

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

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The recombinant endochitinase (rChi21702) from a psychrophilic Antarctic bacterium, *Sanguibacter antarcticus* (KCTC 13143, PAMC 21702), has been produced using *Pichia pastoris* expression system. In this study, the optimum fermentation temperature (20°C) and growth media pH (6) for Chi21702 production was determined using a 2-stage fed-batch culture system. The maximal enzyme activity (178 U/l) of rChi21702 obtained using a glycerol-methanol fed-batch culture system was approximately 6-fold higher than that of unoptimized conditions. In addition, the rChi21702 exhibited cold-active endochitinase activity and higher thermal stability than that of the wild-type Chi21702. This work allows for large-scale production of rChi21702, which could be extended to further application studies using recombinant chitinases.

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

Chitin is an unbranched long-chain homopolymer of *N*-acetylglucosamine (NAG) abundantly found in nature. Although crude chitin is inexpensive, its cleaved products such as chitin oligosaccharides and NAG are expensive and are used in industries related 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, and these 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 chitinase from the Antarctic bacterium, *Sanguibacter antarcticus* PAMC 21702 (KCTC 13143) was characterized⁵, and the production of this cold-active endochitinase Chi21702 was optimized using statistical design methods⁶. In addition, Chi21702 was successfully expressed in the methylotrophic yeast, *Pichia pastoris*, by using codon optimization⁷. However, the recombinant endochitinase expressed in *P. pastoris* had a maximal activity of 30 U/l which was much lower than the maximal activity (90 U/l) of the wild-type Chi21702⁶.

In summary, the optimal conditions for recombinant Chi21702 (rChi21702) production in *P. pastoris* were determined using a 2-stage fed-batch culture mode.

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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 pPICZ α A (Invitrogen, Carlsbad, CA, USA)⁷ following the manufacturer's protocol. The yeast cells were grown in YPD (10 g yeast 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 g glycerol, 0.93 g CaSO₄, 18.2 g K₂SO₄, 14.9 g MgSO₄·7H₂O, 4.13 g KOH, 26.7 ml H₃PO₄ [85%] per liter) supplemented with trace metal solution (6.0 g CuSO₄·5H₂O, 0.08 g NaI, 3.0 g MnSO₄·xH₂O, 0.2 g NaMo₂·xH₂O, 0.02 g H₃BO₄, 0.5 g CoCl₂, 20.0 g ZnCl₂, 65.0 g FeSO₄·7H₂O, 0.2 g biotin, and 5.0 ml H₂SO₄ per liter)⁸.

Fermentation conditions

Fed-batch production of rChi21702 using methanol was performed to optimize the culture conditions such as temperature and pH by using a 1-ljar bottom magnetic drive fermentor (Biostat Q, B. Braun Biotech, Germany). Seed culture (50 ml) was prepared using YPD medium and incubating on a shaker at 30°C, 200 rpm for 20 h. The seed culture was transferred into a 1-l fermentor containing 0.5 l of FBS medium supplemented with 4.4 ml of filter-sterilized trace metal solution per liter. First, the effect of 4 different culture 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 at 5 pH values (pH 5.0, 5.5, 6.0, 6.5, and 7.0) was assessed at the optimal induction temperature. Each pH value was maintained by automated addition of 10% (v/v) ammonium hydroxide (NH₄OH) 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 consumption was 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 of trace metal solution. The methanol feed rate was set to 3.6 ml·h⁻¹·l⁻¹ of the initial fermentation volume.

