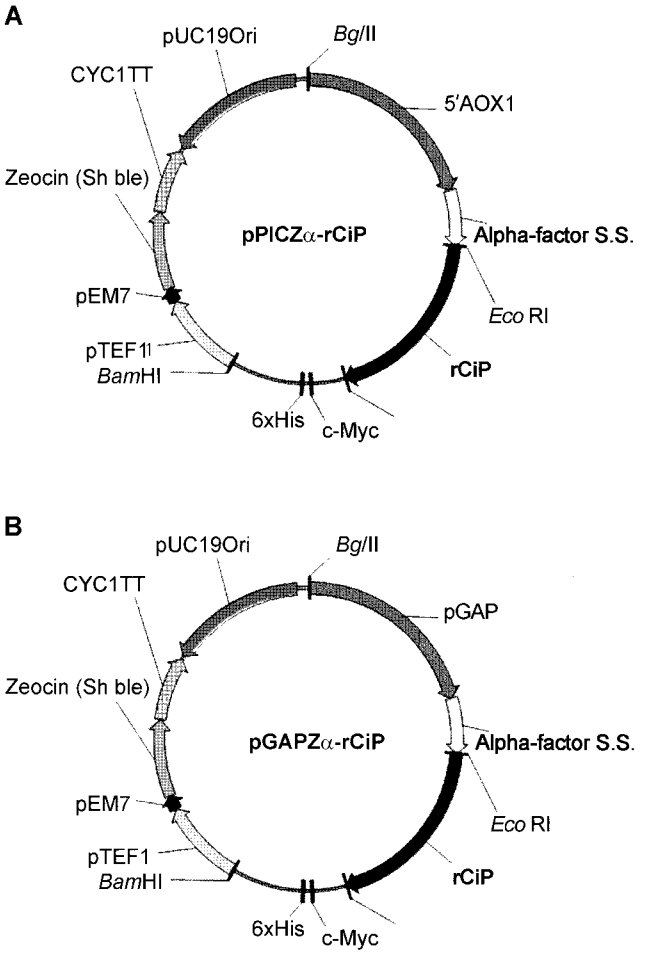

Fig. 1. Construction of peroxidase (CiP) expression vector in *P. pastoris*.
The CiP gene cloned into the vectors pPICZαA (**A**) and pGAPZαA (**B**) with the α-mating factor pre-pro-leader sequence derived from *S. cerevisiae*.

CiP Expression in *P. pastoris* X-33

prCiP-X and prCiP-K strains were inoculated in 5 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 2% glycerol), grown overnight at 30°C in a shaking incubator, and the cultures adjusted to the same OD_{600}. One hundred ml of each culture was used to express CiP in BMMY at 30°C, with sterile methanol added every 24 h to maintain induction conditions. grCiP-X and grCiP-K (Table 1) were used to express CiP in YPD (1% yeast extract, 2% peptone, and 2% glucose) at 30°C.

Cell Density and CiP Activity Assay

Samples collected at different time intervals were analyzed and the cell density was monitored spectrophotometrically at 600 nm.

The CiP peroxidase activity was measured using an ABTS and hydrogen peroxide assay in which the purified protein samples derived from cell suspensions were mixed with 2 ml of ABTS–H_2O_2 (0.5 mM ABTS and 2.9 mM H_2O_2, pH 5.0) in a quartz cuvette and changes in absorbance at 420 nm (molar extinction coefficient of ABTS, 34,700 $M^{-1}\cdot cm^{-1}$) were determined using a UV–Vis spectrometer (Shimadzu, Japan) at 25°C.

Table 1. Yeast strains and plasmids.

