Appl Microbiol Biotechnol (2011) 89:613–621
DOI 10.1007/s00253-010-2890-y

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Optimization of cold-active chitinase production from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702

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Received: 3 August 2010 / Revised: 14 September 2010 / Accepted: 15 September 2010 / Published online: 5 October 2010

Abstract In the present study, cultivation conditions and medium components were optimized using statistical design and analysis to enhance the production of Chi21702, a cold-active extracellular chitinase from the Antarctic bacterium *Sanguibacter antarcticus* KOPRI 21702. Identification of significant carbon sources and other key elements was performed using a statistical design technique. Chitin and glycerol were selected as main carbon sources, and the ratio of complex nitrogen sources to carbon sources was determined to be 0.5. Among 15 mineral components included in basal medium, NaCl, Fe ($C_6H_5O_7$), and $MgCl_2$ were found to have the most influence on Chi21702 production. The optimal parameters of temperature, initial pH, and dissolved oxygen level were found to be 25°C, 6.5, and above 30% of air saturation, respectively. The maximum Chi21702 activity obtained under the optimized conditions was 90 U/L. Through statistical optimization methods, a 7.5-fold increase in Chi21702 production was achieved over unoptimized conditions. Chi21702 showed relatively high activity, even at low temperatures close to 0°C. The information obtained in the present study could be applied to the production of cold-active endochitinase on a large scale, suitable for a process at low temperature in industry.

Keywords Antarctic bacterium · Cold-active · Endochitinase · *Sanguibacter antarcticus* · Statistical optimization

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Introduction

Chitin is a polysaccharide synthesized from units of *N*-acetylglucosamine (*N*-acetyl-D-glucos-2-amine, NAG) and is regarded as a critical carbon source for many Antarctic bacteria. A large amount of chitin was found in penguin *guano,* as penguins consume krill for their diet (Croll and Tershy 1998; Sun and Xie 2001). This chitin-rich source contributes to carbon recycling, and thus makes up a critical part of Antarctic ecosystems (Xiao et al. 2005). Chitin composes the cell walls of some microbes, such as fungi, and exoskeletons of invertebrates, including insects and crustaceans. Chitinases (EC 3.2.1.14; 1,4-β-poly-*N*-acetylglucosaminidase), a group of enzymes capable of degrading chitin directly to chitin oligosaccharides or NAG, are produced by a wide variety of organisms. They have received increasing attention because of their potential application in the bio-control of plant-pathogenic fungi and pests, as well as in the bioconversion of shellfish chitin wastes. Chitinases, in general, are divided into two categories: endochitinases and exochitinases. Endochitinases (EC 3.2.1.14) cleave chitin polymers at random internal sites, whereas exochitinases cleave chitin progressively starting at the non-reducing ends of the chains and release *N*-acetyl-D-glucosamine (GlcNAc) monomers or diacetyl-chitobiose by β-(1,4)-*N*-acetylglucosaminidase activity (EC 3.2.1.30) or 1,4-β-chitobiosidase activity (EC 3.2.1.29), respectively (Graham and Sticklen 1994; Harman et al. 1993).

An extracellular chitinase with cold-active characteristics was recently purified by our lab from the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702 (KCTC 13143) (Park et al. 2009). We obtained the coding sequence for this enzyme from genomic DNA and

described the psychrophilic characteristics of endochitinases. This cold-adapted endochitinase, Chi21702, was successfully expressed in the methylotrophic yeast, *Pichia pastoris*, by codon optimization (Lee et al. 2010). However, the maximal chitinase activity of the recombinant endochitinase in *P. pastoris* reached only 30 U/L at 84 h after induction. Accordingly, the goals of the current study were focused on investigating the effects of medium composition on wild-type Chi21702 production and activity, and developing efficient production conditions using wild-type *S. antarcticus* KOPRI 21702. In the area of bioprocess development, statistical experimental design, including two-factorial design and response surface methodology, have proven effective for optimization of culture medium (Tang et al. 2004; Li et al. 2007; Chen et al. 2010; Guerfali et al. 2010). Therefore, statistical experimental design was carried out to optimize the mineral component in the culture medium. The present study describes a statistical approach to optimize production of cold-active endochitinase.

Materials and methods

Strain and media

Chi21702-producing bacteria were isolated from a sea sand sample collected on the Weaver Peninsula (S 62°13′ 45″, W 58°47′15″) on King George Island, Antarctica (Hong et al. 2008). It was named *S. antarcticus* KOPRI 21702 (KCTC 13143) by morphological, biochemical, and phylogenetic analyses. The seed culture was prepared by inoculation of a flask containing ZoBell medium (ZoBell 1946) from a glycerol stock and incubating for 24 h at 25°C. The seed culture (10%) was transferred to the main culture medium for growth of cells and production of Chi21702. Unoptimized Marine Broth basal medium contained the following (grams per liter): glucose, 5.0; peptone, 5.0; yeast extract, 1.0; $Fe(C_6H_5O_7)$, 0.1; NaCl, 19.45; $MgCl_2$, 5.9; Na_2SO_4, 3.24; $CaCl_2$, 1.8; KCl, 0.55; $NaHCO_3$, 0.16; KBr, 0.08; and (milligrams per liter): $FePO_4$, 10.0; $SrCl_2$, 34.0; H_3BO_3, 22.0; Na_2SiO_3, 4.0; NaF, 2.4; NH_4NO_3, 1.6; Na_2HPO_4, 8.0.