Glycerol-methanol fed-batch fermentation

At the optimal temperature and pH (20°C, pH 6) for rChi21702 production, which was

determined from the above experiments, glycerol fed-batch phase was introduced after the glycerol batch phase to increase the recombinant protein production, using a 5-ljar top impeller drive fermentor (Minifors, Infors HT, Switzerland). The seed culture grown in 200 ml YPD medium was transferred to a 5-l fermentor containing 2 l of FBS medium supplemented with 4.4 ml of filter-sterilized trace metal solution per liter. Fermentation was carried out with the aeration rate set at 10–20 l/min, mixing at 200–900 rpm, 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 ml of trace metal solution per liter). The methanol feed was supplied 0.5 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- β -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 hydrolyze 1 μ mol substrate 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 rChi21702 production, 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 activity were 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 were 41%, 81%, and 62% at 15°C, 25°C, and 30°C, respectively, in comparison to 100% activity at 20°C. These results

indicate that rChi21702 production is influenced by cultivation temperatures between 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, rChi21702 production at cultivation temperatures lower than 30°C has not been well characterized. In this study, both the yeast-cell growth rate and rChi21702 enzyme activity were increased by

lowering the cultivation temperature to 20°C. The lower temperature may have decreased proteolytic 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 the cultivation 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 methanol induction. Although maximal cell density

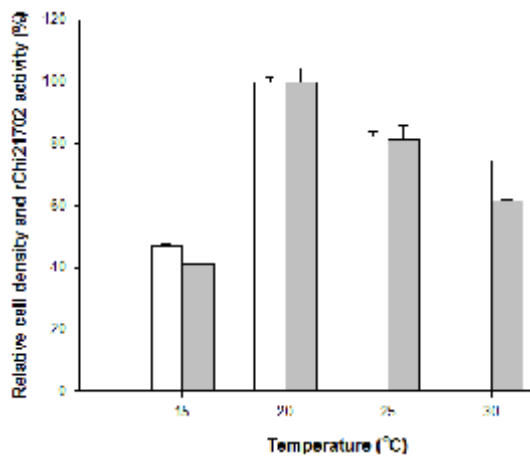


Fig. 1. Effect of temperature on recombinant Chi21702 (rChi21702) production. Cells were cultivated in a 1-l jar fermentor with batch and then methanol fed-batch mode at various culture temperatures, at pH 5.0. At the end of the culture growth, the dry cell weight (white bars; 100%, 80.7 g/L) and rChi21702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

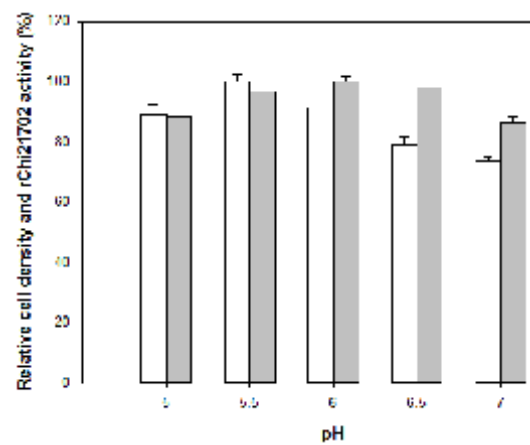


Fig. 2. Effect of pH on rChi21702 production. Cells were cultivated in a 1-l jar fermentor with batch and then methanol fed-batch mode at various culture pH values, at 20°C. At the end of culture growth, the dry cell weight (white bars; 100%, 90.4 g/L) and rChi21702 activity (gray bars; 100%, 91 U/L)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