Strains/plasmids	Genotype/characteristics	Reference
Strains		
P. pastoris X-33	Wild type	
P. pastoris KM71H	arg4, Mut^s	
prCiP-X	pPICZαA-rCiP X-33 transformant	This study
prCiP-K	pPICZαA-rCiP KM71H transformant	This study
grCiP-X	pGAPZαA-rCiP X-33 transformant	This study
grCiP-K	pGAPZαA-rCiP KM71H transformant	This study
Plasmids		
pPICZαA	Zeo^r expression vector, *AOX1* promoter	
pGAPZαA	Zeo^r expression vector, *GAP* promoter	
pPICZαA-rCiP	Zeo^r containing rCiP DNA (secretion form)	This study
pGAPZαA-rCiP	Zeo^r containing rCiP DNA (secretion form)	This study

Immunoblotting and Quantity of Enzyme

After removal of yeast cells by centrifugation of the culture medium, the supernatant was added to a 5× sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), boiled for 10 min at 100°C, and the rCiP separated from the mixture on 12% SDS–PAGE by electrophoresis and transferred to PVDF membranes (Amersham, U.K.). Anti-rCiP antibody (Peptron. Co., Korea) raised in mice by using commercially available peroxidase (Novozyme, Denmark) was used as the primary antibody, followed by an anti-mouse goat IgG-alkaline phosphatase. The quantity of enzyme recovered was determined by measuring the immunoblotting band densities on an IMAGE J (Java-based image processing program developed at the NIH).

Results and Discussion

Comparison of rCiP Expression in *Pichia pastoris* by Varying Hosts

There are many examples of the expression of heterologous protein using *P. pastoris* Mut$^+$ and Mut^s. The expression of human tumor suppressor P53 by Mut^s has shown high yields, compared with expression by Mut$^+$ [1].

To optimize the expression of rCiP according to the host, pPICZαA-rCiP was transformed into *P. pastoris* X-33 (Mut$^+$) and KM71H (Mut^s), and the pPICZαA-rCiP-harboring cells were cultivated in 100 ml of BMMY medium at 250 rpm and 30°C in a shaking incubator. After 24 h of culture, 0.5% methanol was added during the induction period. Methanol was added, over a range of 0.3–2% and every 24 h, to Mut$^+$ and Mut^s cultures and the enzyme activity examined. Consequently, 0.5% methanol was found to attain a high level of cell growth and peroxidase activity (data not shown). The time courses of rCiP expression and cell growth in withdrawn supernatant samples of individual transformants (Fig. 2) showed no differences in rCiP expression levels between Mut$^+$ and Mut^s early in the cultivation, but after 5 days, peroxidase activity of Mut$^+$ progressively increased (Fig. 2A). Fig. 2B shows the peroxidase concentration of culture media from 3 days to 10 days, and similar to changes in peroxidase activity, peroxidase concentrations from Mut$^+$ was also higher than from Mut^s over time. Thus, from observation of