Optimization of carbon source and carbon/nitrogen ratio

The effect of carbon sources on liquid cultures of *S. antarcticus* KOPRI 21702 was studied using 10 g/L of each of the following carbon sources: glucose, galactose, fructose, sucrose, lactose, maltose, glycerol, starch, or cellulose. In experiments to determine the effects of carbon/nitrogen ratios, the levels of complex nitrogen sources, yeast extract, and peptone were changed, while the concentration of carbon source was fixed. Cells were grown with the initial ratios of complex nitrogen sources to carbon sources of 0, 0.1, 0.2, 0.5, 1.0, and 2.0.

Identification of significant mineral components

The Plackett–Burman design (Plackett and Burman 1946), an efficient tool for the screening of medium composition, was used to find the mineral components significantly influencing Chi21702 production from *S. antarcticus* KOPRI 21702. Based on the design, 15 mineral components of basal medium were examined at two levels, low (−) and high (+), resulting in a first-order model, $Y=\beta_0+\Sigma\beta_i X_i$, where Y is the predicted response (Chi21702 production), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. This model did not describe interaction among factors (mineral components) and was used only to screen and evaluate important factors influencing the response.

Optimization of selected mineral components

To optimize the concentration of each mineral component previously selected using the Plackett–Burman design, a Box–Behnken design was applied (Box and Behnken 1960). The amount of each mineral component was coded into three levels: (−), (0), and (+) for low, intermediate, and high concentrations, respectively. For prediction of the optimal concentrations, a second-order polynomial model was designed to describe the relationship between the independent variables (mineral components) and the response: $Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ij}X_iX_j+\Sigma\beta_{ii}X_i^2$, where Y is the predicted response (Chi21702 production), and β_0, β_i, β_{ij}, and β_{ii} are the constant and regression coefficients of the model, with X_i and X_j representing the independent nutrient.

Determination of optimal culture conditions and fermentor study

Optimal Chi21702 production conditions were determined by culturing cells under a range of temperature, pH, and dissolved oxygen (DO) conditions; 5°C, 15°C, 20°C, 25°C, 30°C, and 37°C, 5.0 to 9.0 initial pH, and DO of <10% or >30% air saturation. To study the effect of DO on Chi21702 production, a 1-L jar fermentor (Biostat Q—DCU3, B. Braun Biotech International, Germany) was used with 0.5 L optimized medium. Bioreactor operations were carried out to confirm the optimization steps. Cells were cultivated in different media conditions of each optimization step in a 1-L jar fermentor.

Analytical methods

Growth of the *S. antarcticus* KOPRI 21702 was monitored by measuring the optical density of the culture at 600 nm with a spectrophotometer (S-3100, Scinco, Korea). Cell density was also determined by measuring dry cell weight (DCW). The ratio of DCW (grams per liter) to optical density was 0.32. Chi21702 activity in the broth was measured by using *p*-nitrophenyl-β-D-*N*,*N*′-diacetyl-chitobiose [pNP-$(NAG)_2$] as a substrate. The amount of *p*-nitrophenol formed by the enzyme was determined by measuring the absorbance at 400 nm and using the extinction coefficient, 17,000 $M^{-1}cm^{-1}$. A unit of activity per liter (1 U/L) was defined as 1 μmol substrate hydrolyzed to *N*-acetyl glucosamine and *p*-nitrophenol h^{-1} L^{-1}. The optimum temperature for Chi21702 activity was measured in the standard activity assay at temperatures ranging from 0°C to 60°C. The thermal stability of the Chi21702 was measured by incubating at temperatures ranging from 0°C to 60°C for 1 h.

Results

Effect of culture temperature

To evaluate the effects of culture temperature on cell growth and Chi21702 production, *S. antarcticus* KOPRI 21702 cells were cultured using unoptimized basal medium supplemented with or without swollen chitin (2.0 g/L) at temperatures ranging from 5°C to 37°C for 3 days. As shown in Fig. 1, cell growth indicated a dependency on culture temperature; growth rate decreased at 15°C or less, cell growth was completely inhibited at 37°C, and the maximal cell density was 2.89 g dry cell/L without chitin at 25°C. The activity of Chi21702 reached a maximal level of 57 U/L when cells were cultivated at 25°C with chitin and was only 12 U/L without chitin. These results indicate that chitin was necessary for expression of active Chi21702 and that the optimal growth temperature was 25°C.

