Enhanced chitinase production by *Chitinolyticbacter meiyuanensis* SYBC-H1 using staged pH control

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The pH of a microbiological culture is important for both cell growth and chitinase accumulation, but the optimal pH is not normally the same for both. The objective of this study was to investigate the effect of pH on chitinase production by *Chitinolyticbacter meiyuanensis* strain SYBC-H1 (ATCC BAA-2140) in a mineral medium. The results of batch culture at different pH values showed that the optimum pH for cell growth and chitinase production varied with time, although KOH produced the best results for cell growth and chitinase production, NaOH was chosen because of cost considerations. We designed a three-stage pH control strategy using NaOH as the neutralizing agent. Maximum cell growth (1.07 g dry cell weight/l) and maximum chitinase activity (13.6 U/ml) were observed after culture at 26°C for 72 h in a mineral medium. These values were greater by 129% and 162%, respectively, and the length of time to attain maximum chitinase activity was decreased by 12 h, compared with results from an earlier study (Hao et al., 2011b).

Key Words: chitinase; *Chitinolyticbacter meiyuanensis* SYBC-H1; fermentation; neutralizing agents; pH control strategy

Introduction

Chitinases (EC 3.2.1.14) are enzymes that hydrolyze chitin to its oligomeric and monomeric components by cleaving the β-1,4-N-glycosidic bond (Nakagawa et al., 2011). Chitinases are gaining increasing attention owing to their wide range of biotechnological applications, such as shellfish waste management (Nakagawa et al., 2011), generation of fungal protoplasts (Fleuri et al., 2009), production of single cell protein (Wang et al., 2006), production of N-acetyl-β-D-glucosamine (GlcNAc) and derivatives (Lin et al., 2009) and bio-control of fungal plant pathogens (Joo, 2005).

Despite the potential commercial benefits, the application of chitinases has been limited by low productivity (Liu et al., 2013), which is of particular relevance to the production by the enzymatic degradation of chitin. Thus, increasing the levels of chitinase production is a key to improving GlcNAc production on an industrial scale. In a previous study, increasing the chitinase production, mainly via strain screening (Meena et al., 2013), strain mutation (Li et al., 2007), genetic modification (Iqbal et al., 2012) and medium optimization (Singh et al., 2013). Furthermore, the control of culture conditions, including the pH, was shown to be important for controlling the level of chitinase activity.

The effect of pH on cell growth and enzyme production differs among microorganisms. Chitinase can be divided into alkaline (Bhushan and Hoondal, 1998), acidic (Chang et al., 2014), and neutral (Takeo et al., 2009) chitinase, according to its isoelectric point. The change of the growth medium pH owing to the accumulation of GlcNAc produced by the hydrolysis of chitin (Halder et al., 2013) does not benefit both cell growth and chitinase production in chitinase fermentation. However, there are few reports about the effect of pH on chitinase production in batch fermentation.

A highly efficient chitinolytic bacterium *Chitinolyticbacter meiyuanensis* SYBC-H1 producing alkaline chitinase was isolated in our lab (Hao et al., 2011a, b; Zhang et al., 2016). In the present study, the influence...
of pH and neutralizing agents on the production of chitinase by *C. meiyuanensis* SYBC-H1 were investigated in a 1.4-L fermentor. We proposed a staged pH control strategy based on the recorded effect of pH on cell growth and chitinase formation.

**Materials and Methods**

*Chemicals and the microorganism.* Chitin powder was purchased from Aladdin Co., Ltd, Shanghai, China. Colloidal chitin was prepared from chitin powder as described Inokuma et al. (2013). Other chemicals and solvents were of analytical grade and were purchased from local suppliers. The *C. meiyuanensis* strain SYBC-H1 (ATCC BAA-2140) was isolated previously (Hao et al., 2011b).

