A Novel Three-stage Process for Continuous Production of Penicillin G Acylase by a Temperature-sensitive Expression System of *Bacillus subtilis* Phage phi105

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Original scientific paper
Received: March 23, 2005
Accepted: October 1, 2005

This study is pertaining to the production of penicillin G acylase (PGA) by *Bacillus subtilis* ϕ105MU331 in which PGA gene, under the control of thermal-induced promoter, was integrated. The key process parameters including induced-temperature, induced-time, and culture temperature were optimized in flask culture. A three-stage cultivation process was developed for PGA production with the expression system of *B. subtilis* ϕ105MU331. Furthermore, a bioreactor with a thermal-induced apparatus was designed for continuous production of PGA, where cell growth, induction, and PGA expression could be conducted separately. At a dilution rate of 0.20 h^{-1}, PGA production was taken under continuous cultivation in three-stage process. After continuous feeding, the cell density, pH, and residual glucose in the first- and third-reactor were maintained steady for up to 40 h. These results suggested that the new three-stage process might be feasible and very efficient for production of heterologous proteins.

Keywords:
Penicillin G acylase; *Bacillus subtilis*; prophage; three-stage cultivation process

Introduction

Penicillin G acylase (E.C.3.5.1.11, PGA) can hydrolyze benzylpenicillin to phenylacetic acid (PAA) and 6-aminopenicillianic acid (6-APA), which is the essential intermediate for the production of many kinds of semi-synthetic antibiotics, such as ampicillin and amoxycillin.[1] PGA is a heterodimeric protein consisting of α-subunit and β-subunit and has been isolated from a wide variety of microorganisms including bacteria, yeast, and filamentous fungi. Most of studies on PGA production refer to the intracellular expression by *Escherichia coli*.[2–4] Although, extracellular production of PGA in *B. megaterium* is interesting, there are few studies related to *B. megaterium* PGA are available.[5–7] The production of PGA involves in several complex steps, which are usually regulated by several environmental conditions, such as temperature,[8] catabolite repression by glucose, [9–10] and induction by phenyl acetic acid (PAA).[11]

Recombination technology enabled promotion of PGA production, as well as the production of many other important enzymes and bioactive molecules. As an expression host, *B. subtilis* has several advantages compared with *E. coli*. It is capable of secreting extracellular protein directly into the culture medium, which greatly simplifies the downstream purification process. *B. subtilis* is a non-pathogen and is free from endotoxins and has no significant bias in codon usage.[12] Therefore, *B. subtilis* is an attractive host for production and secretion of proteins of interest.[13–14] Many heterologous genes have been expressed successfully in the *B. subtilis* expression systems.[15]

B. subtilis ϕ105 system has been adopted for efficient expression of heterologous gene, because it is very stable even in the absence of selective pressure and has a strong heat-inducible ϕ 105 promoter, which can reach high-level of expression following induction.[16–18] In this expression system, it is essential that the promoter was induced at 55 °C and the expression was conducted at 37 °C. So it's very difficult to reach high induced-temperature and cultivation temperature in several minutes, especially in large-scale or continuous production process. On the other hand, bioreactor is the heart of many industrial biochemical processes, including constructed host/vector systems. In the development of a bioreactor, parameters including mixing, oxygen transfer, and shear stress need to be optimized in order to reach a highest productivity.

In this study, *B. subtilis* ϕ105MU331 was employed for excretive production of PGA based on the temperature-sensitive promoter of phage ϕ105. In order to enhance foreign protein production, this work focused primarily on optimization of the key parameters related to overproduction of PGA, using thermal-induced *B. subtilis* expression system. Fur-

thermore, the feasibility of using three-step cultivation for PGA production was also evaluated in self-designed bubble column reactor based on the characteristics of this expression system.