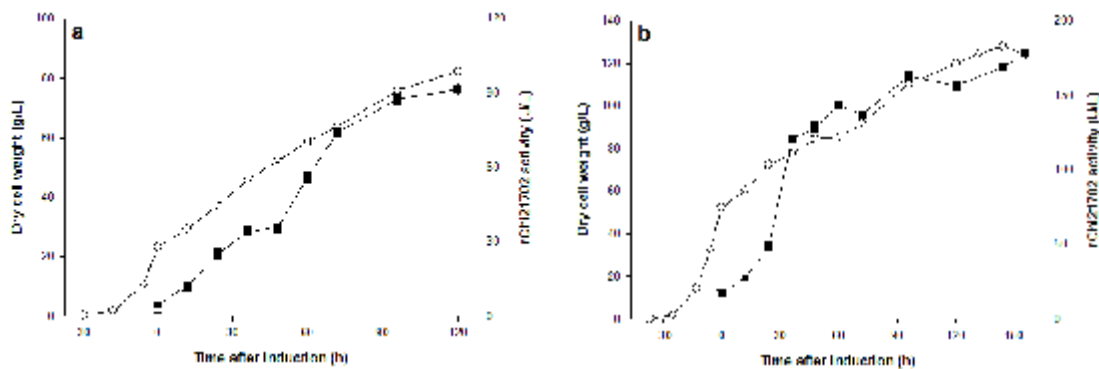


Fig. 3. Production of rChi21702 in the 5-l fermentor with dissolved oxygen-stat fed-batch mode. (a) Time profile of cell density (T^M) and rChi21702 activity (\blacksquare) in methanol-only fed-batch culture. (b) Time profile of cell density (T^M) and rChi21702 activity (\blacksquare) in glycerol-methanol fed-batch culture.

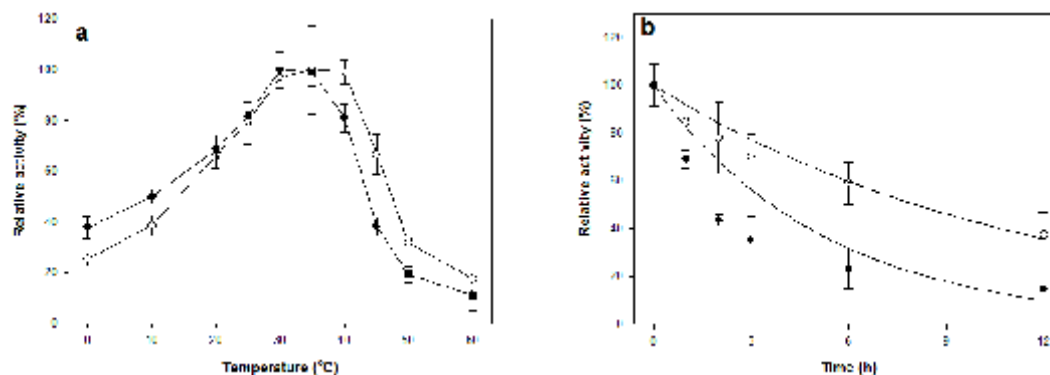


Fig. 4. Effect of temperature on the activity and stability of wild-type Chi21702 and rChi21702. (a) Activity of wild-type Chi21702 (●) and rChi21702 (○) was determined at various temperatures ranging from 0°C to 60°C. (b) Deactivation profile of wild-type Chi21702 and rChi21702 at 40°C for 12h

was obtained at 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 increased rChi21702 production and decreased cell growth (Fig. 2). This could probably be due to the reduced enzyme activity at lower pH values⁷.

Glycerol-methanol fed-batch fermentation

rChi21702 production was carried out in a 5-l fermentor maintained at 20°C and media pH 6.0 by using the following 2 methods: 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/l at 120 h after induction. In addition, the rChi21702 activity in the culture broth increased to 91 U/l at 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 density¹⁴. To increase rChi21702 production, 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 144 h 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 culture⁶.

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) reported in a previous study⁷. The optimal temperature for maximal rChi21702 activity (35°C~37°C) was higher than 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 rChi21702 activity was retained at 0°C. In contrast, 37% of the maximal wild-type Chi21702 activity was retained at 0°C. In addition, both the recombinant and wild-type enzymes had 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 rChi21702 could potentially enhance enzyme activity at a high temperature, but the underlying reasons remain unclear. Furthermore, rChi21702 and wild-type Chi21702 retained 70% and 35% of its original activity after incubating at 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 production in *P. pastoris* provides a suitable and practical basis for large-scale recombinant cold-active chitinase production.

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