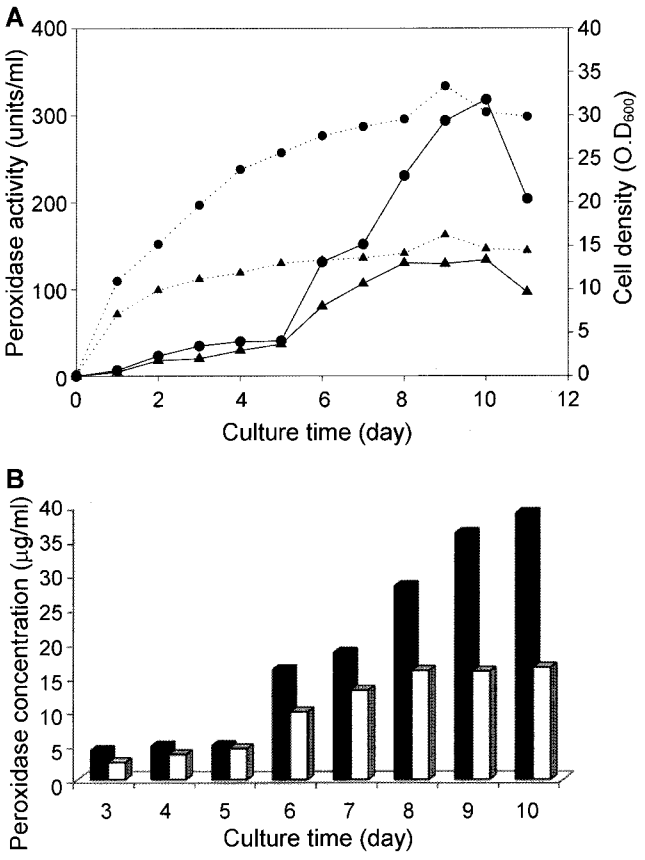

Fig. 2. Effect of *P. pastoris* strains on cell growth and rCiP production based on the *AOX1* promoter expression system.
A. Time course of peroxidase activity (solid line) and cell density (dotted line) between Mut$^+$ (●) and Mut^s (▲). **B.** Quantitative analysis of rCiP production at different cultivation times. Filled bars, peroxidase concentration from Mut$^+$; empty bars, peroxidase concentration from Mut^s.

Table 2. Maximum values

Types	Maximum cell concentration (OD_{600})	Maximum peroxidase activity (U/ml)	Maximum peroxidase concentration (μg/ml)	Specific peroxidase activity (U/ug)
pAOX-Mut$^+$	33.3	318	39.0	8.15
pAOX-Mut^s	16.2	133	16.4	8.16
pGAP-Mut$^+$	14.6	39	4.7892	8.13
pGAP-Mut^s	14.3	38	4.6664	8.09

the expression of rCiP in Mut$^+$ and Mut^s, the former was found to be superior in cell growth and peroxidase activity.

Table 2 shows the maximum cell growth, peroxidase activities, and peroxidase concentrations, etc., in terms of the hosts and promoters.

Comparison of rCiP Expression in *Pichia pastoris* by Different Promoters

Recently, much research has been done examining the expression of heterologous proteins under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter. The *GAP* promoter allows recombinant protein to be expressed constitutively through the promoter of the *GAP* gene, which encodes glyceraldehyde-3-phosphate dehydrogenase. Hohenblum *et al.* [11] have explained that expression of heterologous proteins through the constitutive *GAP* promoter may be superior to the methanol inducible *AOX1* promoter. Thus, the type of promoter employed is an important element in optimizing the expression of heterologous proteins.

After cloning of rCiP into pPICZαA (*AOX1* promoter) and pGAPZαA (*GAP* promoter), the products were transformed into *P. pastoris* and their rCiP expression compared. pPICZαA-rCiP transformants were grown in BMMY and pGAPZαA-rCiP transformants in YPD at 250 rpm and 30°C. Methanol at 0.5% was added to the pPICZαA-rCiP transformants after 24 h of culture for induction. Fig. 3 shows the effect of the different promoters in *P. pastoris* X-33 (Mut$^+$). Over time, prCiP-X increased rapidly in peroxidase activity, whereas grCiP-X slowly decreased (Fig. 3A), and concurrently, the peroxidase concentration showed a growing difference between the two transformants and, at about 10 days, prCiP-X was approximately four times greater than grCiP-X (Fig. 3B). Furthermore, similar results were observed with KM71H (Mut^s) as well as *P. pastoris* X-33 (Mut$^+$) (Fig. 4). As there was a gradual depletion of the glucose carbon source with culture progression, and *GAP* promoter transformants appeared to have retarded cell growth and expression of rCiP, additional 2% glucose was added every 3 days, such that cell growth occurred at levels similar to the *AOX* promoter transformants. However, the peroxidase activity was decreased in *GAP* promoter transformants, and it appeared clear that there was a reduction of peroxidase expression by the *GAP* promoter not related to depletion of the carbon source (data not shown). *P. pastoris* strains containing the *GAP* promoter showed that there is no difference in growth rate between Mut$^+$ and Mut^s strains (Figs. 3 and 4). Although *P. pastoris* host strains have their different phenotypes distinguished by their specific growth rates on methanol [25], in this case, they used the glucose as carbon source such that their growth rate exhibited the same trends. In other words, these results established that the methanol inducible *AOX* promoter was superior to the *GAP* promoter for expression of rCiP.

