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Enhancement of Geldanamycin Production by pH Shock in Batch Culture of *Streptomyces hygroscopicus* subsp. *duamyceticus*

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Various sequences of pH change were applied in a batch bioreactor to investigate pH shock effects on geldanamycin production by *Streptomyces hygroscopicus* subsp. *duamyceticus* JCM4427. In the control culture where the pH was not controlled, the maximum geldanamycin concentration was 414 mg/l. With the pHS1 mode of pH shock, that is, an abrupt pH change from pH 6.5 to pH 5.0 and then being maintained at around pH 5.0 afterward, 768 mg/l of geldanamycin was produced. With pHS2, in which the pH was changed sequentially from pH 6.7 to pH 5.0 and then back to pH 6.0, 429 mg/l of geldanamycin was produced. With pHS3 having a sequential pH change from pH 6.0 to pH 4.0 and then back to pH 6.5 followed by the third pH shock to pH 5.5, no geldanamycin production was observed. Considering that the productivity with pHS1 was about two-fold of that of the control culture with no pH control, we concluded that a more sophisticated manipulation of pH would further promote geldanamycin production.

Keywords: pH shock, geldanamycin, *Streptomyces hygroscopicus*

Geldanamycin is a potent anticancer drug produced as a secondary metabolite by *Streptomyces hygroscopicus* subsp. *duamyceticus* [5, 6]. Many geldanamycin derivatives have been studied for reducing hepatotoxity and for increasing solubility [2, 4, 15, 16]. Amongst those geldanamycin derivatives, 17-AAG (17-allyamino-17-demethoxygeldanamycin) and 17-DMAG (17-dimethylaminoethylamino-17-demethoxy-geldanamycin) are at various stages of clinical trials as novel antitumor agents [13]. It is anticipated that geldanamycin derivatives will be proven as a new anticancer drug in the near future. As its potential as a drug substance increases, the importance of efficient geldanamycin production in quantity also increases.

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Nonnutritional stresses for the promotion of secondary metabolites by *Streptomyces* spp. have been intensively studied. Hayes *et al.* [9] reported that methylenomycin production by *Streptomyces coelicolor* A(3)2 could be promoted by an acidic pH shock. Doull *et al.* [7] reported that the biosynthesis of jadomycin B could be enhanced by ethanol treatment or heat shock. Kim *et al.* [11] reported a 7-fold enhancement of kasugamycin production by pH shock in batch culture of *Streptomyces kasugaensis*.

Here, we describe the effect of pH shock, that is, an abrupt sequential pH change during the course of culture, on geldanamycin production by *Streptomyces hygroscopicus* subsp. *duamyceticus*.

Materials and Methods

Strain and Spore Preparation

Geldanamycin-producing strain *S. hygroscopicus* subsp. *duamyceticus* JCM4427 was obtained from the Japanese Culture Collection of Microorganisms. *S. hygroscopicus* JCM4427 was grown on ISP4 agar plate (Difco, U.S.A.) for spore preparation. The spore stock was stored in 25% glycerol at −70°C.

Media

The seed culture medium, R2YE, contained (per liter) sucrose 103 g, K_2SO_4 0.25 g, $MgCl_2 \cdot 6H_2O$ 10.12 g, glucose 10 g, casamino acids 0.1 g, 1 ml of 0.5% KH_2PO_4, 8 ml of 3.68% $CaCl_2 \cdot 2H_2O$, 1.5 ml of 20% L-proline, 10 ml of 5.73% N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, 1 ml of 1 N NaOH, and 0.2 ml of trace element solution, prepared as described in Kieser *et al.* [10]. The geldanamycin production medium for bioreactor cultures contained (per liter) glucose 5 g, glycerol 25 g, yeast extract 2 g, soytone 4 g, and $CaCO_3$ 0.03 g, and its presterilization pH was 6.7.

Culture Conditions

For seed culture, spore stock was inoculated into 100 ml of R2YE in a 500-ml Erlenmeyer flask and incubated for 24 h at 28°C and 200 rpm in a rotary shaker. The seed culture was inoculated into a 5-l fermenter (Korea Fermenter Co., Ltd., Korea) with a working

volume of 2.5 l with 5% (v/v) inoculum size. The dissolved oxygen was maintained at 25% of air saturation by manipulating agitation at 300–500 rpm and aeration rate of 1.5 vvm. The manipulation of pH was carried out with 1 N HCl and 1 N KOH. All bioreactor cultures were performed twice or three times for a given condition.