*Medium and culture.* The *C. meiyuanensis* strain SYBC-H1, which was kept at –70°C, was transferred to 50 mL of pH 7.0 seed medium (composition (g/l): glucose 4.0, peptone 4.0, KH₂PO₄ 0.7, K₂HPO₄·3H₂O 0.3, MgSO₄ 0.5) in 250-mL shake flasks and incubated at 37°C for 12 h on a rotary shaker at 200 rpm.

The fermentation medium was composed of (g/l): inulin 3.5, urea 4, chitin powder 4, KH₂PO₄ 0.7, K₂HPO₄·3H₂O 0.3, MgSO₄ 0.5 and pH 7.0. Seed with size 5% of inoculation was inoculated into the fermentation medium. Flask fermentation was conducted in a 500-mL shake flask containing 100 mL fermentation medium and kept on rotary shakers at 250 rpm at 26°C.

Batch fermentation was carried out in a 1.4-L fermentor (INFORS HT Multifors) with 800 mL fermentation medium. The culture conditions were: fermentation temperature (26°C +/- 1), aeration ratio of 1vvm (vessel volume per minute), agitation speed of 200–400 rpm, and dissolved oxygen levels were 100% of air saturation at the time of inoculation, and was maintained at 40% of air saturation during cell growth during the rest of the process. The initial pH value and the constant pH value of the fermentation medium were adjusted with 2.0 mol/L HCl and 2.0 mol/L NaOH. The pH was controlled mechanically online. In the two-stage pH control experiment, the pH was controlled at 6.6 during the first 12 h, then increased to 7.8 within 15 min and kept at that value until the end of fermentation. The three-stage pH control strategy involved the pH being kept at 6.6 for the first 12 h, then increased to 7.8 and kept at that value for 12–48 h, and, finally, being increased to 8.4 and kept at that until the end of the fermentation. Other conditions were the same as those of the constant pH experiment. All experiments were carried out in triplicate.

*Analytical method.* The chitinase assay used colloidal chitin as the substrate according to a previous report (Hao et al., 2011b). A mixture containing 0.5 mL of 1% (w/v) colloidal chitin, 1.4 mL of 50 mM sodium phosphate buffer (pH 7.0) and 0.1 mL of the supernatant was incubated for 0.5 h at 37°C. Reducing sugar released during the reaction was quantified using GlcNAc standards at concentrations ranging from 0.25–1.25 μmol/mL by the 3, 5-dinitrosalicylic acid method (Rojas-Avelizapa et al., 1999). One unit (U) of chitinase activity was defined as the amount of enzyme that produced 1 μmol of GlcNAc in 1 min at 37°C.

Cell growth was determined by the measurement of dry cell weight (DCW) as follows: 10 mL of fermentation liquor was passed through a single qualitative filter paper (medium-speed) and added to a dry centrifuge tube of known weight (G1), which was then centrifuged at 3500 g for 5 min and the supernatant was discarded. The sediment was washed with deionized water and then placed with the tube into an oven at 60°C and dried to constant weight (G2). DCW (g/l) was calculated as:

\[
DCW = (G2 - G1)/10.
\]

The specific activity (U/mg) was calculated as:

\[
\text{Specific activity} = \text{Chitinase activity/DCW}.
\]

**Results and Discussion**

*Scale-up of chitinase fermentation from a 500-mL shake flask to a 1.4-L fermentor with different initial pH values.*

The effect of the pH of the initial medium on cell growth and chitinase activity in the shake flask culture was investigated. Figure 1 showed that poor cell growth and a low level of chitinase activity were detected at an initial pH < 6.5. Cell growth and chitinase activity were increased 1.3-fold and 11.4-fold, respectively, when the initial pH was increased from 6.5 to 7.0. Maximum cell growth and maximum chitinase activity of 0.49 g/l and 5.17 U/ml, respectively, were found at an initial pH 7.5. Together, these results suggested that chitinase production with *SYBC-H1* might be optimal at pH > 7.0.

