JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3531-3536

Enhanced Production of Endochitinase from An Antarctic Bacterium, *Sanguibacter antarcticus* PAMC21702, in *Pichia pastoris*

Se Jong Han[1,2]*, Sung Gu Lee[1,2], Heeyong Park[1], Hye Yeon Koh[1,3] and Joung Han Yim[1]

[1]Division of Polar Life Sciences, Korea Polar Research Institute, Incheon 406-840, South Korea.
[2]Department of Polar Sciences, University of Science and Technology, Incheon 406-840, South Korea.
[3]Department of Applied Marine Biotechnology and Engineering, Gangneung-Wonju National University, Gangneung 210-702, South Korea.

(Received: 10 December 2014; accepted: 22 January 2014)

The recombinantendochitinase (rChi21702) from apsychrophilic Antarctic bacterium, *Sanguibacter antarcticus*(KCTC 13143, PAMC 21702),has been produced using *Pichia pastoris*expression system. In this study, theoptimumfermentation temperature (20°C) and growth media pH (6) for Chi21702 production was determinedusing a2-stage fed-batch culturesystem.The maximal enzyme activity (178 U/l)of rChi21702obtained using a glycerol-methanol fed-batch culture system wasapproximately6-fold higher than that of unoptimized conditions. In addition, therChi21702 exhibitedcold-activeendochitinase activity and higher thermal stability than that of the wild-type Chi21702.This work allows forlarge-scale production ofrChi21702, which could be extended to further application studies using recombinant chtinases.

Key words: Antarctica, Cold-active, Endochitinases, Fed-batch, *Pichia pastoris.*

Chitin is anunbranched long-chain homopolymer of *N*-acetylglucosamine (NAG) abundantly found in nature. Althoughcrude chitin is inexpensive, its cleaved products such aschitin oligosaccharides and NAG are expensive and are used in industriesrelated to medicine, food, and agriculture [1, 2]. Chitinases, a group of enzymes capable of degrading chitin to chitin oligosaccharides or NAG, are produced by a variety of organisms, andthese enzymes are divided into 2 categories: endochitinases and exochitinases. Endochitinases (EC3.2.1.14) cleave chitin polymers at random internal sites, whereas exochitinases cleave chitin progressively starting at the non-reducing ends of the chains and release NAG monomers or diacetyl-chitobiose by β-(1,4)-*N*-acetylglucosaminidase activity (EC3.2.1.30) or 1,4-β-chitobiosidase activity (EC3.2.1.29), respectively [3, 4].

Previously, a new extracellular chitinasefrom theAntarctic bacterium, *Sanguibacter antarcticus*PAMC 21702 (KCTC 13143) was characterized [5], and the production of this cold-active endochitinase Chi21702 was optimized using statistical design methods [6].In addition,Chi21702was successfully expressed in the methylotrophic yeast,*Pichia pastoris*, by using codon optimization[7]. However, the recombinant endochitinase expressed in *P. pastoris*had a maximal activity of 30 U/lwhich was much lower than the maximal activity (90U/l)of the wild-type Chi21702[6].

In summary, the optimal conditions for recombinant Chi21702 (rChi21702) productionin*P. pastoris* were determinedusing a2-stage fed-batch culture mode.

* To whom all correspondence should be addressed.
Tel: +82-32-260-6370, Fax: +82-32-260-6301
E-mail: hansj@kopri.re.kr

MATERIALS AND METHODS

Yeast strain and culture media

The chitinase gene was expressed in*Pichia pastoris*X-33 (Invitrogen, Carlsbad, CA, USA) by using the plasmid vector pPICZáA (Invitrogen, Carlsbad, CA, USA)[7] following the manufacturer's protocol. The yeast cellswere grown in YPD (10 gyeast extract, 20 g peptone, and 20 g dextrose per liter) for seed culture preparation. Fermentations were carried out using fermentation basal salts(FBS) medium(40 gglycerol, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \times 7H_2O$, 4.13 g KOH, 26.7 ml H_3PO_4[85%]per liter) supplemented withtrace metalsolution (6.0 g $CuSO_4 \times 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \times H_2O$, 0.2 g $NaMo_2 \times H_2O$, 0.02 g H_3BO_4, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \times 7H_2O$, 0.2 g biotin, and 5.0 mlH_2SO_4 per liter) [8].