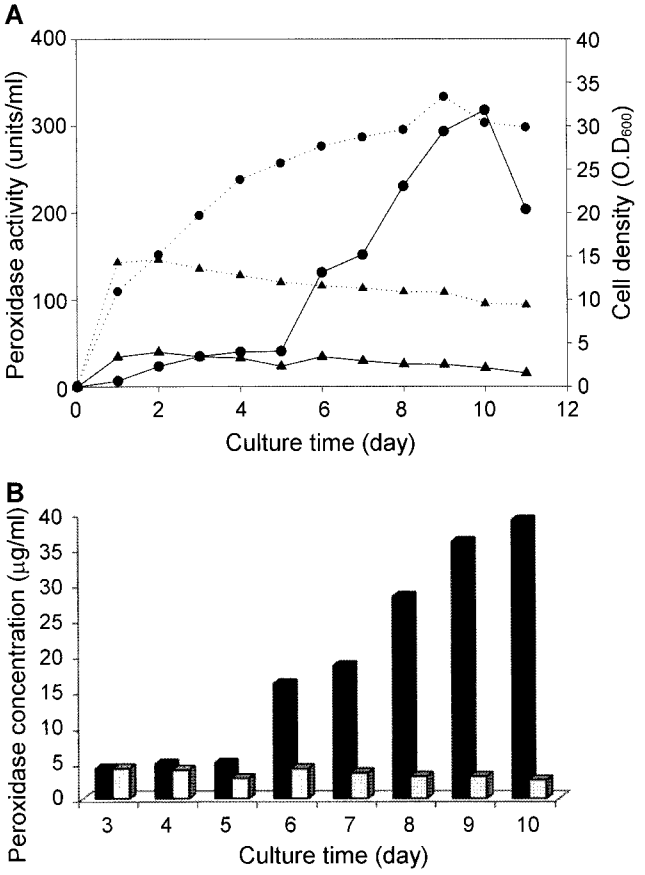

Fig. 3. Effect of promoter on cell growth and rCiP production by *P. pastoris* X-33 (Mut$^+$).

A. Time course of peroxidase activity (solid line) and cell density (dotted line) between the *AOX1* promoter (●) and *GAP* promoter (▲). **B.** Quantitative analysis of the rCiP production at different cultivation times. Filled bars, peroxidase concentration from *P. pastoris* X-33 the using *AOX1* promoter; empty bars, peroxidase concentration from *P. pastoris* X-33 using the *GAP* promoter.

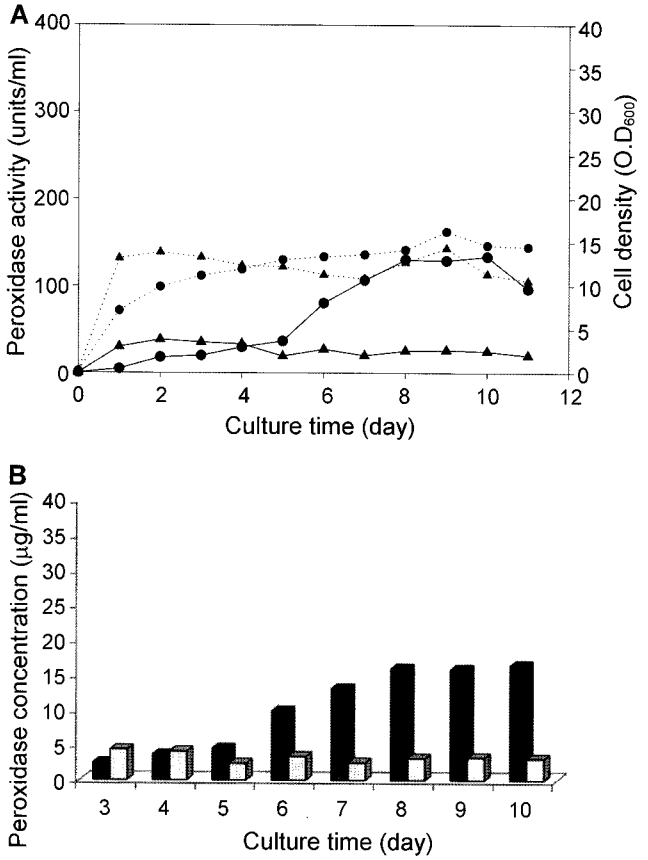

Fig. 4. Effect of promoter on cell growth and rCiP production by *P. pastoris* KM71H (Mut^s).
A. Time course of peroxidase activity (solid line) and cell density (dotted line) between the *AOX1* promoter (●) and *GAP* promoter (▲). **B.** Quantitative analysis of the rCiP production at different cultivation times. Filled bars, peroxidase concentration from *P. pastoris* KM71H using the *AOX1* promoter; empty bars, peroxidase concentration from *P. pastoris* KM71H using the *GAP* promoter

