Enhancement of Kasugamycin Production by pH Shock in Batch Cultures of Streptomyces kasugaensis

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Biosynthesis of kasugamycin could be greatly enhanced by applying a nonnutritional stress of pH shock, that is, sequential pH changes from a neutral pH to an acidic condition and then back to the neutral condition. During the acidic period, cell growth decreased to nil. After recovery of the neutral condition, the cell growth resumed after a time lag concurrently with the biosynthesis of kasugamycin at a greatly enhanced rate compared with the control case without a pH shock. In a series of experiments performed to identify the optimal length of pH shock, four different lengths (6, 12, 24, and 48 h) of pH shock were applied. The best result was obtained when pH shock was applied for 24 h, with kasugamycin productivity approximately 7-fold higher than that of the control.

Introduction

There have been strong interest and a great deal of research activity in the area of secondary metabolite fermentation. Frequently, physiological evidence points to environmental influences, with an emphasis on the effects of nutrition on the triggering of secondary metabolism. It has been demonstrated in many cases that the biosynthesis of a secondary metabolite is initiated as growth rate is reduced (1) or as a specific nutrient (carbon, nitrogen, phosphate, or iron) is depleted (1–4). Recently, the regulatory elements that control gene expression in secondary metabolic pathways, mostly concerned with antibiotics in Streptomyces, are becoming identified with the advent of molecular genetic technique (5).

Although much information is available from previous studies, only little information is available on the use of nonnutritional stresses for the promotion of secondary metabolite production. Doull et al. (6) reported that the synthesis of jadomycin B, a polyketide antibiotic, could be promoted by heat shock or ethanol treatment. Although they showed a 30-fold increase of volumetric productivity by heat or ethanol shock compared with the control culture, the attained maximum concentration, 30 mg/L, was extremely low.

In this study, we investigated the effect of pH shock, sequential pH changes from a neutral pH to an acidic condition and then back to the neutral condition during the course of culture, on the kasugamycin production by Streptomyces kasugaensis and optimized the length of pH shock.

Materials and Methods

Microorganism. Streptomyces kasugaensis (KCCM 11390) was used throughout this study. The stock culture was stored as 10-mL aliquots of mycelium suspension in 30% glycerol at −70 °C.

Media. The seed culture medium, YM broth (Diffco Laboratory, U.S.) contained per liter glucose 10 g, peptone 5 g, yeast extract 3 g, and malt extract 3 g. Its presterilization pH was 7.0. The production medium for shake-flask cultures contained per liter maltose 10 g, glycine 4.5 g, phosphate (as a 7:3 mixture of K2HPO4 and KH2PO4) 0.25 g, NaCl 1 g, CaCl2 0.1 g, and a trace mineral salt solution 20 mL. The composition of trace mineral solution was described by Zhang et al. (7). Its pH was adjusted to 6.4 with NaOH before sterilization. When necessary, 21 g/L of morpholinopropane sulfonic acid (MOPS) buffer was added to this production medium. The production medium for bioreactor culture contained per liter maltose 30 g, glycine 10 g, K2HPO4 1.4 g, NaCl 1 g, CaCl2 0.1 g, PEG 2 g, and the trace mineral salt solution 20 mL.

Cultivation Conditions. A 100-mL portion of the seed medium in a 500-mL Erlenmeyer flask was inoculated with 10 mL of mycelium suspension from the stock culture and incubated at 29 °C and 230 rpm in a rotary shaker. After 60 h, 10 mL of the seed culture was transferred to 100 mL of production medium in a 500-mL Erlenmeyer flask for the subsequent flask culture. The same cultivation conditions as in the seed culture were employed in the flask cultures.

Bioreactor culture was carried out in a 3.4-L fermentor (Korea Fermentor Co., Ltd.) with a working volume of 1.5 L. The inoculum size was 10% (v/v). The dissolved oxygen concentration (DO) was maintained at 30% of air saturation by manipulating the agitation speed under 1 vvm of aeration rate. Oxygen-enriched air (oxygen, 50%) was sparged when DO decreased to below the set point even at a maximum agitation speed of 430 rpm. The temperature was maintained at 29 °C, and pH was controlled with 2 N H2SO4 or 2 N NaOH.

Analytical Methods. Cell concentration was determined by measuring dry cell weight (DCW). Maltose concentration was measured by the dinitrosalicylic acid.
Results and Discussion

Kasugamycin Formation with and without pH Buffer. Maltose was used as the suitable carbon source on the basis of previous reports (8), and glycine was selected as the nitrogen source because it was known to be a precursor (9, 10). Before this study, we investigated the effects of nutrient concentration (nitrogen or phosphate) on kasugamycin production in pH-buffered flask cultures. It was found that the highest kasugamycin concentration was obtained in the culture containing 4.5 g/L glycine and 0.25 g/L phosphate. However, the concentration, 30 mg/L, was considered to be too low. Thus, we tried to improve the productivity and found that a significant improvement of kasugamycin production could be made in a flask culture without buffer as elucidated in Figure 1.