Fermentation conditions

Fed-batch production of rChi21702using methanol was performed to optimize the culture conditions such as temperature and pH by using a 1-ljar bottommagnetic drive fermentor (Biostat Q, B. Braun Biotech,Germany). Seed culture (50ml)was prepared usingYPD medium and incubatingon a shaker at 30°C, 200rpm for 20 h. The seed culture was transferred into a 1-lfermentor containing 0.5lof FBS medium supplemented with 4.4 mlof filter-sterilized trace metal solution per liter. First, the effect of 4differentculture temperatures (15°C, 20°C, 25°C, and 30°C at pH 5.0) on the rChi21702 production was investigated.Next, the effect of culture media at5pH values (pH 5.0, 5.5, 6.0, 6.5, and 7.0) wasassessedat the optimal induction temperature. Each pH value was maintained by automated addition of 10% (v/v) ammonium hydroxide (NH_4OH) solution.The batch-phase culture was carried out under varying temperatures (at pH 5) or at varying pH values (at 20°C)until glycerol was completely consumed. Glycerol consumptionwas indicated by a sudden increase in the dissolved oxygen content. Following this,a methanol fed-batch culture was started by adding 100% methanol feed containing 12 ml/lof trace metal solution. The methanol feed rate was set to 3.6 $ml \cdot h^{-1} \cdot l^{-1}$ of the initial fermentation volume.

Glycerol-methanol fed-batch fermentation

At the optimal temperature and pH (20°C, pH 6) for rChi21702production,which was determined from the above experiments, glycerol fed-batch phase was introduced after the glycerolbatch phase to increase the recombinant protein production,using a 5-ljar top impeller drive fermentor (Minifors, Infors HT, Switzerland). The seed culture grown in200 ml YPD medium was transferred to a 5-lfermentor containing 2 lof FBS medium supplemented with 4.4 mlof filter-sterilized trace metal solution per liter. Fermentation was carried out with the aeration rate set at 10~20l/min, mixing at 200~900rpm, and maintenance of dissolved oxygen levels at 20~30%. The dissolved oxygen (DO)-stat glycerol-methanol fed batch was initiated by supplying the 200 ml of glycerol feed medium (50% glycerol [w/v] and 12 mlof trace metal solution per liter). The methanol feed was supplied0.5 h after the glycerol feed was exhausted, and it was controlled as follows: the feeding was paused when the DO value dropped below the set point, 20% of air saturation,and it was continued when the DO value recovered to above the set point.

Analytical methods

The growth of *P. pastoris* was monitored by measuring dry cell weight (DCW). The chitinase activity in the fermented broth was measured using *p*-nitrophenyl-*N*-acetyl-b-D-glucosaminide as a substrate. The amount of *p*-nitrophenol formed by enzyme reaction was determined by measuring the absorbance at wavelength of 405 nm and using the extinction coefficient, 17,000/M/cm. One unit of enzyme activity per liter (1 U/l) was defined as the amount of enzyme required to hydrolyze1 µmolesubstrate to *N*-acetyl glucosamine and *p*-nitrophenol in 1 h.

RESULTS AND DISCUSSION

Effect of temperature on rChi21702 production

To examine the effect of temperature on rChi21702production, the cells were cultivated in a 1-ljar fermentor at 15°C, 20°C, 25°C, and 30°C with methanol induction. As shown in Fig. 1, the DCW and rChi21702 activitywere dependent on the culture temperature; when the DCW at 20°C was set to 100%, the DCWs measured at 15°C, 25°C, and 30°C were 47%, 82%, and 75%, respectively. The relative rChi21702 activities were41%, 81%, and 62% at 15°C, 25°C, and 30°C, respectively, in comparison to 100% activity at 20°C. These results

indicate that therChi21702production is influenced by cultivation temperaturesbetween 15°C and 30°C.Thus, 20°C was chosen as the optimal operating temperature for rChi21702 production. Previous studies have reported that recombinant chitinase production could be carried out using methylotrophic *P. pastoris*.However, rChi21702production at cultivation temperatures lower than 30°C has not been wellcharacterized. In this study, both the yeast-cell growth rateand rChi21702 enzyme activity wereincreased by lowering the cultivation temperature to 20°C. The lower temperature may have decreasedproteolytic degradation of recombinant proteins [9,11] and thereby increased the viability of *P. pastoris*[8].