Materials and methods

Strain and storage

B. subtilis ϕ105 MU331 was used as a host strain for PGA production, in which PGA gene from *B. megaterium* was integrated into its chromosome by gene recombination technology. ϕ105MU331 is a thermal-inducible vector and a derivative of prophage ϕ105 with an insertion of *lacZ*-cat cartridge near the *cos* site of the prophage. [18–19]

Media and cultivation condition

Seed medium was composed of 35 g l^{-1} of beef extract, 5.0 g l^{-1} of yeast extract, 3.0 g l^{-1} of NaCl, pH 7.0. The standard medium for PGA production was comprised of the following composition (g l^{-1}): Yeast extract 50.0, NaCl 3.0, $NaCO_3$ 2.0, $NaHCO_3$ 1.5, pH 7.5. The production medium was modified on the basis of standard medium.

The stock culture was maintained in a 15 % of glycerol solution in Luria-Bertani broth and was stored at –70 °C. A loopful of stock culture was streaked on nutrient agar (beef extract 1 g l^{-1}, peptone 10 g l^{-1}, NaCl 5 g l^{-1} and agar 15 g l^{-1}) with selective pressure of chloramphenicol. The plate was incubated at 37 °C for about 24 h. A single colony was picked and inoculated into 25 ml of seed medium in 250 ml flask. Seed culture was incubated at 37 °C for approximately 9–10 h.

For flask cultures, cells were grown in 250 ml conical-flasks containing 30 ml medium supplemented with 5 μg ml^{-1} chloramphenicol. The culture was incubated in an incubator shaker with a shaking velocity of n = 200 min^{-1} at 34 °C. All experiments have been performed at least in triplicate to ensure the reproducibility.

Expression of penicillin G acylase

For PGA expression, thermal induction was carried out for a short time by putting the flask into water-bath or starting the temperature-controlled system in bioreactor, when cells were in the mid-log phase, at about 5–5.5 h cultivation after inoculation. Cells were continued to be cultivated for another 9–10 h to ensure sufficient expression of PGA gene.

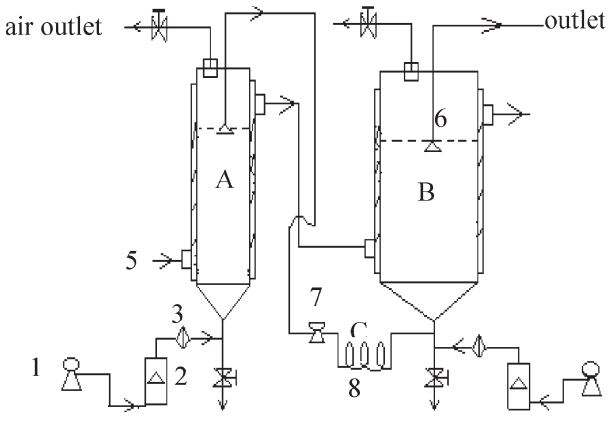
Analytical methods

Cell biomass was determined by measuring the cell density at 600 nm using a spectrophoto-meter (Model HP8452A) and was shown as OD_{600}. The culture sample was appropriately diluted with physiological saline solution.

The enzymatic activity of PGA was determined using NIPAB as a substrate in culture supernatant of fermentation broth after centrifugation at 5000 r min^{-1}, as described by *Kutzbach* and *Rauenbusch*.[20] The color intensity of the culture supernatant was determined by measuring the absorbance at 590 nm using UV-visible spectrophotometer (Model HP8452A). The enzyme activity of 1 U is defined as the amount of enzyme producing 1 μmol of 6-aminopenicillanic acid per minute at 37 °C. The total volumetric activity is expressed in U ml^{-1} of culture broth. All assays were conducted in triplicate and the results presented as the average values.

Bioreactor scheme

Cylindrical glass bioreactors equipped with sampling ports at the top, middle and the bottom of the bioreactor were used for the study of PGA production as shown in Fig. 1. When batch-cultures were conducted, both, working volume of column A and column B were 500 ml in series. In continuous fermentation, the volume of column B was 1000 ml instead of 500 ml. The ratio of height to diameter of column A and column B is 4.0, 2.5 separately. The bottom outlet was used for forced aeration using a room-aquarium aeration apparatus. The thermal-induced unit (unit C) is a pipe-line whose length and diameter can be regulated according to the demand and induced temperature was controlled at constant value by water-bath.