Determination of best carbon source and concentration optimization

To identify a suitable carbon source for Chi21702 production by flask cultivation of *S. antarcticus* KOPRI 21702, 10 g/L of different carbon sources were tested. Of the carbon sources, lactose, sucrose, maltose, glycerol, starch, and cellulose showed a positive effect on final cell density, while glucose had a negative effect (Fig. 2a). In the case of Chi21702 production, only glycerol and lactose showed a positive effect, while the other carbon sources appeared inhibitory (Fig. 2b). When glycerol was used as the carbon source, dry cell weight and Chi21702 activity were 6.4 g/L and 77 U/L, respectively, representing about 35% higher enzyme activity.

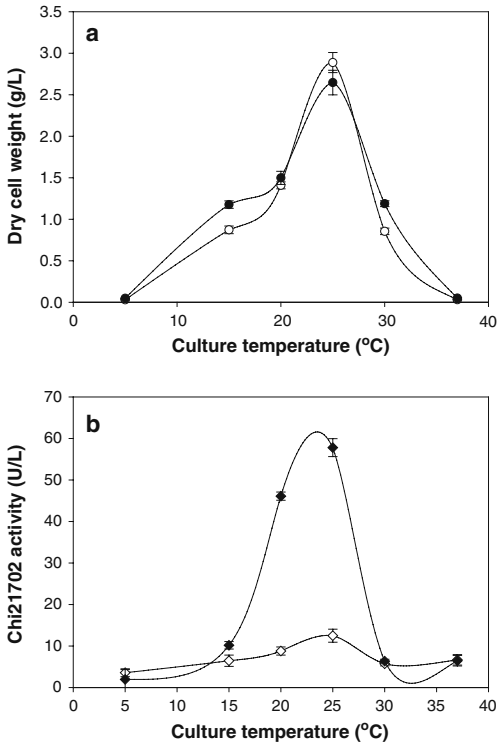

Fig. 1 Effect of culture temperature on (**a**) cell growth and (**b**) Chi21702 activity. Cells were grown at various temperatures in medium with or without chitin. After 3 days of cell cultivation, final cell densities (*empty circle*, without chitin; *filled circle*, with chitin) and Chi21702 activities (*empty diamond*, without chitin; *filled diamond*, with chitin) were measured

Effect of initial concentrations of chitin and glycerol

The effect of initial concentrations of chitin and glycerol in the basal medium on Chi21702 production was also investigated by varying the amounts of these components. Maximum cell density and Chi21702 activity were obtained at 10 g/L of glycerol when tested initially (Fig. 3a). However, Chi21702 activity was found to increase as the initial concentration of chitin increased and maximum activity (84 U/L) was achieved with 5 g/L chitin supplemented with 10 g/L glycerol (Fig. 3b). Chitin (5 g/L) resulted in only a 10% increase in activity over 2 g/L chitin. This result indicated that production of Chi21702 increased by adding chitin, while the yield decreased, and 2 g/L of chitin was more efficient than 5 g/L. Therefore, the best carbon source was determined as a combination of 2 g/L chitin and 10 g/L glycerol, and this combination was supplied to the medium in the following studies.

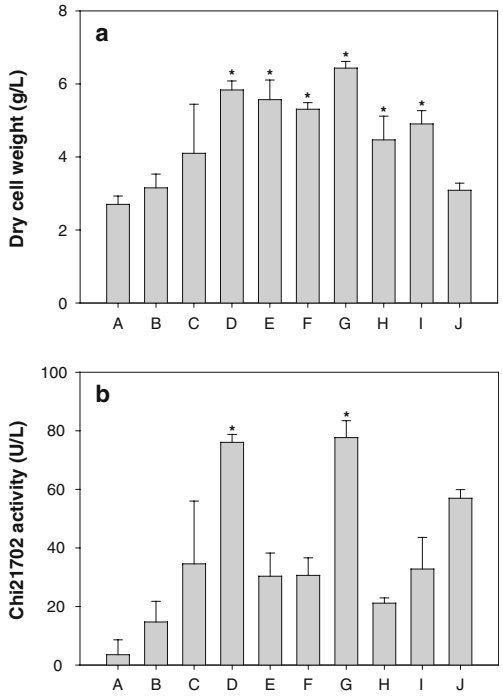

Fig. 2 Effect of carbon sources on cell growth and Chi21702 production. Cells were grown in the medium containing 2 g/L of chitin and 10 g/L of different carbon sources: *A*, glucose; *B*, galactose; *C*, fructose; *D*, lactose; *E*, sucrose; *F*, maltose; *G*, glycerol; *H*, starch; *I*, cellulose; and *J*, not added. After 3 days of cultivation, cell density (**a**) and Chi21702 activity (**b**) were measured. *Asterisks* denote $P<0.05$