Effect of pH on the rChi21702 production

The recombinant protein production in *P. pastoris*is known to be affected by thecultivation medium pH [12, 13].To determine the optimal pH for rChi21702 expression, *P. pastoris* was cultivated in media with pH in the range of 5.0–--7.0 along with methanolinduction. Althoughmaximal cell density

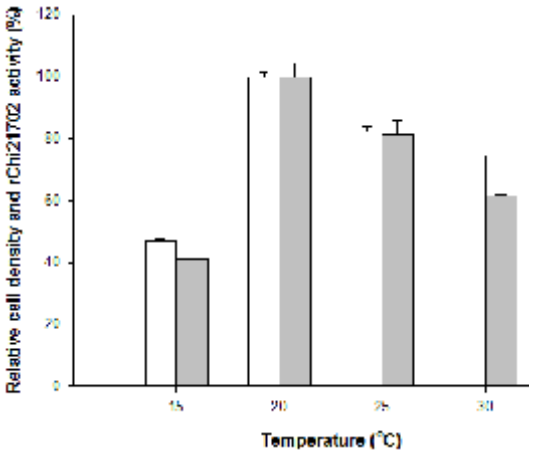

Fig. 1. Effect of temperature on recombinant Chi21702 (rChi21702) production. Cells were cultivated in a 1-ljar fermentor with batch and then methanol fed-batch mode at various culture temperatures, at pH 5.0. At the end of the culture growth, the dry cell weight (white bars; 100%, 80.7 g/L) and rChi21702activity (gray bars; 100%, 80.6 U/L) were measured

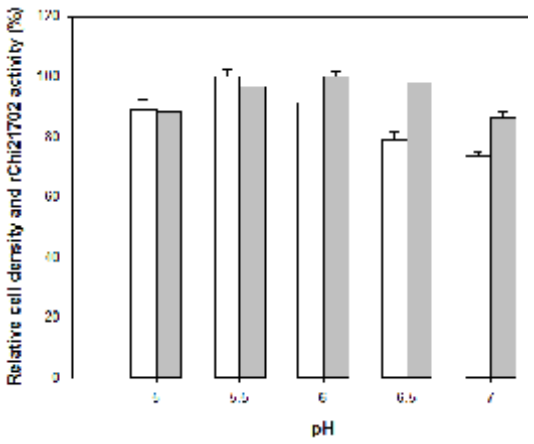

Fig. 2. Effect of pH on rChi21702 production. Cells were cultivated in a 1-ljar fermentor with batch and then methanol fed-batch mode at various culture pH values, at 20°C. At the end of culture growth, the dry cell weight (white bars; 100%, 90.4 g/L) and rChi21702activity (gray bars; 100%, 91 U/L) were measured

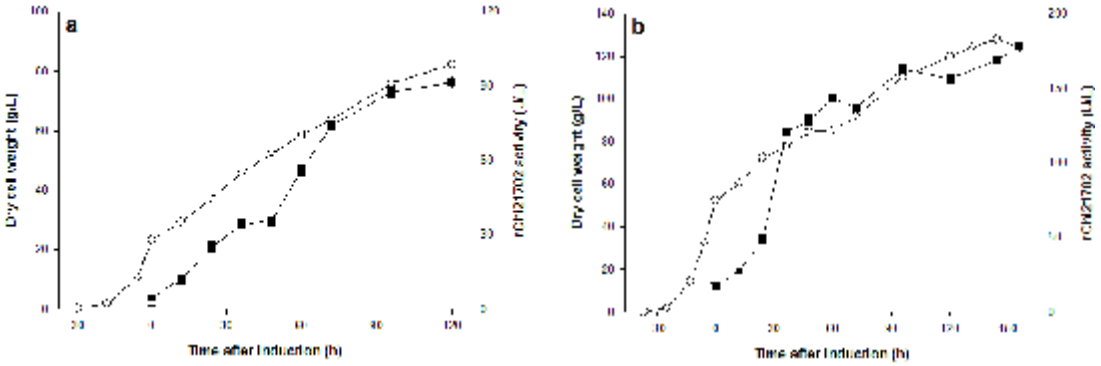

Fig. 3. Production of rChi21702 in the 5-l fermentor withdissolved oxygen-stat fed-batch mode. (a)Time profile of cell density (™) andrChi21702 activity (■)in methanol-only fed-batch culture. (b) Time profile of cell density (™) andrChi21702 activity (■)in glycerol-methanol fed-batch culture.