1- air pump
2- airflow meter
3- air filter
4- sample outlet
5- temperature-controlled system
6- liquid surface level valve
7- flow meter
8- thermal-induced unit

F i g . 1 – *Diagram of three-stage process system for continuous fermentation of PGA*

Results and discussion

Effect of induced-temperature and induced-time on cell growth of the recombinant *B. subtilis* ϕ105MU331 and PGA expression

Temperature and time were the important factors for the thermal-induced expression system of PGA, which was under the control of thermal-sensitive promoter. The effect of induced-temperature range, varying from 48 °C to 60 °C on cell growth and PGA expression, was examined. As shown in Table 1, the expression of PGA gene is very sensitive to temperature higher than 50 °C. It was proved that cells induced at 50 °C produced a high level of PGA.

Table 1 – *Effect of the induced-temperature on cell growth and PGA production*

Induced temperature/°C	Cell density/OD_{600}	PGA/U ml^{-1}
48	6.37	0.55
50	5.39	1.05
53	4.11	0.73
56	3.62	0.6
60	2.72	0.36

Effect of induced-time on PGA gene expression was further investigated at 50 °C. Fig. 2 showed that PGA activity was enhanced with increasing the induced-time for up to 4 min, but decreased when the induced-time was longer than 4 minutes. Keeping the cells at 50 °C for longer time was detrimental to the cell growth and also decreased PGA production. Although, decreasing induced-time can benefit cell growth, PGA activity was not at the highest level.

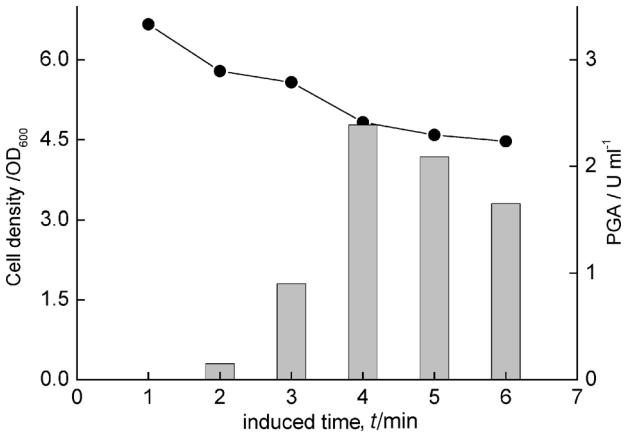


Fig. 2 – *Effect of induced-time on cell growth and PGA expression at 50 °C*

Effect of nitrogen source

As shown in Fig. 3, five nitrogen sources were added to the medium at concentrations of 50 g l^{-1}. All tested nitrogen sources did not have distinct effect on cell growth, although PGA expression varied significantly. When tryptone or beef extract were used as nitrogen source, recombinant cells can produce a high level of PGA. Taking economic cost into account, beef extract was selected as nitrogen source in further study. In order to determine the optimum concentration of beef extract for PGA production, different mass concentrations (20–75 g l^{-1}) of beef extract were added to the medium. Results showed that beef extract at 35 g l^{-1} was the optimal mass concentration in terms of PGA activity (data not shown). Higher mass concentration did not increase PGA expression significantly.