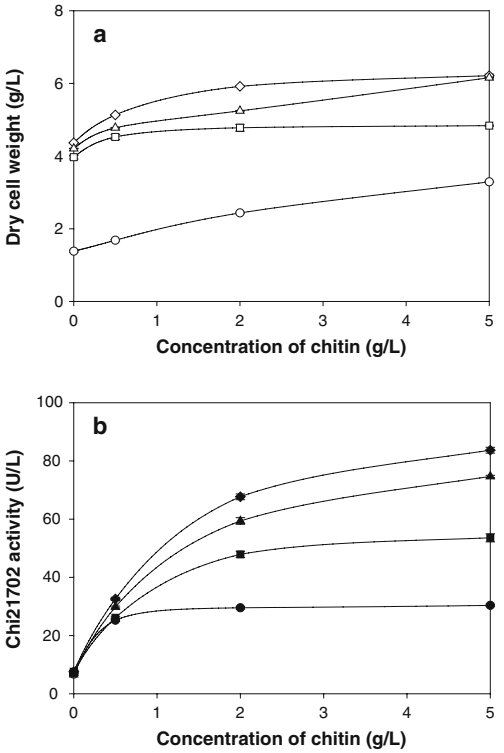

Fig. 3 Effect of initial concentration of chitin and glycerol on cell growth and Chi21702 production. Cells were cultured at different concentrations of glycerol, 0, 5, 10, and 20 g/L with various concentrations (0, 0.5, 2.0, and 5.0 g/L) of chitin. After 3 days of cell cultivation, cell densities (**a**) (*empty circle*, *empty square*, *empty diamond*, and *empty triangle* for 0, 5, 10, and 20 g/L of glycerol, respectively) and Chi21702 activities (**b**) (*filled circle*, *filled square*, *filled diamond*, and *filled triangle* for 0, 5, 10, and 20 g/L of glycerol, respectively) were measured

Selection of the most suitable ratio of complex nitrogen source to carbon source

The production of Chi21702 was conducted in basal medium containing chitin (2 g/L) and glycerol (10 g/L) as carbon sources, with varying amounts of peptone and yeast extract added as complex nitrogen sources. Cell density obtained a maximum value of 7.5 g dry weight/L at a ratio of 0.5, and Chi21702 activity continued to increase up to 84 U/L (Fig. 4). The increase in Chi21702 activity seen with ratios above 0.5 was negligible, and therefore 6.0 g/L was chosen as an input amount of complex nitrogen sources.

Selection of significant mineral components in basal medium

To eliminate unnecessary mineral components for Chi21702 production, each of the 15 different elements included in the basal medium preparation was tested. Table 1 shows the test levels, effect, *t* statistics, and *P* value for each mineral component. The mineral components showing a *P* value of <0.05 were accepted as significant factors affecting the production of Chi21702. It was found that the *P* values of $NaHCO_3$, $FePO_4$, $SrCl_2$, Na_2SiO_3, NaF, and Na_2HPO_4 were >0.05, indicating that these six elements were not significant factors. Three mineral components, KCl, KBr, and NH_4NO_3, were excluded from further studies because of negative effects on Chi21702 production. However, $Fe(C_6H_5O_7)$, NaCl, $MgCl_2$, Na_2SO_4, $CaCl_2$, and H_3BO_3 showed positive effects on Chi21702 production with *P* values of <0.05;

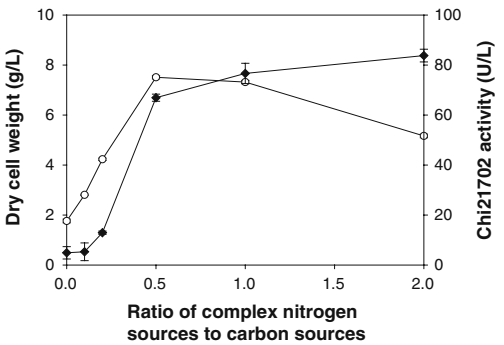

Fig. 4 Optimization of complex nitrogen/carbon ratio for Chi21702 production. After 3 days of cultivation in a flask, final cell density (*empty circle*) and Chi21702 activity (*filled diamond*) were measured

Table 1 Statistical analysis of mineral components using the initial Plackett–Burman experiment

Variable	Mineral component	+ Value (g/L)	− Value (g/L)	Effect	*t* Statistics	*P* value
X_1	$Fe(C_6H_5O_7)$	0.1	0	13.762	3.70	0.002
X_2	NaCl	19.45	0	19.256	5.17	0.000
X_3	$MgCl_2$	5.9	0	8.539	2.29	0.036
X_4	Na_2SO_4	3.24	0	9.319	2.50	0.024
X_5	$CaCl_2$	1.8	0	10.002	2.69	0.016
X_6	KCl	0.55	0	−9.427	−2.53	0.022
X_7	$NaHCO_3$	0.16	0	−3.478	−0.93	0.364
X_8	KBr	0.08	0	−17.554	−4.72	0.000
X_9	$FePO_4$	0.01	0	−3.370	−0.91	0.379
X_{10}	$SrCl_2$	0.034	0	−6.534	−1.76	0.098
X_{11}	H_3BO_3	0.022	0	8.940	2.40	0.029
X_{12}	Na_2SiO_3	0.004	0	−0.087	−0.02	0.982
X_{13}	NaF	0.0024	0	0.823	0.22	0.828
X_{14}	NH_4NO_3	0.0016	0	−14.965	−4.02	0.001
X_{15}	Na_2HPO_4	0.008	0	−1.961	−0.53	0.606

consequently, these six components were selected as significant mineral sources for Chi21702 production.