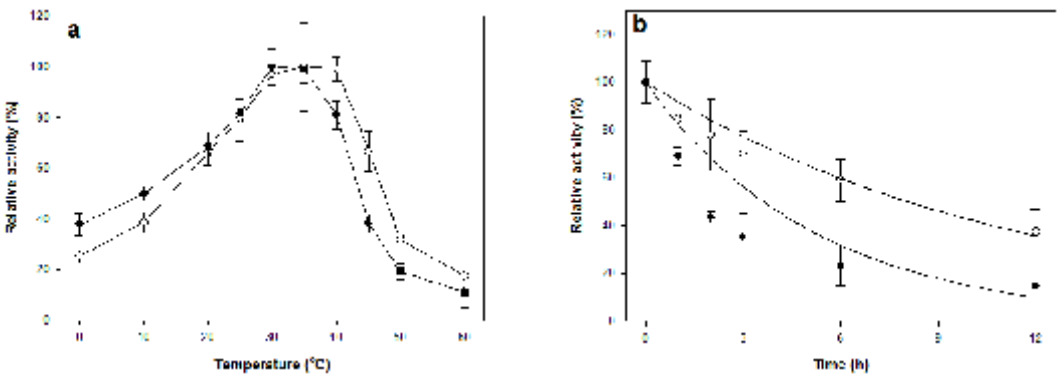

Fig. 4. Effect of temperature on the activityand stabilityof wild-type Chi21702and rChi21702. (a)Activity of wild-type Chi21702 (●) and rChi21702 (O) was determined at various temperatures ranging from 0°C to 60°C. (b) Deactivation profile of wild-type Chi21702 and rChi21702 at 40°Cfor 12h

was obtainedat pH 5.5, rChi21702 activity was the highest at pH 6.0 (Fig. 2). Thus, pH 6.0 was selected as the optimal pH for rChi21702 production. In this study, the optimal pH for cell growth did not ensure maximal production of target recombinant protein. At high pH values, there was increasedrChi21702 production and decreased cell growth (Fig. 2). This could probably be due to the reduced enzymeactivity at lower pH values[7].

Glycerol-methanol fed-batch fermentation

rChi21702 production was carried out in a5-l fermentor maintainedat 20°C and mediapH 6.0 by usingthe following 2methods: methanol-only and glycerol-methanol fed-batches (Fig. 3a and 3b, respectively). First, cells were grown without using the glycerol fed-batch mode. The cell density gradually increased and reached a maximum value of 82.5 g dry cell/lat 120 hafter induction. In addition, the rChi21702 activity in the culture broth increased to 91U/lat 120 h after induction, and it was 19-fold higher than the enzyme activity before induction (Fig. 3a).A high cell density during fermentation is generally desirable because the amount of recombinant protein secreted into the medium often increases in direct proportion to the cell density [14]. To increase rChi21702production, glycerol fed-batch phase was introduced between the batch and methanol-induction phases. Cell density reached 52 g dry cell/l at the end of the glycerol fed-batch phase. During the methanol-induction phase, cell density increased continuously and reached the maximal DCW of 128 g/l after 144h of induction (Fig. 3b). The maximal rChi21702 activity was 178 U/l, which was approximately 2-fold higher than that of batch culture that was not supplied glycerol or of the wild-type *S. antarcticus* cell culture [6].

Temperature effect on the activity and stability

The activity of rChi21702 was analyzed at various temperatures and compared with that of the wild-type Chi21702 from *S. antarcticus*. The optimal temperature for rChi21702 activity was determined to be 35°C (Fig. 4a), which was similar to the optimal temperature (37°C) reportedin a previous study [7]. The optimal temperature formaximal rChi21702 activity (35°C~37°C) was higherthan those reported for the following enzymes: recombinant cold-adapted chitinase from *Moritella marina* (28°C)[15], *Alteromonas* sp. strain O-7 (30°C)[16], *Vibrio* sp. strain Fi7 (35°C)[17], and *Glaciozyma antarctica* PI12 (15°C)[18]. However, only 25% of the maximal rChi21702activity was retained at 0°C.In contrast,37% of the maximal wild-type Chi21702 activity was retainedat 0°C. In addition, both the recombinant and wild-type enzymeshad similar patterns of temperature sensitivity except for the slightly higher recombinant enzyme activity at high temperature, above 40°C (Fig. 4a). The higher thermal stability of rChi21702could potentially enhance enzyme activity at a high temperature, butthe underlying reasons remain unclear. Furthermore, rChi21702 and wild-type Chi21702 retained 70% and 35% of its original activity after incubatingat 40°C for 3h, respectively (Fig. 4b).

CONCLUSIONS

The methylotrophic yeast *Pichia pastoris* was used to produce a recombinant Antarctic chitinase, rChi21702. To improve expression of cold-active rChi21702, culture temperature and pH were optimized, and a 2-fold increase in enzyme activity was noted using a glycerol-methanol fed-batch culture. This high-level expression strategy for rChi21702 productionin*P. pastoris* provides a suitable and practical basis for large-scale recombinant cold-active chitinase production.

ACKNOWLEDGMENTS

This research was supported by grants from the Korea Polar Research Institute (PK09040, PE13040andPE13150).