Analysis

Cell concentration was determined as dry cell weight. Glucose concentration was determined by using a glucose assay kit (Asan Pharmacia, Korea). Glycerol concentration was determined by the isocratic HPLC method with a Bio-Rad HPX 87H (300×7.8 mm; Bio-Rad, U.S.A.) column. As mobile phase, 0.01 N H_2SO_4 solution was used at a flow rate of 0.6 ml/min. An ELSD detector (Sedex 75; Sedere, France) was used for glycerol analysis. Culture broth was extracted with an equal volume of ethyl acetate. The extracted sample was centrifuged at 6,500 rpm and 4°C for 10 min, and then the upper phase was withdrawn and evaporated. The evaporated sample was resuspended in methanol for HPLC analysis. Geldanamycin was measured by an isocratic HPLC method with a mobile phase of 60% (v/v) acetonitrile at 0.5 ml/min. A sample of 10 µl was loaded onto a Luna 5u C_{18} column (250×4.60 mm; Phenomenex, U.S.A.) and geldanamycin was analyzed at 315 nm by a UV detector (Waters, U.S.A.).

Results and Discussion

A batch culture with no pH control as control culture was carried out in the bioreactor containing the geldanamycin production medium. The results are given in Fig. 1. The initial pH of 6.7 was slightly increased to near 7.0 after inoculation owing to NaOH contained in the seed culture medium.

The pH showed a large fluctuation in the range of pH 4.7–7.3. It quickly dropped to around pH 4.7 after 24 h of cultivation and then increased to a neutral pH at 48 h. Cell concentration started to increase at 12 h of cultivation and reached a maximum of 7.2 g/l at 120 h, and then started to decline with the depletion of glycerol. Glucose was exhausted after 48 h of cultivation (data not shown). Geldanamycin production started at 48 h, and its concentration reached a maximum of 414 mg/l at 144 h.

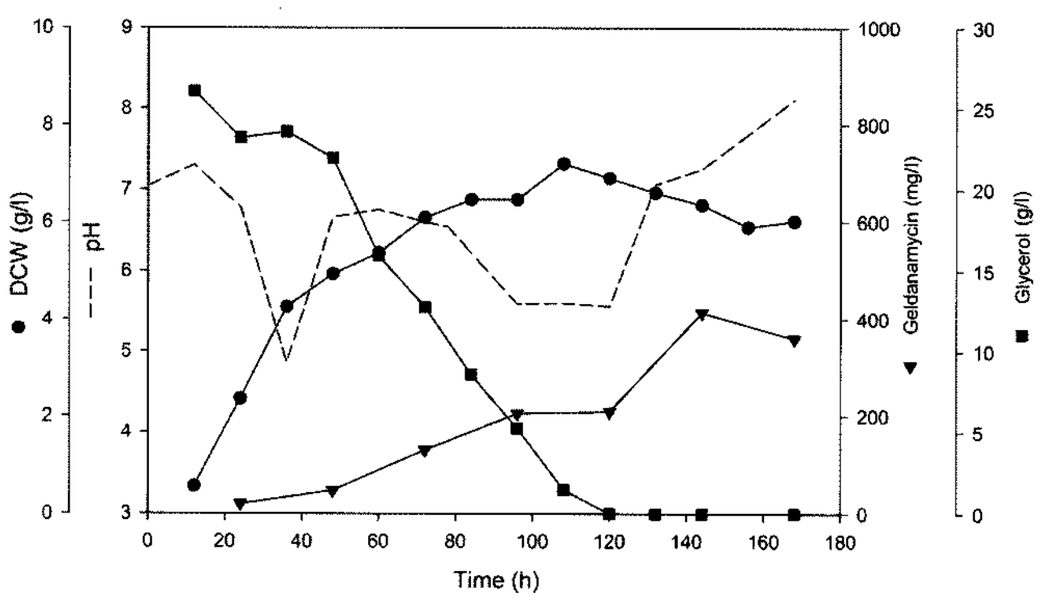

Fig. 1. Time profiles of batch culture with no pH control.