To select the mineral components having optimal effects on Chi21702 yield, the six mineral components showing positive effects on yield were retested using a secondary Plackett–Burman design. As a result, the variables with two levels of concentrations for each variable were obtained, and the effect values, *t* statistics, and *P* value for the six components were calculated (Table 2). Analysis of the regression coefficients of the six mineral components showed *P* values for Na_2SO_4, $CaCl_2$, and H_3BO_3 above 0.1, indicating that these components were insignificant for Chi21702 production (Table 2). Finally, three mineral components, $Fe(C_6H_5O_7)$, NaCl, and $MgCl_2$, were selected as the most effective components for the production of Chi21702.

Optimization of mineral components for Chi21702 production

The significant mineral components for Chi21702 production were studied further using a Box–Behnken design. Table 3 shows the mineral components tested for Box–Behnken optimization and the values, which were calculated by linear multiple regression using Minitab software. As a result, the following equation was obtained: $Y=54.67-219.14X_1+2.80X_2+2.63X_3+1,111.11{X_1}^2-0.07{X_2}^2-1.13{X_3}^2+0.62X_1X_2+0.00X_1X_3+0.14X_2X_3$; where Y is the predicted response (Chi21702 production), and X_1, X_2 and X_3 are the concentrations of $Fe(C_6H_5O_7)$, NaCl, and $MgCl_2$, respectively. The value of R^2, a measure of the fit of the model, was 0.961 for Chi21702 production, which indicates that 3.9% of the total variation is not explained by Chi21702 production. A three-dimensional response plot shows the behavior of Chi21702 production. The main effect and interaction effect of three factors, $Fe(C_6H_5O_7)$, NaCl, and $MgCl_2$, at different concentrations were shown in Fig. 5. The expected Chi21702 production was determined to be 89 U/L when the optimal concentrations of Fe $(C_6H_5O_7)$, NaCl, and $MgCl_2$ were 0.01, 23, and 2.5 g/L, respectively.

Effect of initial pH and dissolved oxygen level on the Chi21702 production

The effect of initial pH on cell growth and Chi21702 production was observed in flasks containing optimized medium. Varying the initial pH of the medium between 5.0

Table 2 Statistical analysis of selected mineral components using the secondary Plackett–Burman design

Variable	Mineral component	+ Value (g/L)	− Value (g/L)	Effect	*t* Statistics	*P* value
X_1	$Fe(C_6H_5O_7)$	0.2	0.02	−10.023	−2.07	0.052
X_2	NaCl	38.9	3.89	22.083	4.56	0.000
X_3	$MgCl_2$	11.8	1.18	−9.891	−2.04	0.055
X_4	Na_2SO_4	6.48	0.648	6.151	1.27	0.219
X_5	$CaCl_2$	3.6	0.36	−0.435	−0.09	0.929
X_6	H_3BO_3	0.044	0.0044	4.352	0.90	0.380

Table 3 Box–Behnken optimization of selected significant mineral components

Variable	Mineral component	+ Value (g/L)	0 Value (g/L)	− Value (g/L)
X_1	$Fe(C_6H_5O_7)$	0.1	0.055	0.01
X_2	NaCl	40.0	22.0	4.0
X_3	$MgCl_2$	6.0	3.3	0.6

and 9.0 had an effect both on growth and Chi21702 production. Maximal enzyme activity was obtained when the initial pH of the culture medium was adjusted to 6.5, while maximal cell density was obtained at an initial pH of 7.0 (Fig. 6a). The effect of DO level on cell growth and Chi21702 production was also investigated by adjusting DO level to high (>30%) or low (<10%) using a bioreactor.

