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Production of a fusion protein of sweet potato sporamin from recombinant *E. coli* XL1 Blue by fed-batch fermentations

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Abstract

Glucose-stat and pH-stat control strategies were employed in order to culture a recombinant *E. coli* XL1 Blue to produce a fusion protein of sweet potato sporamin (SPA) and glutathione S-transferase (GST) from the recombinant *E. coli* XL1 Blue. Cell densities up to 25 g l^{-1} and 28.9 mg fusion protein (GST-SPA) g^{-1} cell dry weight (CDW) was achieved from a fed-batch fermentation controlled by glucose-stat strategy. A pH-stat control fermentation using glycerol as a carbon source gave *E. coli* up to 27 g l^{-1} and 31.5 mg GST-SPA g^{-1} CDW. Additionally, a pH-stat control strategy using glucose as a carbon source gave *E. coli* up to 15 g l^{-1} and about 22.7 mg g^{-1} CDW of GST-SPA.

Introduction

Sporamin is the major storage protein in sweet potato tuberous roots. Sporamin is a Kunitz-type trypsin inhibitor which accounts for about 60–80% of the total protein in sweet potato tubers. A very low amount of sporamin is expressed in stem and no expression is shown in leaves. Expression of sporamin gene can be induced in leaves after being wounded by insect infestations, and results in indigestability in insects. These observations were thought to be the most convincing evidence for a direct defensive role of plants against insect infestations (Green & Ryan 1972). Transfer of these protein inhibitor genes into other plant species is effective as an endogenous insecticide against pest damage (Hilder *et al*. 1987).

Yeh *et al.* (1997) have cloned a cDNA encoding sporamin from tuberous root of sweet potato (*Ipomoea batatas* Lam.). The full length of 0.54 kb cDNA was ligated to 3′ end of open reading frame of glutathione S-transferase (GST) encoded in the expression vector pGEX-2T. The recombinant plasmid was transformed to *E. coli* XL1 Blue to produce the fusion protein of GST-SPA.

High cell density culture of recombinant *E. coli* is effective methods for mass production of target proteins (Lee 1996). Various strategies of nutrient feeding were reported to achieve high cell density culture (Lee & Chang 1993, Cutayar & Poillon 1989, Kim *et al.* 1994). The aim of this investigation is to employ the *E. coli* XL1 Blue to produce GST-SPA in large amount for possible application to vegetables to protect against insect infestations. A glucose-stat fermentation was performed using a commercial biosensor for monitoring glucose concentration during fermentation in this study. A pH-stat fermentation using glycerol or glucose as a carbon source is another alternative method for the culturing of recombinant *E. coli* in high cell density.

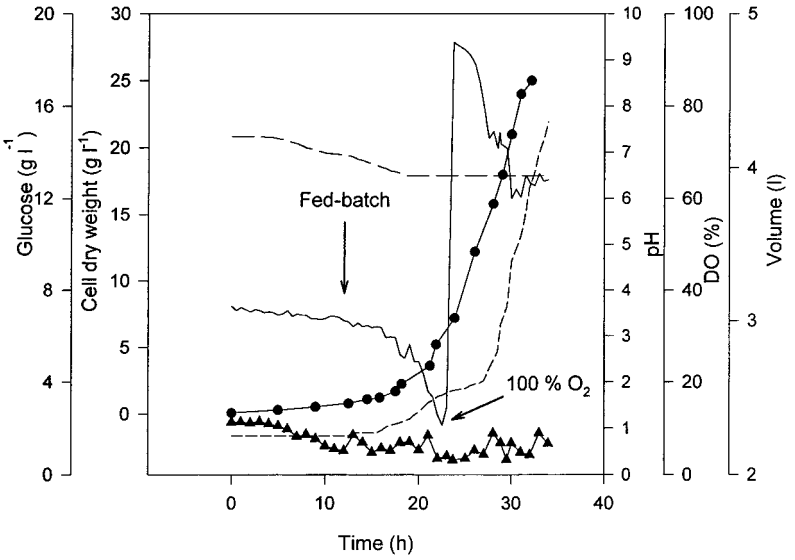

Fig. 1. Fed-batch fermentation by glucose-stat control strategy. Glucose was controlled at 1 g l^{-1} by on-line analysis of glucose sensor. ●, Dry cell weight; ▲, glucose concentration; — — , pH; —, DO (dissolved oxygen); – – –, volume (broth volume).

Materials and methods

Materials

Glutathione Sepharose 4B was from Pharmacia. Ampicillin was from Boehringer Mannheim.