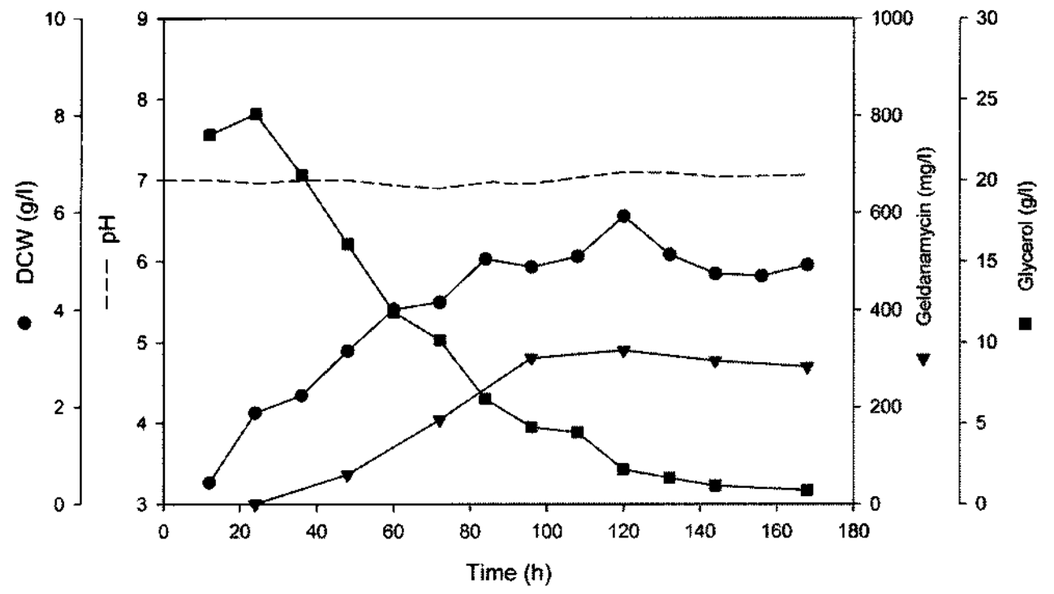

Fig. 2. Time profiles of batch culture with pH control (pH 6.9–7.0).

We speculated that the rapid spontaneous pH drop-and-recovery in the beginning might have positive effects on geldanamycin production. To prove our speculation, a pH-stat culture, in which the pH was maintained at 6.9–7.0, was carried out. Cell concentration, geldanamycin synthesis, glycerol concentration, and pH profile are shown in Fig. 2. The cell growth and glycerol consumption had similar patterns to those of the control with no pH manipulation. The maximum cell concentration was 5.9 g/l at 120 h. The geldanamycin concentration reached a maximum of 317 mg/l at 120 h, a significantly lower value than that of the control. Those results support that the rapid pH drop-and-recovery in the beginning of the culture positively affects geldanamycin production, as we had speculated.

On the basis of this result, we hypothesized that an abrupt artificial pH change, so-called pH shock, could promote geldanamycin production. To prove the hypothesis, we investigated the effects of pH shock on geldanamycin production. Three different modes of artificial pH shock were applied to the culture.

In the first mode of pHS1, the pH spontaneously dropped to pH 5.0 at 36 h of cultivation, and then recovered to pH 6.5 at 48 h of cultivation. At this point, the pH was quickly decreased to 5.0, and then maintained at this level. Time profiles of the pHS1 culture are shown in Fig. 3. No adverse effects of the artificial pH shock were observed, and geldanamycin productivity was slightly retarded compared with the control culture after the pH shock until 96 h. After this period, however, the productivity rapidly increased, and the geldanamycin concentration reached 768 mg/l at 168 h, almost twice as high as that of the control culture. Glycerol consumption rate was retarded in this pH-shocked culture, and the geldanamycin productivity was significantly higher than that of the control culture. It is well known that environmental signals, including pH shock, can stimulate and promote the biosynthesis of secondary metabolites [1, 7, 9, 11, 12, 14]. On the contrary, a strong pH shock inhibits cell growth and primary metabolism. As