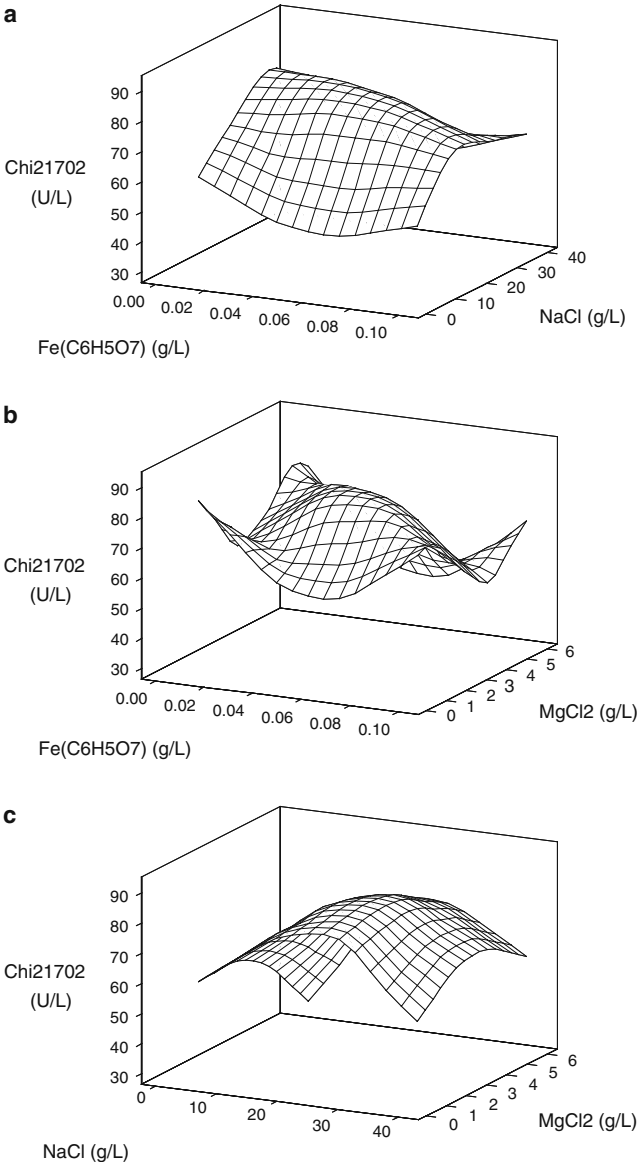

Fig. 5 Three-dimensional response plot showing the effect of (**a**) Fe ($C_6H_5O_7$) and NaCl; (**b**) Fe($C_6H_5O_7$) and $MgCl_2$; (**c**) NaCl and $MgCl_2$ on Chi21702 production

Adjusting the DO level was performed by changing stirring speed or airflow rate. The DO level was controlled 24 h after inoculation because it was difficult to adjust the DO level to lower than 30% in the early stage of fermentation. Under these conditions, the DO levels had no effect on the Chi21702 production (Fig. 6b).

Bioreactor operations under optimized steps

To confirm our predictions and optimized conditions, Chi21702 production from *S. antarcticus* KOPRI 21702 was performed in a bioreactor. Cells were cultivated at 25°C under different media conditions: basal medium without chitin, basal medium with chitin, carbon and nitrogen source-optimized medium, and mineral source-optimized medium. The initial pH was adjusted to 6.5 and DO level was maintained above 30% of air saturation during fermentation. Figure 7 shows the time profiles of

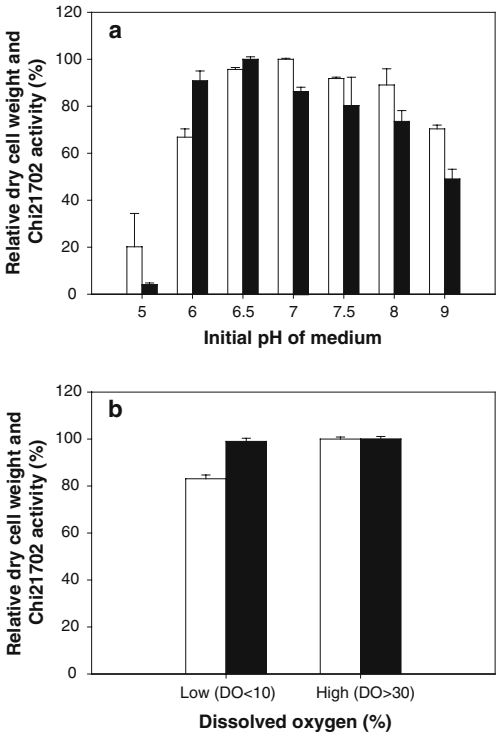

Fig. 6 Effect of (**a**) initial pH and (**b**) DO level on Chi21702 production. Cells were cultivated in flasks containing optimized medium adjusted to pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0, respectively. The effect of DO was observed by adjusting the DO level to high (>30%) or low (<10%) using a bioreactor. After 3 days of cultivation, cell density (*white bar*) and Chi21702 activity (*black bar*) were measured

cell growth and Chi21702 production using different media and same operation conditions. The maximal cell densities of 2.0, 2.5, 5.1, and 5.5 g dry cell/L were attained and the maximal Chi21702 productions reached 12, 65, 79, and 90 U/L in basal medium without chitin, basal medium with chitin, carbon and nitrogen source-optimized medium, and mineral source-optimized medium, respectively. These results indicate that the optimization enhanced the Chi21702 production.

Effects of temperature on enzyme activity and stability

Chi21702 activity was analyzed at various temperatures and compared with that of a commercially available mesophilic chitinase from *Streptomyces griseus*. The chitinolytic activity was highest at 30°C and 40°C for Chi21702 and the mesophilic chitinase, respectively (Fig. 8a). Chi21702 exhibited relative activities of 37% and 59% at 0°C and 10°C, respectively, in comparison to 100% at 30°C. The mesophilic chitinase from *S. griseus* exhibited relative activities of 13% and 30% at 0°C and 10°C, respectively, in comparison to 100% at 40°C.