Medium composition and culture conditions

E. coli was grown in basal medium containing (g l^{-1}): glucose, 2.5; yeast extract, 0.5; Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; $CaCl_2$, 0.015, $MgSO_4 \cdot 7H_2O$, 0.05; ampicillin, 0.05. Ampicillin was filter-sterilized. The basal medium was used for seed culture and batch fermentation. The feeding medium for fed-batch fermentation contained (g l^{-1}): yeast extract, 56; glucose, 112; Na_2HPO_4, 11.2; KH_2PO_4, 5.7; NH_4Cl, 22.5; $CaCl_2$, 0.25; $MgSO_4 \cdot 7H_2O$, 11.1. A seed culture of 250 ml was transferred to a 5 l fermenter with a working volume of 2 l. The culture conditions for fermenter were: agitation, 500 rpm; air flow rate, 10 l min^{-1}; temperature 37 °C; initial pH was 7.3–7.4; dissolved oxygen (DO) was above 10% saturation (referred to pure O_2 as 100%). Pure O_2 was used whenever necessary.

Computer control

The control strategy diagram was constructed in the Genie (Advantech Co.). Measured signals of DO, pH, and glucose concentration was connected to a PCLD-8115 wiring terminal board (Advantech), connected with a PCL-818 L data aquisition card and communicated with a Genie's program. The control signal was connected as output to a PCLD-885 power relay board in order to control NaOH and glucose (or glycerol) pump. An IBM compatable personal computer was used for data processing and recording.

On-line analysis

Fermentation broth was continuously circulated outside the fermenter to a microfilter, and the minimum volume of filtrate was pumped to the glucose sensor (YSI 2700). Glucose concentration was measured and recorded by the control system for batch fermentation. In the fed-batch fermentation with glucose-stat, glucose was controlled at 1 g l^{-1}. DO was controlled manually above 10% saturation (refer to pure O_2 as 100%) by feeding pure O_2 if necessary. In the pH-stat control strategy, substrate feeding was based on increase of pH to a set point of 6.55 when carbon source is exhausted. NaOH was used to adjust the pH above 6.5.

Purification of GST-SPA fusion protein using Glutathione Sepharose 4B column

The cell slurry was sonicated in an extraction buffer solution (50 mM Tris/HCl, 150 mM NaCl, 1 mM

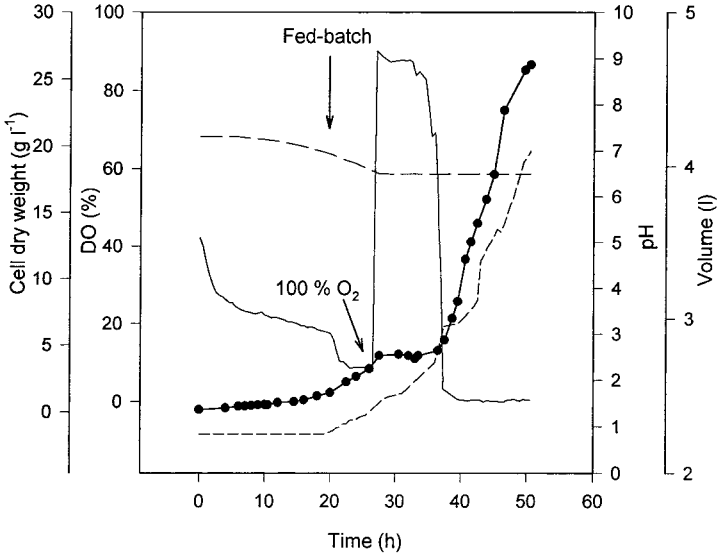

Fig. 2. Fed-batch fermentation by pH-stat control strategy using glycerol as a carbon source. The pH of fermenter was controlled above pH 6.5 by NaOH. Feeding medium was added to fermenter as glycerol was consumed completely causing pH rose to a set-point of 6.55. ●, Dry cell weight; — —, pH; —, DO (dissolved oxygen); – – –, volume (broth volume).

Table 1. Effects of control strategies and carbon sources to cell growth and GST-SPA production.

Control strategy	Carbon source	Final cell density (g l^{-1})	GST-SPA (mg g^{-1} dry wt)	GST-SPA (%)[a]
Glucose-stat	Glucose	25	28.9	8.5
pH-stat	Glycerol	27	31.5	9.5
pH-stat	Glucose	15	22.7	6.6

[a]Percentage of the total protein in crude extract is GST-SPA

PMSF, pH 8). The supernantant of crude extract (centrifuged) was applied to a glutathione-Sepharose 4B gel (400 μl) in a disposable column and washed with 4 ml PBS buffer (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM, KH_2PO_4, pH 7.3). The fused GST-SPA was eluted with 400 μl 10 mM glutathione in 50 mM Tris/HCl (pH 8.0) four times. The eluted fusion protein was collected. Protein of crude extract and purified fusion protein was measured by dye-binding method using Bio-Rad reagent, and BSA as a standard.