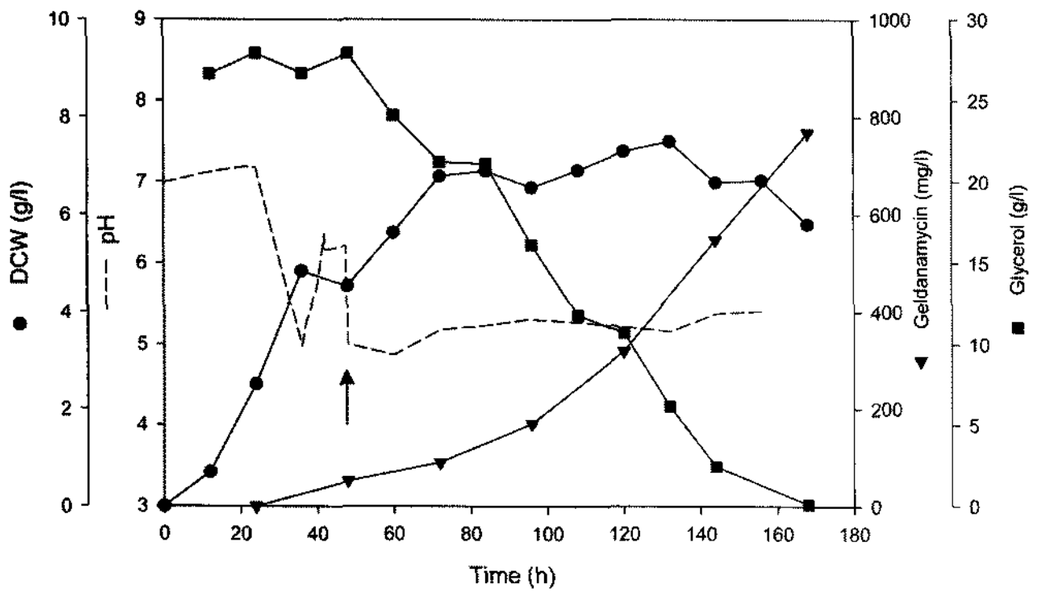

Fig. 3. Time profiles of batch culture with pHS1. The arrow indicates a pH shock applied.

discussed above, however, cell growth was only slightly retarded after the pH shock in this study, indicating *S. hygroscopicus* JCM4427 could effectively propagate even at pH 5.0, a relatively mild acidic condition. This pH level seems to be low enough to stimulate geldanamycin biosynthesis by *S. hygroscopicus* JCM4427 with no seriously harmful effects on cell growth.

In the second mode of pHS2, the pH was decreased to 5.0 at 40 h of cultivation after a spontaneous pH drop-and-recovery, and then maintained until 100 h before it was increased to pH 6.0. The cell concentration, glycerol concentration, and geldanamycin concentration are shown in Fig. 4. After the first pH shock down to 5.0, cell growth was slightly retarded as in the pHS1 culture. Retardation of glycerol consumption was also observed. The maximum geldanamycin concentration was as low as 428 mg/l at 172 h of cultivation, a comparable value to that of the control culture. The second pH shock from pH 5.0 to 6.0 seemed to have inhibited the recovery of geldanamycin productivity, causing the low geldanamycin concentration.

In the third and the last mode of pHS3, pH shock was applied three times to mimic and exaggerate the multiple spontaneous pH fluctuations in the control culture, as can be seen in Fig. 1. An abrupt pH shift from pH 6.0 to 4.0 at

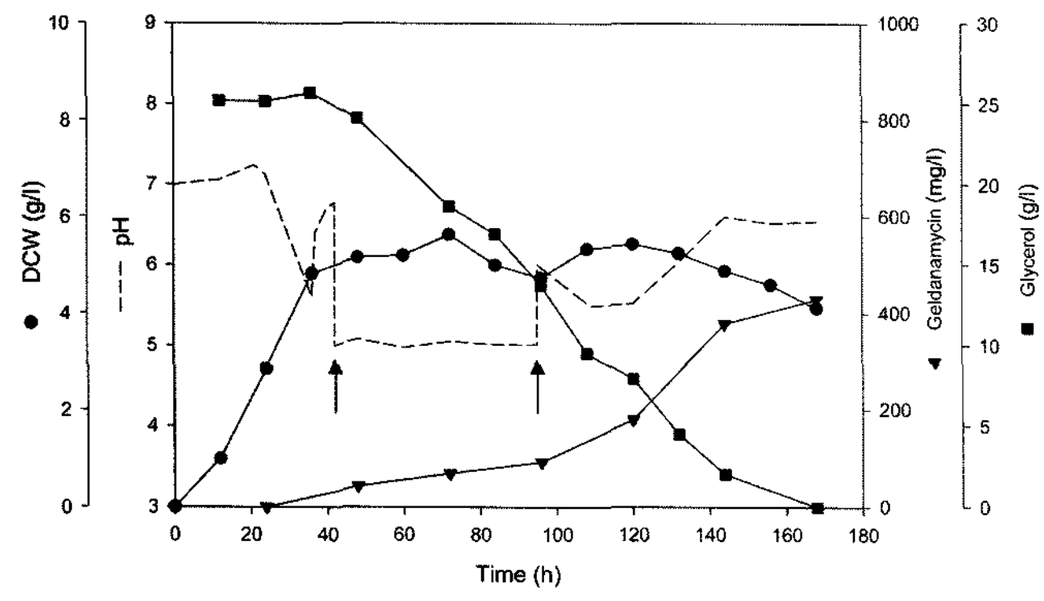

Fig. 4. Time profiles of batch culture with pHS2. Arrows indicate a pH shock applied.