In this flask culture with no pH buffering, the pH showed a large fluctuation as expected in the range of 4.0 – 8.6, while it was maintained fairly constant in the range of 6 – 7 in the pH-buffered cultures. The pH quickly dropped down to around 4.0 after 24 h of cultivation. A variety of organic acids were produced, including mostly pyruvate and α-ketoglutarate as shown in Figure 2. The organic acids were produced until 48 h of cultivation, keeping the pH low during this period (Figure 1). By 72 h of cultivation, the amount of organic acids had decreased to a very low level, indicating their consumption by the microorganism, and thus the pH increased to 7.5. The change in pH due to the production and consumption of organic acids by the microorganism is supported by a previous report (11). The pH further increased over the neutral pH and then reached 8.6 at 96 h when organic acids were completely consumed. It is considered that the consumption of organic acids and the consumption of glycine accompanied by ammonia formation result in the increase of pH after 72 h. Especially, the formation of ammonia seems to have caused the culture pH to become alkaline (12). A similar phenomenon was reported by Doull et al. (1) in a fermentation using glutamate as the nitrogen source. Kasugamycin production began after 96 h of cultivation, and 114 mg/L of kasugamycin was obtained at 144 h.

A batch culture with no pH buffering was also run in the fermentor containing the same medium as in the flask cultures, and the results are given in Figure 3. The pH was initially adjusted to 7.0. During the culture, it showed a fluctuation in a relatively small range of 6.2 – 8.3 without dropping to a very low level, i.e., 4.0 as in the flask culture with no pH buffering. The kasugamycin concentration was merely 9.5 mg/L at the end of the fermentation. We speculated that the rather mild change in the pH was not very effective in enhancing kasugamycin production.

On the basis of the experimental results described above, we hypothesized that a sufficiently large pH change might be necessary to trigger and promote kasugamycin formation.

Effect of pH Shock on Product Synthesis in Fermentor. To prove the above hypothesis, we carried out batch cultures in the fermentor with pH control. First, as the control, a fermentation was carried out at a neutral pH (controlled at 6.8 ± 0.4). The time profiles of cell growth and product synthesis are shown in Figure 4. Cell concentration started to increase after 24 h of cultivation, reached a maximum of 9.8 g/L at 87 h, and then declined, indicating cell lysis due to the exhaustion of nutrients. Kasugamycin biosynthesis began after cell growth had stopped. The maximum product concentration was only 12.6 mg/L.

In the subsequent run of fermentation, a pH control strategy mimicking the pH profile in the flask cultures without pH buffering was employed. Fermentation was started at a neutral pH, and then the pH was switched to 3.5 – 4.0 at 36 h of cultivation. After the fermentation was run in this acidic condition for 48 h, the pH was shifted back to the neutral pH and then maintained to the end of fermentation. We called such an abrupt pH switching procedure a “pH shock”. The profiles of cell
concentration and product concentration are shown in Figure 5. The cells entered growth phase after 18 h of lag phase, and the cell growth stopped as pH was lowered. After the pH was shifted back to the neutral level, cell concentration started to slightly decrease with the exhaustion of maltose. To quantify the effect of pH shock on cell growth, specific growth rate \((\mu)\) was calculated as shown in Figure 6. The specific growth rate decreased from 0.11 h\(^{-1}\) at the neutral condition to 0.04 h\(^{-1}\) after the culture was affected by the pH shock. The onset of product synthesis took place concurrently with the resumption of cell growth. The cells retained production activity even after the cell growth stopped. The kasugamycin concentration reached 116 mg/L at 165 h of cultivation.

The cellular yield \((Y_{x/s})\) and product yield \((Y_{p/s})\) were calculated on the basis of maltose concentration. The maximum cell concentration and cellular yield for both cultures showed a relatively small difference. However, the culture with pH shock showed a remarkably efficient utilization of the carbon source for kasugamycin production and thus a product yield 9.8-fold higher than that of the control.

HPLC diagrams for broth samples taken under the two different culture conditions were compared (Figure 7). Kasugamycin in the authentic standard was eluted at 15.34 min (Figure 7A). For a sample taken at 165 h from the control culture, a small kasugamycin peak was detected, while an unidentified compound was eluted at 16.66 min, which was quite close to the retention time of kasugamycin. Additionally, it appeared that a considerable amount of side products was eluted at 1.8, 2.4, and 6.5 min. For a sample taken at 159 h from the culture with pH shock, a relatively large kasugamycin peak was detected. Additionally, the peak area of the unidentified compound significantly decreased. In the control culture, a greater portion of the carbon source was observed to be utilized for the biosynthesis of side products than for kasugamycin formation. The specific growth rate of the microorganism was greatly reduced by the pH shock. This reduction of cell growth may be due to the suppression of primary metabolisms caused by pH shock. Generally, it is well-known that the formation of secondary metabolites is a response to reduced growth opportunities and is normally suppressed when the microorganism is growing at its full potential. In this connection, the slow growth due to pH shock is considered to be responsible for the enhanced biosynthesis of kasugamycin.

