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Strategy for the improvement of prodigiosin production by a *Serratia marcescens* mutant through fed-batch fermentation

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Summary

A *Serratia marcescens* mutant for prodigiosin production was obtained by u.v. mutation with rational screening methods and a two-step feeding strategy was used to increase its productivity. In flasks, the mutant strain B6 gave a 2.8-fold higher prodigiosin production than that of the parent strain with glycerol as a carbon source. In a 5-l bioreactor, with a two-step feeding strategy in which glucose was selected as the initial carbon source in the fermentation media and glycerol was fed as a 'prodigiosin inducer', it gave a 7.8 times higher prodigiosin production (583 mg/l) than the parent stain with the original cultivation mode.

Introduction

Serratia marcescens produces a red pigment, prodigiosin, when grown under the appropriate conditions. In recent years, prodigiosin was found to be a potent anticancer agent which could induce apoptosis of several cancer cell lines *in vitro* including haematopoietic cancer cells, colon cancer cells, B-cell and chronic lymphocytic leukemia cells (Campas *et al.* 2003).

Many researches have been done to improve prodigiosin production. Using a classical syntrophic cross-feeding method, Williams (1973) showed that prodigiosin was formed *via* a bifurcated pathway which depended upon a number of genes coding for the enzymes involved (Block 1961). Many factors, such as temperature, pH, dissolved oxygen levels, light and medium composition influence the production of prodigiosin (Heinemann *et al.* 1970; Rjazantseva *et al.* 1994; Sole *et al.* 1994; Ryazantseva *et al.* 1995). There are different fermentation skills that have been applied for the high production of prodigiosin. Feng *et al.* enhanced the prodigiosin secretion by adding SDS into the cultures (Feng *et al.* 1982); Bae *et al.* used an integrated fermentation-separation system to eliminate the metabolites' toxic effects on cell growth and alleviate the feedback repression by end product (Bae *et al.* 2001).

In this paper, a new feeding strategy with a mutant of *Serratia marcescens* selected for high production of prodigiosin in a normal stirred bioreactor is reported. This is a two-step culture process separating cell growth and prodigiosin biosynthesis artificially and two kinds of carbon source were fed in the broth separately in these two steps. Compared with those strategies developed in the previous paragraph, such as a complex fermentation-separation system (Kim *et al.* 1999), our feeding strategy was more practical and manageable.

Materials and methods

Medium

Agar slants and plates medium containing (g/l): glucose 2.0, tryptone (Oxoid) 2.0, yeast extract (Oxoid) 1.0 and agar 2.0. Growth medium for seed culture containing (g/l): glycerol 10; tryptone 2; yeast extract 1; $(NH_4)_2SO_4$ 6; K_2HPO_4 10; NaCl 0.5; $MgSO_4$ 0.5. The standard medium for fermentation containing (g/l): glycerol 10; tryptone 10; yeast extract 10. The initial pH of the medium was adjusted to 7.2 before sterilization.

Culture conditions

Seed medium (30 ml) in 250-ml Erlenmeyer flasks was inoculated with inoculas grown on agar slant or plate and cultivated at 28 °C for 12 h on a rotary shaker at 220 rev/min. For the production of prodigiosin, 3 ml

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seed culture was transferred to a 250-ml Erlenmeyer flask containing 30 ml fermentation medium. The flasks were incubated at 220 rev/min on a rotary shaker at 28 °C for 24 h. For batch fermentation, a 5 l stirred bioreactor (B. Braun, Germany) was used. 2.5 l production medium was inoculated with 250 ml seed culture in its late exponential phase. The temperature, aeration and stirring rate was controlled at 28 °C, 3 v/v/m in and 500 rev/min, respectively. The pH was automatically controlled at 7.0 in the first 16 h and 7.4 for the rest of 20 h. Glucose and glycerol was fed in respectively in different cultivation phase. A stepwise increase of the feeding rate was used in the feeding process of glucose. The flow rate of glycerol was adjusted to keep the glycerol concentration in the medium being always about 5 g/l.