There is a published example that the production of heterologous protein is more efficient under the *AOX1* than *GAP* promoter using *P. pastoris* [5, 11, 23]. According to Gasser *et al.* [10], however, antibody fragments have not been expressed under the control of the strong inducible *AOX1* promoter, which may cause cellular stress by excessive transcription, such that the production rate of polypeptides can be rapid and interfere with correct protein folding. Gasser *et al.* [10] could not achieve expression of antibody fragments with the *AOX1* promoter, and Bibila and Flickinger [4] considered that folding is a limiting factor in antibody secretion in mammalian cells. In our study, using the *AOX1* promoter did not cause a major problem with the production and secretion of rCiP proteins, but further work is needed to ascertain the protein folding problem of CiP and to improve the production of the peroxidase.

In the last decade, *P. pastoris* has become one of the favorite expression systems for the production of various proteins of interest. This report described the functional heterologous expression of *Coprinus cinereus* peroxidase (CiP) in the methylotrophic yeast *Pichia pastoris*. It is well known that *P. pastoris* secretes very little native protein, providing an advantageous means to recover foreign secreted protein from the supernatant. Thus, the *S. cerevisiae* α-factor pre-pro-signal was fused to CiP cDNA and the collected supernatant from the rCiP recombinant analyzed. rCiP expression systems were constructed in *P. pastoris* and the rCiP expression followed according to strain type and kind of promoter to optimize the expression system. Comparing the cultures and rCiP expression from *P. pastoris* Mut$^+$ and Mut^s, the former strain showed faster cell growth and higher peroxidase activities and concentrations. The methanol inducible *AOX1* promoter exhibited superior cell growth, peroxidase activity, and peroxidase production in all *P. pastoris* Mut$^+$ and Mut^s strains. Therefore, to optimize rCiP expression using *P. pastoris*, the expression system of Mut$^+$ under the control of the *AOX1* promoter was most suitable.

Acknowledgments

This work was supported by a Korea Research Institute of Chemical Technology (KRICT), Kwangwoon University Research Grant (2009) and by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-314-D00081).

References

1. Abdelmoula, S., L. Rekik, A. Gargouri, and R. Mokdad. 2007. High-level expression of human tumor suppressor P53 in the methylotrophic yeast: *Pichia pastoris*. *Protein Expr. Purif.* **54:** 283–288.
2. Arnold, R. R., J. E. Russell, W. J. Champion, M. Brewer, and J. J. Gauthier. 1982. Bactericidal activity of human lactoferrin: Differentiation from the stasis of iron deprivation. *Infect. Immun.* **35:** 792–799.
3. Arnold, R. R., M. Brewer, and J. J. Gauthier. 1980. Bactericidal activity of human lactoferrin: Sensitivity of a variety of microorganisms. *Infect. Immun.* **28:** 893–898.
4. Bibila, T. and M. C. Flickinger. 1991. A structured model for monoclonal antibody synthesis in exponentially growing and stationary phase hybridoma cells. *Biotechnol. Bioeng.* **37:** 210–226.
5. Boer, H., T. T. Teeri, and A. Koivula. 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. *Biotechnol. Bioeng.* **69:** 486–494.
6. Conesa, A., P. J. Punt, and C. A. van den Hondel. 2002. Fungal peroxidases: Molecular aspects and applications. *J Biotechnol.* **93:** 143–158.