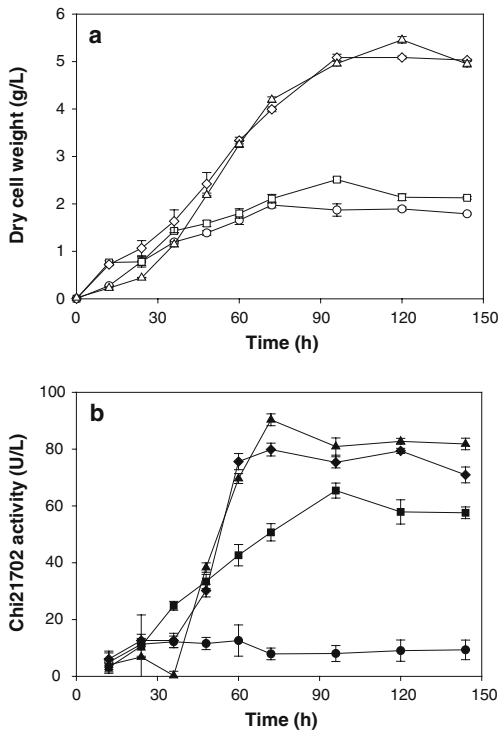

Fig. 7 Time profile of cell growth by dry weight (**a**) (*empty circle*, basal medium without chitin; *empty square*, basal medium with chitin; *empty diamond*, carbon and nitrogen source-optimized medium; *empty triangle*, mineral source-optimized medium) and Chi21702 production (**b**) (*filled circle*, basal medium without chitin; *filled square*, basal medium with chitin; *filled diamond*, carbon and nitrogen source-optimized medium; *filled triangle*, mineral source-optimized medium) during cultivation of *S. antarcticus* KOPRI 21702 in a jar fermentor

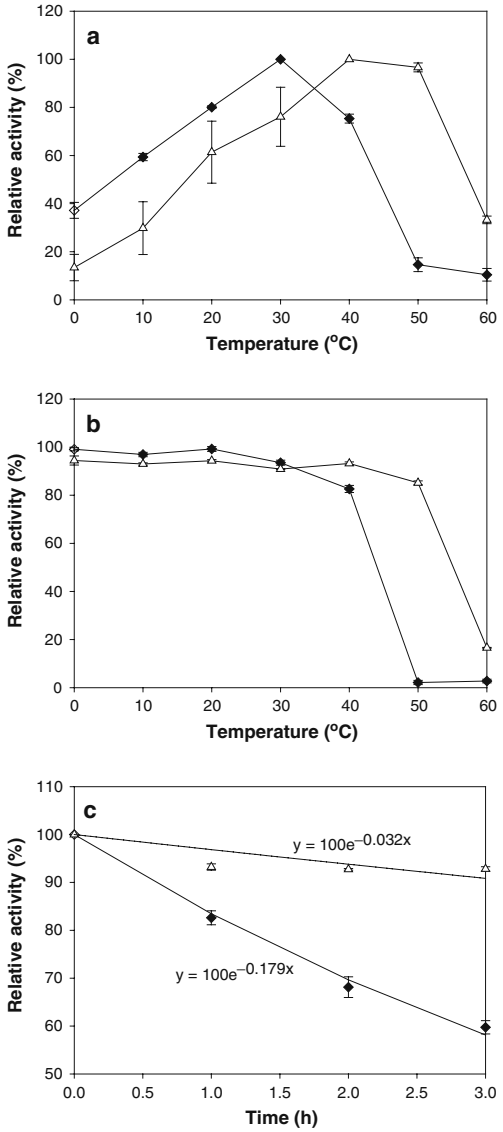

Fig. 8 Effects of temperatures on the enzyme activity and stability. (**a**) Temperature dependence of Chi21702 (*filled diamond*) and mesophilic chitinase from *S. griseus* (*empty triangle*) was determined at various temperature ranging from 0°C to 60°C for 1 h. (**b**) Thermal stability of Chi21702 (*filled diamond*) and mesophilic chitinase from *S. griseus* (*empty triangle*) was measured after 1 h incubating at various temperature. (**c**) Deactivation profile of Chi21702 (*filled diamond*) and mesophilic chitinase from *S. griseus* (*empty triangle*) at 40°C for 3 h

To compare temperature stability, Chi21702 and the mesophilic chitinase were incubated at various temperatures (0−60°C) for 1 h, and then activity was determined. When Chi21702 was incubated at 40°C for 1 h, activity slowly decreased (Fig. 8b), whereas activity of mesophilic chitinase decreased after 1 h incubating at above 50°C. Chi21702 retained only 2% of the original activity after 1 h of incubation at 50°C, whereas mesophilic chitinase retained 85% of its original activity after 1 h incubation at the same temperature. Chi21702 retained 60% of its

original activity after incubating at 40°C for 3 h, and the half-life of inactivation was estimated to be 3.87 h. On the other hand, after preincubation for 3 h at 40°C, mesophilic chitinase retained above 90% of its original activity (Fig. 8c).