To investigate further the whole process of cell growth and chitinase production, fermentation at initial pH 7.0–8.0 was scaled up in a 1.4-L fermentor. Figures 2a–c showed that the pH decreased after 12 h and then increased...
from 12–36 h, but there was no distinct change after 48 h. Moreover, cell growth increased before 48 h but there was no distinct change after 48 h, as in the case of the pH; however, chitinase activity increased by 84 h. Changes of pH and cell growth might be related to two carbon sources, inulin and chitin, in the medium. Inulin was metabolized first and the pH decreased sharply owing to the production of lactic acid (data not shown). Next, chitinase activity was induced and the degradation of chitin released GlcNAc, which served as a carbon source for cell growth. The pH increased, likely due to the accumulation of GlcNAc, which contain an amine group (Halder et al., 2013). Changes of cell growth and pH stopped when all the chitin was metabolized. The maximum chitinase activity and the maximum cell growth achieved in the 1.4-L fermentor were 9.85 U/ml and 0.97 g/l at pH 7.5, which were increases of 90.5% and 93.9%, respectively, compared with the 500-mL shake flask. Sufficient oxygen was shown to be beneficial to cell growth and the synthesis of chitinase in this aerobic microorganism (Abd-Aziz et al., 2008). One shortcoming of an uncontrolled pH culture is that an optimal pH for cell growth cannot be maintained. Thus, the effect of a constant pH on cell growth and chitinase production was investigated.

**Fig. 2.** Process of chitinase fermentation for initial pH values. (▲, Initial pH 7.0; ■, Initial pH 7.5; ○, Initial pH 8.0). Error bars show one standard deviation, as determined from triplicate experiments.

**Fig. 3.** Effect of constant pH on chitinase fermentation. (■, pH 6.0; ○, pH 6.6; ●, pH 7.2; ▼, pH 7.8; △, pH 8.4; ▼, pH 9.0). Error bars show one standard deviation, as determined from triplicate experiments.
The effect of constant pH on cell growth and enzyme production

The effect of constant pH in the range 6.0–9.0 on chitinase production was studied using NaOH as a neutralizing agent. Figures 3a and 3b showed that cell growth was in a rapid growth phase during the first 12 h of culture, little chitinase activity was detected and the optimum pH for cell growth was 6.6. During 12–48 h of culture, however, both cell growth and chitinase activity increased. There was no change in the rate of cell growth after 48 h, but chitinase activity continued to increase. Furthermore, an excessively high or low pH led to poor cell growth and chitinase production. Chitinase activity at pH 7.2–8.4 was higher compared to pH < 6.6. Among these values, pH 7.8 was most suitable for chitinase accumulation, the maximum cell growth was 0.97 g/l and the maximum chitinase activity was 10.5 U/ml at 84 h. Chitinase activity at pH 7.8 was increased by 6.5% and the cell growth rate was similar compared with that at the initial pH 7.5.

The specific activity increased with increasing pH from 6.0–8.4 and maximum specific activity (12.3 U/mg) was attained at pH 8.4 (Fig. 3c). Total chitinase activity at pH 8.4, however, was lower compared to pH 7.8 owing to poor cell growth. The optimal pH for cell growth and chitinase production varied with the length of culture time and it is clear that a staged control of pH at different times is required for optimal cell growth and chitinase production.

Effect of different neutralizing agents

Neutralizing agents used to control the pH of fermentation often affects production in the culture of microorganisms (Qin et al., 2010). Thus, in this study, we investigated the effect of neutralizing agents on chitinase production. We used NaOH, KOH, NH₃·H₂O, or 4MgCO₃·Mg(OH)₂·5H₂O to maintain a constant pH 7.5 during chitinase production.