Mutagenesis

The wild-type strain of *Serratia marcescens* 02 was used as parent strain for mutation and ultraviolet radiation was selected as the mutagen. *S. marcescens* 02 was first incubated on the agar plate at 28 °C for 24 h. Cells were collected and suspended in sterile saline, and then diluted to a concentration range of 10^5–10^7 cells/ml. After such pretreatment, the diluted cell suspensions under agitating were exposed to u.v. radiation from a 15 W, u.v. lamp for 20 s at a distance of 30 cm to give a survival rate of about 15%. The treated suspensions were laid on the agar plate and incubated at 28 °C for 24 h. The high-production mutants were roughly screened out according to the colony colour and further examined by shake flask cultivation.

Analytical methods

Prodigiosin in the broth was extracted by acidic methanol (pH 3). The concentration of prodigiosin was determined by measuring the absorbance at 535 nm, and then calculating with a standard correlation curve between absorbance and dry weight of prodigiosin. The standard prodigiosin used in this research was prepared through silica gel and reverse-phase chromatography and characterized by ESI-MS and NMR (data not shown). The purity was determined by HPLC (Agilent, diode-array u.v. detector, Palo Alto, CA) at 466 nm. Acetonitrile and 10 mM ammonium acetate (9:1 v/v) were used as a mobile phase at a flow rate of 1.0 ml/min through the column (Agilent, Zorbax Eclipase XDB-C18) at 40 °C. The cell concentration was measured at a wavelength of 600 nm and dry cell weight (DCW) was calculated from the standard curve relating absorbance at 600 nm values to DCW. Glucose was measured using a Bioanalysis Kit (GOD-PAP, Kehua, Shanghai) according to the instructions.

Results and discussion

Selection of high prodigiosin-production strains

After the u.v. treatment, the cell suspension of *Serratia marcescens* was transferred to the plate for culturing and screening. This procedure was performed repeatedly for 12 rounds and eight mutants were finally selected for further testing. These eight mutants could grow well on the plates and all gave their colonies a deep red colour with metallic sheen. They were then inoculated into the shake flasks respectively and their prodigiosin concentrations after 24 h cultivation are shown in Table 1. Strain B6 apparently had the highest prodigiosin production and the production was 2.8-fold higher than that of the parent strain under the same culture condition. The high-production hereditary feature of B6 was tested to be stable (data not shown) and thus it was used as a production strain in the following fermentations.

Carbon source optimization

In order to improve the production of prodigiosin, fermentation experiments and other work were necessary. Single factor experiments with different kinds of 10 factors (carbon source, nitrogen source, temperature, pH, light, etc.) proved that the carbon source is more important for production of prodigiosin by the mutant strain than other factors (date not shown). So, various carbon sources of 1% (w/v) concentration including glycerol, maltose, sucrose, citrate, lactose and glucose were tested respectively in fermentation flask, with the same other nutrients as the production medium. The results after 24 h are shown in Figure 1.

Figure 1 shows the effect of different carbon sources on the prodigiosin production of B6 strain. Apparently, glycerol gave the highest production when it was used as the main carbon source in the medium. We also found that some bacterial cells would often begin to autolyse (observed with microscope) at about 12–14 h during the fermentation and the amount of prodigiosin reached a plateau at about 14 h if glycerol was used as sole carbon source. This phenomenon did not occur when other carbon sources were used. The inhibition by final products was tested by addition of purified prodigiosin

Table 1. Comparison of Prodigiosin concentration of eight mutants.

Strain	Concn. of prodigiosin (mg/l)
B1	83.1
B2	81.9
B3	92.7
B4	80.3
B5	88.2
B6	96.5
B7	92.5
B8	86.6
Control	34.7

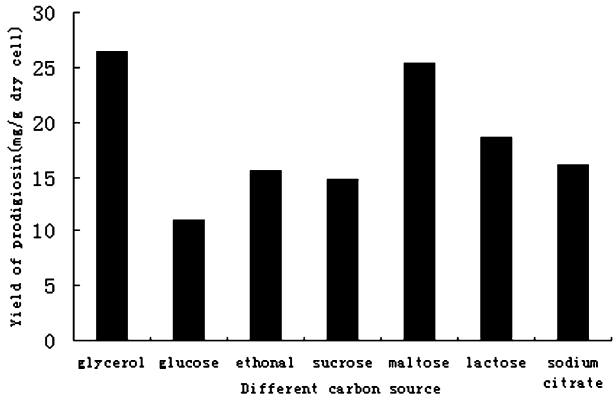

Figure 1. Effect of different carbon sources on prodigiosin production (■).

in shake flask culture, and the results indicated that prodigiosin produced by B6 itself inhibited its further production.

