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Effects of Phosphate and Asparagine on Streptomycin Formation by *Streptomyces griseus* in pH-Stat Cultures

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Effects of glucose, asparagine, phosphate and pH on streptomycin (SM) formation by *Streptomyces griseus* HUT 6037 on a defined medium were studied in pH-stat batch cultures. SM formation occurred in both the trophophase and the idiophase at pH 6.0 or 6.5, but terminated at the end of the trophophase at pH 7.0 or 8.0. No SM formation occurred at pH 9.0 due to the lack of cellular growth. At the optimum pH of 6.5, SM accumulation reached 225 mg/*l*, compared with 70 at pH 6.0, 50 at pH 7.0 and 32 at pH 8.0. At the optimum pH, glucose and L-asparagine but not phosphate were required for efficient SM formation in the idiophase. When phosphate remained at the end of the trophophase, low SM formation and high cellular growth occurred. Cellular lysis was observed on glucose depletion, while SM formation continued. L-Asparagine seemed to act as an amino donor for SM biosynthesis, since it stimulated SM formation when supplied in the idiophase. The decrease of cell mass was not prevented by L-asparagine.

Knowledge of the regulatory mechanisms of secondary metabolism is still limited compared with that of primary metabolism. This is restricting technical improvements of the production of secondary metabolites, in contrast with the achievements in production of such primary metabolites as amino acids.

In industrial production of streptomycin (SM) by *Streptomyces griseus*, complex media have been widely used, mainly for reasons of economy. However, defined media are convenient and useful for studying the effects of nutrients on SM formation, since levels of chemical ingredients as well as growth, product formation and so on can be monitored during cultivation.

From this point of view, some workers have used defined media for SM formation.[1–3] A defined medium containing asparagine and ammonium sulfate as nitrogen source was used by Nimi *et al.*[3] in the study of streptomycin biosynthesis by *S. griseus* HUT 6037. They suggested that SM formation on the asparagine-supplemented medium reached almost the same level as on complex media.

The effects of phosphate on secondary metabolite formation have been studied by many workers, and are reviewed by Martin.[4] These include changes of carbohydrate catabolism, inhibition of antibiotic precursor formation, and inhibition or regulation of phosphatase. Miller and Walker[5] found in SM formation on a complex medium, that phosphorylated streptomycin (SMP) increased with the increase of phosphate concentration. In these and other works[5,6] on SM formation by *S. griseus*, pH was not controlled during the fermentation, in spite of its wide variation between 6.0 and 9.0, which caused the increase of SMP formation particularly at acidic pH.[6]

In this paper, the effects of phosphate, glucose and asparagine on SM formation by *S. griseus* on a defined medium were studied in pH-stat batch culture.

Materials and Methods

Microorganism *Streptomyces griseus* HUT 6037[3] used was given by Prof. R. Nomi, Faculty of Engineering, Hiroshima University.

Composition of media The Bennet's agar medium[7] used for stock culture was composed of (g/*l*, deionized water): mannite, 10; Polypepton, 2; meat extract, 1; yeast extract, 1; $MgSO_4 \cdot 7H_2O$, 5; agar, 20. Initial pH was adjusted to 7.0 with NaOH solution before autoclaving. The complex medium used for seed culture was composed of (g/*l*, deionized water): glucose, 5; Polypepton, 4; meat extract, 2; yeast extract, 2; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 0.25. Initial pH was adjusted to 7.0 with NaOH solution before autoclaving. The defined medium[3] used for preculture was composed of (g/*l*, deionized water): glucose, 25; L-asparagine·H_2O, 7; $(NH_4)_2SO_4$, 2; K_2SO_4, 6; KH_2PO_4, 0.4; NaCl, 1; $CaCO_3$, 2; $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.05. Initial pH was adjusted to 7.0 with NaOH solution before autoclaving. The same defined medium except for the substitution of $CaCl_2 \cdot 2H_2O$ (0.04 g/*l*) for $CaCO_3$ (2.0 g/*l*) was used for SM formation in fermentor experiments. Initial pH was adjusted to 6.0 before autoclaving, and after inoculation pH was controlled at the desired value between 6.0 and 9.0 with a pH-stat.