Discussion

To enhance the productivity of cold-active chitinase Chi21702, which was originally found in the Antarctic microorganism *S. antarcticus* KOPRI 21702, optimized conditions were investigated. In general, no defined medium has been established for chitinase production from different microorganisms. Each organism has its own requirement of special conditions for maximum chitinase production (Dahiya et al. 2005). The chemical and physical parameters affecting on the production of Chi21702 were optimized by using statistical designs.

Glycerol and chitin were selected as carbon sources, with chitin having a critical effect on Chi21702 production. Chitinases are inducible enzymes secreted in the presence of chitin (Singh et al. 2009). The Chi21702 activity when chitin was included in medium reached 57 U/L, which was 4.7-fold higher than when chitin was not included. Chi21702 production was repressed by addition of glucose to the medium. It has been reported that *chiA*, *chiC*, and *chi63* of *Streptomyces* sp. showed a glucose repression effect (Delic et al. 1992; Fujii and Miyashita 1993). A statistical approach was found to be very effective for optimizing the mineral components in a manageable number of experimental runs. The final optimized medium composition was as follows (grams per liter): glycerol, 10.0; chitin, 2.0; peptone, 5.0; yeast extract, 1.0; Fe ($C_6H_5O_7$), 0.01; NaCl, 23.0; $MgCl_2$, 2.5; Na_2SO_4, 3.24; $CaCl_2$, 1.8; $NaHCO_3$, 0.16; and (milligrams per liter): $FePO_4$, 10.0; $SrCl_2$, 34.0; H_3BO_3, 22.0; Na_2SiO_3, 4.0; NaF, 2.4; Na_2HPO_4, 8.0. Previous studies using statistical optimization of chitinase production have been carried out using *Streptomyces* sp. (Nawani and Kapadnis 2005), *Paenibacillus* sp. (Patel et al. 2007; Singh et al. 2009), and *Enterobacter* sp. (Dahiya et al. 2005). However, until recently, only a few psychrophilic exochitinases have been discovered (Fenice et al. 1998; Lonhienne et al. 2001; Mavromatis et al. 2003; Orikoshi et al. 2003; Stefanidi and Vorgias 2008).

The physical parameters, temperature, initial pH, and DO level were optimized to be 25°C, 6.5, and above 30% air saturation, respectively. The cell growth was completely inhibited at 37°C. This is not very surprising since *S. antarcticus* KOPRI 21702 was from a cold region. The cell growth rate decreased at 15°C or less. The microorganism used in this study was able to grow well in the neutral pH range of 6.0–8.0 and produced Chi21702 with optimal activity at pH 6.5. Under pH 6.0, the cell growth was inhibited, which was slightly a different result from Hong et al. (2008), which showed optimal pH of 5–6. The DO level of medium substantially had no effect on the Chi21702 production, except for slightly low cell growth at low DO level. The cells are facultatively anaerobic (Hong et al. 2008). Finally, bioreactor operation performed under different media conditions resulted in the maximal Chi21702 production of 90 U/L in the final optimized conditions, which was 7.5 times higher than that of unoptimized conditions. It was difficult to compare the Chi21702 activity with other reported chitinase activities because of using the different substrate and unit definitions. Our maximal Chi21702 activity of 90 U/L corresponds to about 0.022 μmol/min/mg protein, which is slightly higher than that of MmChi60 reported as a psychrophilic chitinase from *Moritella marina* (Stefanidi and Vorgias 2008).

In general, cold-adapted enzymes display an apparent optimal activity shifted toward low temperatures (Orikoshi et al. 2003). The optimal temperature for activity of Chi21702 was over 10°C lower than that of mesophilic chitinase from *S. griseus*. The relative activity of Chi21702 at 0°C, 37%, was found to be higher than those of the previously characterized, cold-adapted, chitinase from several bacterial strains: *Alteromonas* sp. (28%) (Orikoshi et al. 2003) and *M. marina* (10%) (Stefanidi and Vorgias 2008). The Chi21702 half-life at 40°C, 3.87 h, is 32 times higher than that of cold-adapted chitinase ChiB from *Alteromonas* sp. (Orikoshi et al. 2003) and 18 times lower than that of MnChi60 (Stefanidi and Vorgias 2008) at the same temperature of 40°C. These results indicate that Chi21702 has typical features of cold-active enzymes: relatively high catalytic activity at low temperature and remarkable thermosensitivity (Feller et al. 1996).

To date, no practical information has been available on optimization of cold-active chitinase production by *Sanguibacter* species. The present study provides a suitable medium and operating conditions for improving chitinase production by *S. antarcticus* KOPRI 21702. Consequently, the optimal conditions obtained in the present study would provide a practical basis for the production of cold-active Chi21702 at a large scale.

Acknowledgements This research was supported by a grant from the Korea Polar Research Institute (PE10050).

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