Figures 4a and 4b showed that when NH₃·H₂O was used, the maximum chitinase activity of 8.2 U/ml was decreased by 12.3% compared to NaOH (9.35 U/ml). This was a negative effect for cell growth owing to the accumulation of NH₄⁺ in the medium, which led to lower levels of chitinase production. The original culture medium contains about 0.13 mol/l nitrogen (4 g/l urea). We added about 0.24 mol/L NH₄⁺ (40 ml/l NH₃·H₂O (10%)) to adjust the pH. However, cell growth just needs about 0.1 mol/l nitrogen (DCW (0.8 g/l) × 12%). According to the conservation of nitrogen, about 0.27 mol/l NH₄⁺ might be accumulated. Moreover, experiments have implied that cell growth is inhibited when the concentration of NH₄⁺ reaches 0.15 mol/l. In conclusion, the concentration of NH₄⁺ was great enough to affect the growth of Chitinolyticbacter meiyuanensis. In addition, the published paper shows that the cell membrane has a greater perme-
ability for NH$_4^+$ (Kleiner, 1985) and the infiltration of NH$_4^+$ can cause the intracellular pH to change because cells need more energy to pump out NH$_4^+$. When there is a shortfall of energy, the intracellular pH changes owing to the presence of NH$_4^+$, which affects the normal metabolism of the cell, leading eventually to death (Buurman et al., 1991). However, chitinase activity was only 6.83 U/ml when 4MgCO$_3$·Mg(OH)$_2$·5H$_2$O was used. Although there are reports of Mg$^{2+}$ promoting chitinase activity (Brzezinska and Jankiewicz, 2012; Jiang et al., 2012), the solubility of 4MgCO$_3$·Mg(OH)$_2$·5H$_2$O is poor and it cannot maintain pH well, which might inhibit chitinase production. When KOH was chosen as the neutralizing agent, a maximum cell growth of 1.02 g/l and a maximum chitinase activity of 10.0 U/ml was achieved, which were a little greater than in the case of NaOH. These results suggest that K$^+$ promotes chitinase activity, as has been reported for the chitinase of Halobacterium salinarum (Garcia-Fraga et al., 2014). Thus, more GlcNAc was produced by the hydrolysis of chitin and made available for cell growth and more chitinase was accumulated. However, the industrial relevance of cost (raw materials) and practicality were considered, and NaOH was chosen as the neutralizing agent in chitinase production.

**Staged pH control batch fermentation process for optimal chitinase production**

The results mentioned above have shown that the optimal pH for cell growth and for chitinase formation are different. A relatively low pH at the early stage of fermentation is advantageous to cell growth, whereas a higher pH can support strong cell growth and chitinase formation at the mid- and later stages.

It was therefore necessary to use a staged pH control process, instead of an uncontrolled pH or constant pH control process, for optimal chitinase production. We investigated two types of pH control strategy: (1) A two-stage pH control strategy in which the pH was kept at 6.6 for the first 12 h and then increased to 7.8 and kept at that value until the end of fermentation. (2) A three-stage pH control strategy in which the pH was kept at 6.6 for the first 12 h then increased to 7.8 and kept at that value for 12–48 h and, finally, the pH was increased to 8.4 and kept at that value until the end of fermentation. NaOH was chosen as the neutralizing agent for both strategies.

Figure 5 showed that there was no significant difference in cell growth between the two pH control strategies. Chitinase activity was higher after 48 h of culture with the three-stage strategy, compared with the two-stage strategy. The maximum chitinase activity achieved with the three-stage strategy was 13.6 U/ml, which was 11.8% higher compared with the two-stage strategy (12.1 U/ml). Moreover, maximum chitinase activity with the three-stage pH control strategy was attained at 72 h, which was less by 12 h compared with the two-stage strategy (84 h). Thus, the three-stage pH control strategy was optimal for chitinase production.

Table 1 compares the results of the staged pH control, the uncontrolled pH (initial pH 7.5) and the constant pH 7.8 strategies. Chitinase activity with the three-stage pH control strategy was greater by 162%, 37.6%, 29.1% and 11.8% compared to an earlier study (Hao et al., 2011a), the uncontrolled pH (initial pH 7.5), the constant pH 7.8, and the two-stage pH control strategy, respectively. Moreover, the length of time taken to achieve maximum chitinase activity was decreased by 12 h. We conclude that the three-stage pH control strategy could improve chitinase production greatly. Hence, this study has demonstrated a successful approach to improving the level of chitinase production.

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