The patterns of kasugamycin production and cell growth were investigated under a prolonged acidic condition. After the fermentation was run for 36 h at a neutral pH, the pH was switched to 3.5–4.0, and this acidic condition was maintained to the end of cultivation (data not shown). In the beginning, cell concentration increased with the consumption of maltose under the neutral condition. Switching to the acidic condition, however, completely inhibited cell growth. It was observed that no kasugamycin synthesis occurred during the entire cultivation period in this condition. This result clearly indicates that an abrupt drop of pH is necessary to induce kasugamycin synthesis but the pH should be switched back later.

**Optimization of pH Shock Interval.** It was proved that product formation could be triggered by a pH shock. However, the cells could not retain the production activity for a significant length of time. Thus, it was considered that 48 h of pH shock might have been too long, thus giving the cells unnecessarily severe stress and damage. In this respect, it was required to determine the optimal length of pH shock. The pH was lowered after 36 h of cultivation as before and then it was maintained for four different time intervals, 48, 24, 12, and 6 h, in a series of cultures.
With a pH shock for 48 h, cell concentration stopped increasing after the pH was lowered to 4.0 at 36 h. After the pH was switched back to around 7.0, it declined for the subsequent 36 h and then temporarily leveled off. At about 180 h, it started to increase with a rapid consumption of maltose. It reached a maximum of 14.8 g/L at 203 h of fermentation. Kasugamycin production began at 131 h, and the production rate significantly increased as the cell concentration increased. A maximum product concentration of 120 mg/L was obtained at 215 h of fermentation.

When a pH shock for 24 h was applied, cell concentration declined, as in the case of 48 h of pH shock, after the pH was switched back to the neutral pH. However, it started to increase at about 110 h with no stagnant period in contrast to the previous case. A maximum product concentration of 120 mg/L was obtained at 215 h of fermentation.

When a pH shock for 12 h was applied, the cell concentration profile showed a trend similar to the case of 24 and 48 h of pH shock, but there was no decline observed in cell concentration even after switching back to the neutral pH. A kasugamycin concentration of 14.8 g/L was obtained at 203 h. A kasugamycin concentration of 78 mg/L was obtained at 143 h.

When a pH shock for 6 h was applied, the cell concentration profile showed a trend similar to the case of 12 h of pH shock. Cell concentration reached a maximum of 15.6 g/L at 156 h. The maximum kasugamycin concentration was 59 mg/L.

With a pH shock for 6 h, we observed a significant increase in product formation related to the retardation of cell growth due to pH shock and that the producer microorganism changed its metabolism toward a production mode during this growth-retardation period. Maximum kasugamycin production was obtained in the culture with a pH shock for 24 h. We believe that the proposed pH-shock method is readily applicable to industrial fermentation because a pH control system is already equipped in most industrial scale fermentors and its operation is quite simple.

Table 1. Comparison of Cultures with Different Durations of pH Shock

<table>
<thead>
<tr>
<th>pH shock interval (h)</th>
<th>maximum cell mass (g cell/L)</th>
<th>maximum kasugamycin (mg KSM/L)</th>
<th>(Y_{x/s}) (g cell/g maltose)</th>
<th>(Y_{p/s}) (mg KSM/g maltose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>15.6</td>
<td>58.6</td>
<td>0.57</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>13.2</td>
<td>77.5</td>
<td>0.46</td>
<td>2.7</td>
</tr>
<tr>
<td>24</td>
<td>16.5</td>
<td>182.6</td>
<td>0.57</td>
<td>6.2</td>
</tr>
<tr>
<td>48</td>
<td>14.8</td>
<td>120.2</td>
<td>0.52</td>
<td>4.2</td>
</tr>
</tbody>
</table>

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Table 1 summarizes the effects of pH-shock length in terms of cell and product yields. It is interesting to note that significant variations were observed only in \(Y_{p/s}\).

In summary, in this study, we demonstrated that the biosynthesis of kasugamycin could be turned on and its productivity could be significantly enhanced by imposing a nonnutritional stress, a pH shock. We observed that a significant increase in product formation was related to the retardation of cell growth due to pH shock and that the producer microorganism changed its metabolism toward a production mode during this growth-retardation period. Maximum kasugamycin production was obtained in the culture with a pH shock for 24 h. We believe that the proposed pH-shock method is readily applicable to industrial fermentation because a pH control system is already equipped in most industrial scale fermentors and its operation is quite simple.
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References and Notes


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