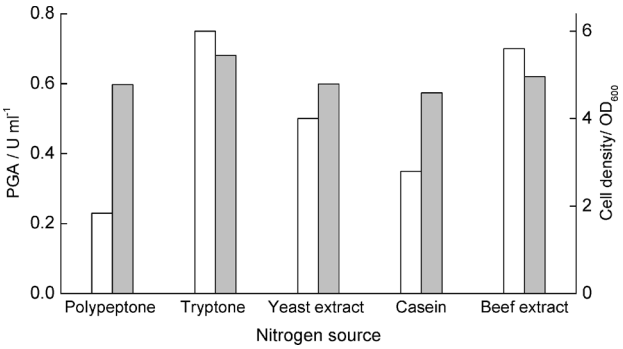


Fig. 3 – *Effect of nitrogen source on cell growth (■) and PGA production (□)*

Effect of carbon source

Various carbon sources were provided separately at mass concentration of 3 g l^{-1} in order to investigate the effect of carbon sources on the production of PGA. It is obvious from Fig. 4 that glucose showed the highest PGA activity whereas repressed cell growth. From all carbon sources tested,

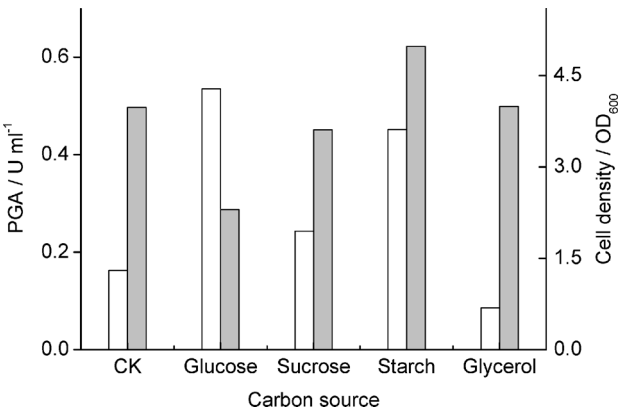


Fig. 4 – *Effect of carbon source on cell growth (■) and PGA production (□)*

starch could support cell growth efficiently but did not increase expression of PGA gene significantly. Sucrose also had negative effect on cell growth. Other carbon source also exhibited PGA activity but the yield was lower than when glucose was used.

Glucose and starch were provided at different concentrations in order to further test the combination of carbon source from glucose and starch for obtaining maximum PGA production. It can be seen from Table 2 that the medium containing 3 g l^{-1} of glucose and 6 g l^{-1} starch was optimal for PGA expression, leading to highest PGA activity at 2.55 U ml^{-1} which was 2.04 times higher then that when using glucose as unique carbon source. The results showed mixed carbon resource of glucose and starch could increase PGA gene expression efficiently.

Table 2 – *Effect of mixed-carbon source on cell growth and PGA production*

Starch γ/g l^{-1}	Glucose γ/g l^{-1}	Cell density OD_{600}	PGA U ml^{-1}
0	2	3.77	1.11
0	3	2.5	1.25
0	4	2.0	0.93
3	2	4.59	1.45
3	3	3.02	1.71
3	4	2.56	1.0
6	2	4.57	1.64
6	3	3.42	2.45
6	4	3.19	1.04
9	2	6.57	1.38
9	3	5.45	2.55
9	4	4.17	2.05

Effect of airflow rate on PGA expression in bubble column reactor in batch-culture

Oxygen availability was one of the most important environmental parameters in the production of PGA fermentation. Many researchers have investigated the effect of dissolved oxygen on expression of PGA by recombinant *B. subtilis* ϕ105MU331, but no general rules can be derived, since the various proteins and host/vector systems reported thus far behaved differently. [21–22] To determine the effect of oxygen availability on the synthesis of PGA and cell growth, recombinant cells were further examined in three-phase fludized bed bubble column, which have been widely employed for chemical and biochemical processes because of their simple structure, low shear stress and ease of maintenance. Dissolved oxygen was regulated by importing regular air mixture through air-pump. The scheme of bioreactor is shown in Fig. 1, and the volume of column A was the same as that of column B in batch-culture.