Results

Optimization of inducing condition

The optimum amount of IPTG needed to induce GST-SPA was determined by growing recombinant *E. coli* XL1 Blue in basal medium to about 0.45 g dry wt l^{-1} and then adding various concentrations of IPTG. A concentration of 0.05 mM IPTG was sufficient to induce GST-SPA and gave about 35 mg GST-SPA g^{-1} CDW when cells were harvested at 0.96 g l^{-1}. The purified GST-SPA was about 10% of the total protein in crude extract. A minimum of 10 h of inducing time is required to obtain high expression of GST-SPA. It was important to maintain sufficient nutrient during the inducing period in order to obtain high expression of the GST-SPA. It was also very important to feed sufficient ampicillin during fermentation to suppress growing of the cells having no recombinant plasmid.

Fed-batch culture with glucose-stat

Basal medium was used as initial medium for fed-batch culture. The maximum specific growth rate

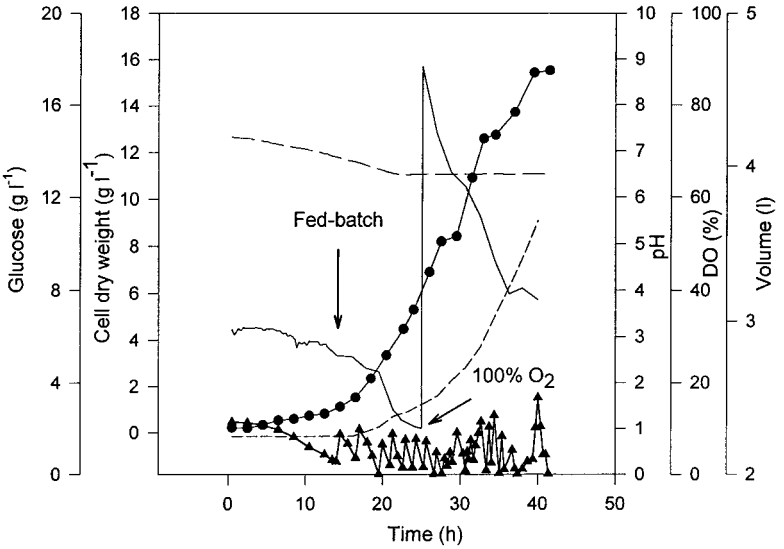

Fig. 3. Fed-batch fermentation by pH-stat control strategy using glucose as a carbon source. The pH of fermenter was controlled above pH 6.5 by NaOH. Feeding medium was added to fermenter as glucose was consumed completely causing pH rose to a set-point of 6.55. ●, Dry cell weight; ▲, glucose concentration ; — —, pH; —, DO (dissolved oxygen); – – –, volume (broth volume).

(μ_{max}) was about 0.66 h^{-1} at 21 h after inoculation. The dry cell density was about 25 g l^{-1}after 32 h of fermentation (Figure 1). IPTG (5.5 mM) was added to the fermenter to induce the GST-SPA, and glucose was controlled at 1 g l^{-1} during inducing period. The cells were harvested at 10 and 12 h after IPTG was added. The average GST-SPA was about 28.9 mg g^{-1} dry wt as shown in Table 1.

Fed-batch culture with pH-stat

Glycerol (2.5 g l^{-1}) was used in steady of glucose in the basal medium initially for fed-batch culture with pH-stat control strategy. The dry cell density was about 27 g l^{-1} after 50 h (Figure 2). IPTG (6 mM) was added to the fermenter to induce GST-SPA, and the fed-batch control of glycerol with pH-stat continued during inducing period. The cells were harvested at 10 and 12 h after IPTG was added. Average GST-SPA was about 31.5 mg g^{-1} dry wt as shown in Table 1.

Another pH-stat fermentation was performed using glucose as a carbon source, and basal medium was used initially. The dry cell density was 15 g l^{-1} after 40 h (Figure 3). Glucose has been depleted frequently in the pH-stat control strategy, and resulting in a slow specific growth rate. The GST-SPA was about 22.7 mg g^{-1} dry wt after induced with IPTG.

Discussion

The culture with glycerol as a carbon source required more time for the cells to grow into exponential phase. Decrease of pH, when glycerol was used as a carbon source, was not so significant as compared to that of glucose. Acetate formation can be reduced or prevented by employing glycerol as a carbon source (Korz *et al.* 1995, Holms *et al.* 1986). The pH did not reach the set-point of 6.5 so often using glycerol, instead, it varied in the range of 6.53–6.55 most of the time of culture. Shortage of nutrients was not so often. Cells grew very well and expression of GST-SPA was satisfactory.

When glucose was used as a carbon source in the pH-stat fermentation, acetate formation caused decrease in pH to 6.5 very often, and it took more time for pH to increase to 6.55, the set-point of feeding nutrients. This resulted in frequent depletion of glucose during fed-batch process, and expression of GST-SPA was not satisfactory. Sufficient nutrients are necessary during the GST-SPA production stage.

Acknowledgement

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