Feeding strategy

According to Figure 1, glucose could give the same dry cell weight as glycerol, but it only gave a little prodigiosin. So we design a two-step culture process dividing the cell growth and prodigiosin biosynthesis artificially. In most secondary metabolite production processes, this method is often performed to increase the production. In our ideal two-step process, the biomass would be accumulated in first stage with using glucose as the main carbon source and the prodigiosin would be produced in the next stage by switching glucose to glycerol feeding in the culture broth.

To validate our expectation, three different two-step feeding strategies in a 5-l bioreactor were compared: (A) first fed with glucose, and then fed also with glucose; (B) first fed with glycerol, and then fed also with glycerol; (C) first fed with glucose, and then fed with glycerol. In the first cultivation stage of these three feeding strategies, the primal carbon source was fed into the broth in a stepwise increasing rate. The feeding was stopped when the cell growth rate began to decrease. The carbon source used in the following step would not be added until the primal carbon source was completely exhausted, and then was kept at 5g/l by adjusting the flow rate of feeding. The pH was controlled at 7.0 in the first stage of all strategies to fit the cell growth and 7.4 in second one to fit the secretion of prodigiosin by automatic feeding of 5% (v/v) $NH_3 \cdot H_2O$. Dissolved oxygen level was kept above 25% of air saturation by stirrer cascade.

As shown in Figure 2, different experiments were carried out according to the above design. In all three kinds of experiments, cell density increased very fast in the first 12 h and almost reached a maximum at the same time. This was defined as the first stage of the whole process. With strategy (B) in which glycerol was the initial carbon source, prodigiosin also increased fast

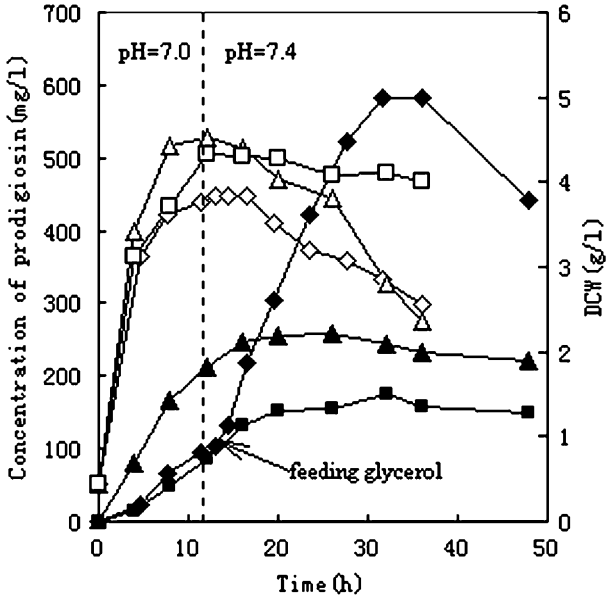

Figure 2. Prodigiosin concentration (PG) and dry cell weight (DCW) in different feeding strategy: (A) PG(■) and DCW(□) when feeding with only glucose, (B) PG (▲) and DCW(△) when feeding with only glycerol, (C) PG (♦) and DCW(◇) when feeding with glucose as the first carbon source and glycerol as the second one.

in the first stage but cells autolysed quickly after that stage without the increase of pigment. With strategy (A) and (C), prodigiosin did not accumulate much in first stage. In strategy (A), pigment concentration always maintained a relatively low level and cells did not autolyse.

These results were consistent with that in shake flask. It seemed that prodigiosin had some negative effects on its production. In strategy (C), when cell concentration reached to its maximum, glucose feeding was stopped. The glucose remaining in the media was exhausted in about an hour, and at this point glycerol was fed to 'induce' prodigiosin synthesis. The maximum prodigiosin level of the mutant B6 obtained with this two-step feeding strategy reached 583 mg/l in 30 h, which was 7.8 times higher than the parent strain (75 mg/l) with glycerol as sole carbon source in a 5-l bioreactor. Thus, the two-step feeding strategy that developed with two different carbon sources has been shown to be very effective for the prodigiosin biosynthesis of the *Serratia marcescens* mutant.

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