Cultivation One loopful of aerial mycelia from the agar slant was inoculated into 10 ml of seed medium in a test tube (20×200 mm) and cultivated on a reciprocal shaker (115 rev./min, 7 cm amplitude) at 28°C for 36 h. A 2-ml portion of the broth was inoculated to 100 ml of defined medium in a 500-ml Sakaguchi flask. The flask was then shaken for 48 h on a shaker (115 rev./min, 7 cm amplitude). To reduce the influence of the residual organic materials carried over from the seed medium upon SM formation, a second 500-ml flask culture was carried out. This culture broth (30 ml) was used as the inoculum for fermentor experiments.

A 2.6-*l* fermentor (MD 260, L. E. Marubishi Co. Ltd., Tokyo) with a working volume of 1.5 *l* was used. Aeration rate and agitation rate were fixed at 1 vvm and 500 rpm throughout the cultivation to maintain a sufficient oxygen supply in the culture. Culture temperature was maintained at 28°C and pH was maintained at a level between 6.0 and 9.0.

Analysis Cell mass was measured as follows: 10 ml of culture broth was centrifuged at 10,000×*g* for 20 min, and the wet cells were washed twice with deionized water and dried for 24 h at 105°C. Glucose concentration was analyzed by the glucostat method. The concentrations of NH_4^+ and PO_4^{3-} were measured by the Nessler method and the molybdenum blue method, respectively.[8] The concentration of asparagine (L-Asn) was determined with an amino acid analyzer (Hitachi KLA-5, Hitachi Co. Ltd., Tokyo). SM and SMP were assayed biologically by the agar diffusion method[9] with *Bacillus subtilis* IFO 3134 as the test organism. Dissolved oxygen concentration in the culture was measured with an oxygen analyzer (Type RA, Oriental Electric Co. Ltd., Tokyo).

Results and Discussion

Effect of pH on streptomycin formation Figure 1 shows a typical time course of SM formation by *S. griseus* HUT 6037 in a defined medium without pH control. The formation of streptomycins [streptomycin (SM) and phosphorylated streptomycin (SMP)] was associated with mycelial growth on the defined medium, in contrast to the non-growth-associated formation of streptomycins by *S. griseus* on a complex medium.[6] The value of pH fell to 6.0 with the start of growth, then gradually rose to 8.5 at the end of culture, as in the case of *S. griseus* grown on a complex medium.[6] Formation of streptomycins stopped in the middle of the growth phase, when glucose and asparagine remained but phosphate had been exhausted in the medium. It appears that alkaline pH above a critical level (e.g. 7.5 at 48 h) may have caused cessation of formation of streptomycins.

To investigate the effect of pH on the formation of streptomycins, five batch cultures with pH-stat were carried out at different pHs between 6.0 and 9.0. No cellular

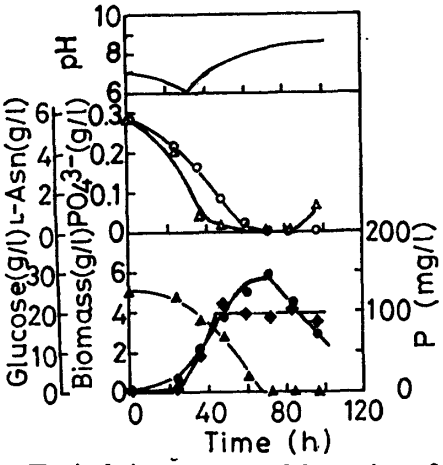

Fig. 1. Typical time courses of formation of streptomycins by *Streptomyces griseus* on a defined medium. pH was adjusted to 7.0 initially and was not controlled later.
●, biomass; ▲, glucose; ◆, P[=streptomycin (SM) +phosphorylated streptomycin(SMP)]; △, PO_4^{3-}; ○, L-asparagine (L-asn)