REFERENCES

1. Dahiya, N., Tewari, R., Tiwari, R.P., and Hoondal, G.S. Chitinase production in solid-state fermentation by *Enterobacter* sp. NRG4 using statistical experimental design. *Curr. Microbiol*., 2005; **51**: 222-228.
2. Hoster, F., Schmitz, J.E., and Daniel, R. Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. *Appl. Microbiol. Biotechnol*., 2005; **66**: 434-442.
3. Graham, L., and Sticklen, M. Plant chitinases. *Can. J. Bot*., 1994; **72**: 1057-1083.
4. Harman, G.E., Hayes, C.K., Lorito, M., Broadway, R.M., diPietro, A., Peterbauer, C., and Tronsmo, A. Chitinolytic enzymes of Trichoderma harzianum: purification of chitobiosidase and endochitinase. *Phytopathol*. 1993; **83**: 313-318.
5. Park, H.J., Kim, D., Kim, I.H., Lee, C.E., Kim, I.C., Kim, J.Y., Kim, S.J., Lee, H.G., and Yim, J.H. Characteristics of cold-adaptive endochitinase from Antarctic bacterium *Sanguibacter antarcticus* KOPRI 21702. *Enzyme Microb. Technol*., 2009; **45**: 391-396.
6. Han, S.J., Park, H., Lee, S.G., Lee, H.K., and Yim, J.H. Optimization of cold-active chitinase production from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702. *Appl. Microbiol. Biotechnol*., 2011; **89**: 613-621.
7. Lee, S.G., Koh, H.Y., Han, S.J., Park, H., Na, D.C., Kim, I.C., Lee, H.K., and Yim, J.H. Expression of recombinant endochitinase from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 in *Pichia pastoris* by codon optimization. *Protein Expr. Purif*., 2010; **71**: 108-114.
8. Lee, J.H., Lee, S.G., Do, H., Park, J.C., Kim, E., Choe, Y.H., Han, S.J., and Kim, H.J. Optimization of the pilot-scale production of an ice-binding protein by fed-batch culture of *Pichia pastoris*. *Appl. Microbiol. Biotechnol*., 2012; **97:** 3383-3393.
9. Hong, F., Meinander, N.Q., and Jonsson, L.J. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnol. Bioeng*., 2002; **79**: 438-449.
10. Li, Z., Xiong, F., Lin, Q., d'Anjou, M., Daugulis, A.J., Yang, D.S., and Hew, C.L. Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. *Protein Expr. Purif*., 2001; **21**: 438-445.
11. Wang, Y., Z., W., Xu, Q., G., D., Hua, Z., Liu, L., and Li, J. Lowering induction temperature for enhanced production of polygalacturonate lyase in recombinant *Pichia pastoris*. *Proc. Biochem*., 2009; **44**: 949-954.
12. Clare, J.J., Romanos, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Payne, M.M., Sreekrishna, K., and Henwood, C.A. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. *Gene*, 1991; **105**: 205-212.
13. Zhu, A., Monahan, C., Zhang, Z., Hurst, R., Leng, L., and Goldstein, J. High-level expression and purification of coffee bean alpha-galactosidase produced in the yeast *Pichia pastoris*. *Arch. Biochem. Biophys*., 1995; **324**: 65-70.
14. Pal, Y., Khushoo, A., and Mukherjee, K.J. Process optimization of constitutive human granulocyte-macrophage colony-stimulating factor (hGM-CSF) expression in *Pichia pastoris* fed-batch culture. *Appl. Microbiol. Biotechnol*., 2006; **69**: 650-657.
15. Stefanidi, E., and Vorgias, C.E. Molecular analysis of the gene encoding a new chitinase from the marine psychrophilic bacterium *Moritella marina* and biochemical characterization of the recombinant enzyme. *Extremophiles*, 2008; **12**: 541-552.
16. Orikoshi, H., Baba, N., Nakayama, S., Kashu, H., Miyamoto, K., Yasuda, M., Inamori, Y., and Tsujibo, H. Molecular analysis of the gene encoding a novel cold-adapted chitinase (ChiB)

from a marine bacterium, *Alteromonas* sp. strain O-7. *J. Bacteriol*., 2003; **185**: 1153-1160.

17. Bendt, A., Huller, H., Kammel, U., Helmke, E., and Schweder, T. Cloning, expression, and characterization of a chitinase gene from the Antarctic psychrotolerant bacterium *Vibrio* sp. strain Fi:7. *Extremophiles*, 2001; **5**: 119-126.

18. Ramli, A.N., Mahadi, N.M., Rabu, A., Murad, A.M., Bakar, F.D., and Illias, R.M. Molecular cloning, expression and biochemical characterisation of a cold-adapted novel recombinant chitinase from *G. antarctica* PI12. *Microb. Cell Fact*., 2011; **10**: 94.