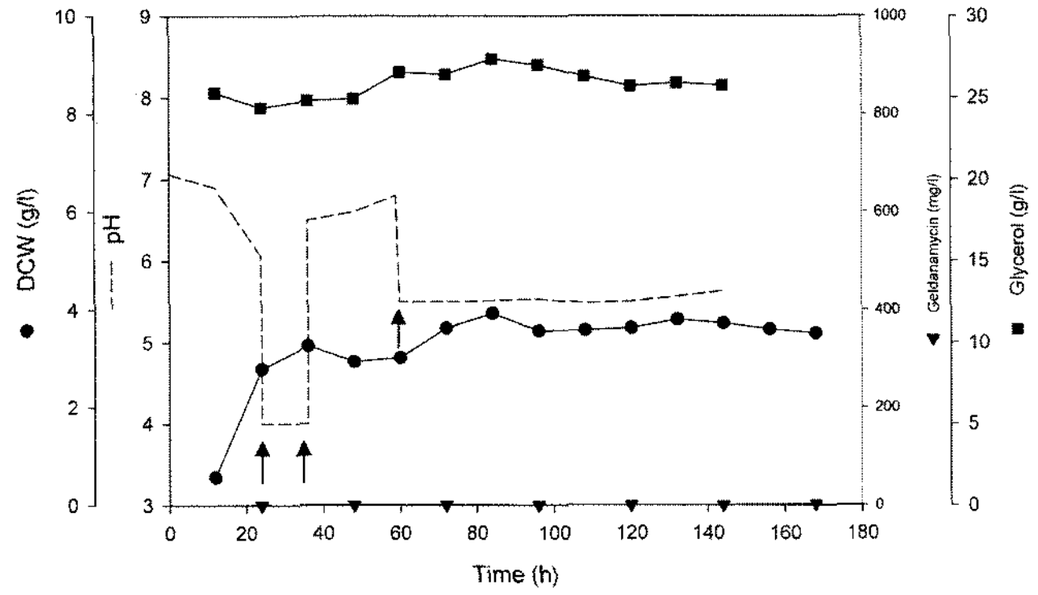

Fig. 5. Time profiles of batch culture with pHS3. Arrows indicate a pH shock applied.

24 h was applied (the first pH shock). Then, the pH was maintained at 4.0 for 12 h before being shifted back to 6.5 (the second pH shock). The third pH shock was applied at 60 h by lowering the pH from 6.9 to 5.5 (Fig. 5). Cell growth was severely inhibited, glycerol was not consumed at all, and no geldanamycin was produced in this culture, probably because too many and/or too strong pH shocks were applied.

Glycerol (25 g/l) and glucose (5 g/l) were the major carbon sources in this study. Of the two, glycerol was observed to have the main contribution to geldanamycin synthesis. It is reported that glycerol-derived precursors can be effectively utilized in the biosynthesis of geldanamycin and immunomycin, which are polyketide metabolites produced by *S. hygroscopicus* [3, 8]. It is considered that, in this study, glycerol in the production medium more effectively provided precursors for the polyketide backbone of geldanamycin, resulting in a higher geldanamycin production. The yield of geldanamycin based on glycerol ($Y_{p/s}$) and cell mass ($Y_{p/x}$) was calculated (Table 1). The pHS1 condition showed a significant enhancement of geldanamycin yield also in addition to the improved productivity, as mentioned earlier, indicating nutrients flux had been improved for geldanamycin biosynthesis by this type of pH shock. The lowest yield was obtained in the pH-stat culture, indicating that regulation of pH at the neutral level during the culture was not effective for geldanamycin biosynthesis.

In this study, we found that an appropriate mode of pH shock could significantly enhance the geldanamycin production by promoting secondary metabolism without detrimental effects on primary metabolism and cell growth. However, the application of too frequent or too strong pH shocks ruined the cellular metabolism overall, resulting in a very low cell growth and no geldanamycin production. Although the mode of pH shock still has room for improvement, the pH shock method proposed here could be a simple and easy-to-implement but very useful tool for industrial fermentation processes to produce geldanamycin and other secondary metabolites.

Table 1. Geldanamycin yields based on glycerol and cell mass for different pH shock modes.

pH shock mode	$Y_{p/s}$ (mg geldanamycin/g glycerol)	$Y_{p/x}$ (mg geldanamycin/g cell)
Control (no pH control)	14.40	74.07
pH-stat (pH 6.9–7.0)	11.32	57.46
pHS1	30.86	132.4
pHS2	17.20	104.4
pHS3	N/A	N/A

Acknowledgment

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