As shown in Fig. 5, final PGA activity, varying from 2.4 U ml^{-1} to 4.0 U ml^{-1}, increased with the increase of oxygen availability when airflow rate was from 2 vvm to 4 vvm, accordingly. From the data it can be concluded that higher level of dissolved oxygen could enhance the accumulation of PGA and stimulate cell growth. The cell growth curve showed that recombinant cells still kept growing instead of lysate immediately after thermal-induction at 50 °C. The holin gene, which could inhibit lysogenic state, was integrated into *lacZ*-cat cartridge of expression cassette.[19] Thermal induction activated the transcription of PGA gene and induced its expression. PGA had the maximum enzyme activity through another 10 h of cultivation after thermal-induction.

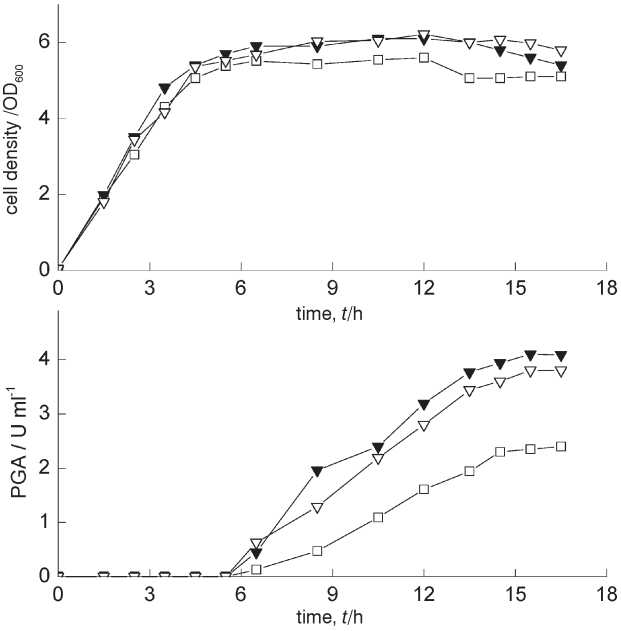


Fig. 5 – *Time-course of recombinant B. subtilis cell growth and PGA expression at different airflow rate*

Three-stage continuous process for production of PGA in *B. subtilis* ϕ105MU331 integrated with PA gene

At 34 °C with airflow rate of 3 vvm, continuous production of PGA by recombinant *B.subtilis* ϕ105MU331 was further investigated in three-stage process bioreactor system where the stages of cells including growth, thermal-induction, and expression

proceeded independently. The scheme of bioreactor is shown in Fig 1, the first reactor (column A) was a 0.85 l bubble column with 0.5 l working volume, and the second reactor (column B) was a 2.0 l with 1.0 l working volume. In our previous study, we have shown that as an immobilized supporter matrix, polyurethane foam (PUF) can enhance hEGF expression and plasmid stability for recombinant *E. coli* efficiently.[7] Using PUF as immobilized supporter matrix, in the 1st stage, PGA-producing recombinant cells were conducted in batch-culture at first. When cells in first bioreactor were in the middle of exponential phase, about 5.5 h after cultivation, the feed pump and intermediate pump between column A and column B started to work simultaneously and at the same flow rate to maintain continuous cultivation at a dilution rate of 0.2 h^{-1}. At the same time, thermal-induction was carried out at 50 °C for exactly 4 min by controlling thermal-induced unit in water bath. The residence time of recombinant cells in the second reactor was designed to be for 9.5 h, while its working volume was regulated by liquid surface level valve.

Fig. 6 shows the profiles of cell density, residual glucose and pH in three-step cultivation system. At a dilution rate of 0.20 h^{-1}, cell density in the first cultivation stage had a decreased fluctuation and reached a stable state after 5 h of continuous cultivation. Recombinant cells were still at constant growth for another 30 h, and residual glucose varied from 0.2 g l^{-1} to 1.5 g l^{-1}. In the first bioreactor, pH was maintained at normal range, which was between pH 6.0 and pH 8.0, when the pH of initial feed broth was 8.0. In the first reactor, (column B), PGA gene didn't express as measured by PGA activity because of lack of thermal induction.