7. Cregg, J. M., J. L. Cereghino, J. Shi, and D. R. Higgings. 2000. Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* **16:** 23–52.
8. Doring, G., T. Pfestrof, K. Botzenhart, and M. A. Abdallah. 1988. Iron-chelating substances and inflammation. *Scand. J. Gastroenterol.* **23:** 68–69.
9. Ellison III, R. T., and T. J. Giehl. 1991. Killing of Gram-negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest.* **88:** 1080–1091.
10. Gasser, B., M. Maurer, J. Gach, R. Kunert, and D. Mattanovich. 2006. Engineering of *Pichia pastoris* for improved production of antibody fragments. *Biotechnol. Bioeng.* **94:** 353–361.
11. Hohenblum, H., B. Gasser, M. Maurer, N. Borth, and D. Mattanovich. 2004. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. *Biotechnol. Bioeng.* **85:** 367–375.
12. Joo, H., H. J. Chae, J. S. Yeo, and Y. J. Yoo. 1998. Depolymerization of phenolic polymers using horseradish peroxidase in organic solvent. *Process Biochem.* **32:** 291–296.
13. Kim, S. J., J. A. Lee, Y. H. Kim, and B. K. Song. 2009. Functional expression of Coprinus cinereus peroxidase in *Pichia pastoris*. *Process Biochemistry* **44:** 731–735.
14. Lee, K., S. Lim, and D. Kim. 2006. Effect of various additives on the production of recombinant HBsAg during methanol induction in *Pichia pastoris*. *Biotechnol. Bioeng.* **21:** 260–266.
15. Lokman, B. C., V. Joosten, J. Hovenkamp, R. J. Gouka, C. T. Verrips, and C. A. van den Hondel. 2003. Efficient production of *Arthromyces ramosus* peroxidase by *Aspergillus awamori*. *J Biotechnol.* **103:** 183–190.
16. Nakayama, T. and T. Amachi. 1999. Fungal peroxidase: Its structure, function, and application. *J. of Molec. Catal. B Enzym.* **6:** 185–198.
17. Petersen, J. F., J. W. Tams, J. Vind, A. Svensson, H. Dalboge, K. G. Welinder, and S. Larsen. 1993. Crystallization and X-ray diffraction analysis of recombinant *Coprinus cinereus* peroxidase. *J. Mol. Biol.* **232:** 989–991.
18. Romanos, M. A., C. A. Scorer, and J. J. Clare. 1992. Foreign gene expression in yeast: A review *Yeast* **8:** 423–488.
19. Ryan, B. J., N. Carolan, and C. O'Fagain. 2006. Horseradish and soybean peroxidases: Comparable tools for alternative niches? *Trends Biotechnol.* **24:** 355–363.
20. Ryu, K., J. P. McEldoon, and J. S. 1995. Kinetic characterization of a fungal peroxidase from *Coprinus cinereus* in aqueous and organic media. *Biocatal. Biotransform.* **13:** 53–63.
21. Sawai-Hatanaka, H., T. Ashikari, Y. Tanaka, Y. Asada, T. Nakayama, H. Minakata, *et al.* 1995. Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Biosci. Biotechnol. Biochem.* **59:** 1221–1228.
22. Schorer, C. A., J. J. Clare, W. R. McCombie, M. A. Romanos, and K. Sreekrishna. 1994. Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Biotechnology* **12:** 181–184.
23. Sears, I. B., J. O'Connor, O. W. Rossanese, and B. S. Glick. 1998. Versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. *Yeast* **14:** 787–790.
24. Sreekrishna, K., J. F. Tschopp, and M. Fuke. 1987. Invertase gene *(SUC2)* of *Saccharomyces cerevisiae* as a dominant marker for transformation *Pichia pastoris*. *Gene* **59:** 115–125.
25. Stratton, J., V. Chiruvolu, and M. M. Meagher. 1998. High cell-density fermentation. *Methods Molec. Biol.* **103:** 107–120.
26. Tschopp, J. F., G. Dverlow, R. Kosson, W. Craig, and L. Grinna. 1987. High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. *Biotechnology* **5:** 1305–1308.
27. Woo, S., J. Cho, B. Lee, and E. Kim. 2004. Decolorization of melanin by lignin peroxidase from *Phanerochaete chrysosporium*. *Biotechnol. Bioprocess Eng.* **9:** 256–260.