In the second reactor (column B), PGA gene started expression and continued expression after recombinant cells were induced in the thermal-induced unit. PGA accumulation continued to rise and reached the maximum yield, which varied in 2.3 U ml^{-1} to 2.7 U ml^{-1} after 15 h of cultivation after thermal-induction, which remained at constant level for another 40 h. Using PUF as supporter matrix, recombinant cells in the first reactor were able to maintain vigorous growth at high speed in self-designed three-step cultivation, even at a high dilution rate of 0.2 h^{-1}. However, cell density in the column B was in the decline trend after induction for 15 h. This might be the possible reason for resulting the decrease of PGA expression.

Conclusions

After optimizing the key quantities for production of PGA by *B. subtilis* ϕ105MU331, the preliminary productivity was enhanced about two times in flask cultures. Furthermore, a three-step cultivation process (growth-induction-expression) was devel-

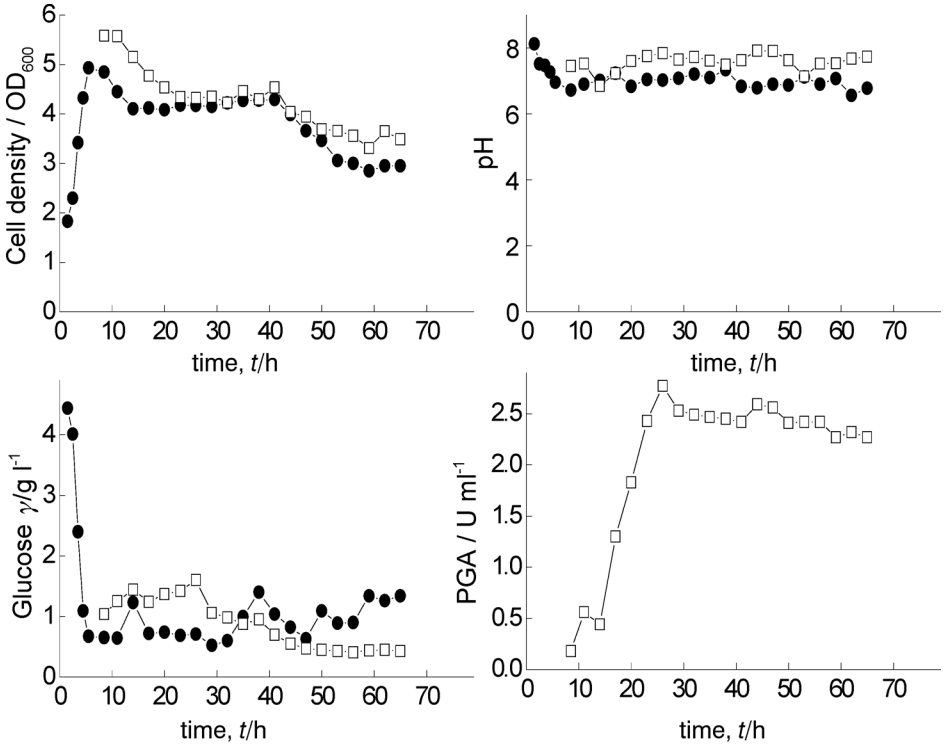


Fig. 6 – *Time-course of B. subtilis ϕ105MU331 in continuous cultivation at a dilution rate of 0.20 h^{-1}*

oped and used in PGA production according to characteristics of host/vector system. Batch culture and continuous fermentation was conducted successfully under the control of the unit for thermal-control. The results showed that cells can be controlled in separate unit and maintained at their own optimal level at each step without interaction and interference. At air flowrate of 3 vvm at 34 °C, the recombinant cells could maintain steady state for over 40 h in three-step continuous cultivation. It can be concluded from this study that it is feasible to utilize thermal-induced expression system and the bioreactor with a separate apparatus for thermal induction of PA gene in three-step cultivation, in order to reach high level of PGA activity.

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