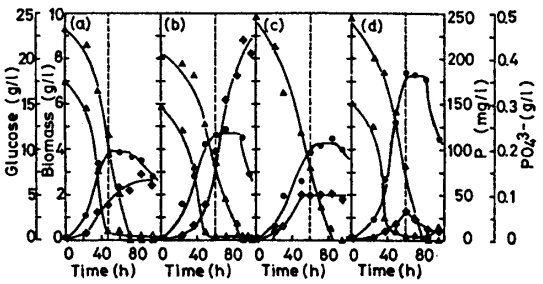

Fig. 2. Effect of pH on formation of streptomycins by *Streptomyces griseus* on a defined medium. pH was controlled at 6.0 (a), 6.5 (b), 7.0 (c) or 8.0 (d). Phosphate was not determined in (c).
The transition from trophophase to idiophase was assumed to be at the time indicated by broken line.
●, biomass; ▲, glucose; ◆, P[=streptomycin (SM) +phosphorylated streptomycin(SMP)]; △, PO_4^{3-}

growth occurred at pH 9.0, and the results obtained in the other four runs are depicted in Fig. 2. The cellular growth seemed to cease when phosphate was almost completely consumed (phosphate was not determined in Fig. 2–c) even though glucose, asparagine and NH_4^+ (not shown in Fig. 2) remained in the culture broth. However, after glucose exhaustion, cellular lysis occurred in all the cases, as reported by other workers for *S. griseus*.[10,11] The cellular growth yields from glucose in the trophophase were 0.28, 0.39, 0.25 and 0.42 g of cells/g of glucose at pH 6.0, 6.5, 7.0 and 8.0, respectively.

Formation of streptomycins was greatly affected by pH. At pH 6.0 (Fig. 2–a) and 6.5 (Fig. 2–b), formation of streptomycins occurred in both the trophosphase and the idiophase until glucose was almost consumed, whereas at pH 7.0 (Fig. 2–c) and 8.0 (Fig. 2–d), it took place only in the trophophase. Here, the trophophase and idiophase correspond to the growth phase until the biomass concentration reached its maximum level, and the non-growth phase. It is notable that formation of streptomycins reached a maximum of 225 mg/*l* at pH 6.5 at the end of the idiophase, three times higher than that at pH 6.0.

To further evaluate the effect of pH on the kinetics of formation of streptomycins, the amounts of streptomycins formed in the trophophase and the idiophase were calculated from Fig. 2. Formation of streptomycins per unit biomass in the trophophase, $P_T/(X_m-X_i)$, the cellular potential for formation of streptomycins in the idiophase, $(P_I-P_T)/X_m$, and the ratio of SM to (SM+SMP) calculated are summarized in Table 1.

The levels of growth-associated formation of streptomycins in the trophophase, $P_T/(X_m-X_i)$, were almost the same at pH 6.0, 6.5 and 7.0 but lower at pH 8.0. However, the cellular potential of formation of strepto-

Table 1. Effect of pH on formation of streptomycins by *Streptomyces griseus* in batch culture on a defined medium (see Fig. 2).

pH	X_m[1] (g/*l*)	P_T[2] (mg/*l*)	P_I[3] (mg/*l*)	SMP[4] (mg/*l*)	$\frac{P_T}{X_m-X_i}$[5] (mg/g)	$\frac{P_I-P_T}{X_m}$[6] (mg/g)	$\frac{\text{SM}}{\text{SM+SMP}}$[7] (—)
6.0	3.8	45	70	20	11.8	6.6	0.71
6.5	4.7	82	225	32	17.5	31.0	0.86
7.0	4.3	50	50	0	11.9	0	1.00
8.0	7.3	32	8	0	5.1	0[8]	1.00

1) Biomass concentration at the end of trophophase.
2) (SM+SMP) at the end of trophophase.
3) (SM+SMP) at the end of idiophase.
4) SMP at the end of idiophase.
5) (SM+SMP) yield for biomass in trophophase. Initial biomass concentration, X_i, was 0.1 g/*l* in each run.
6) Cellular potential of formation of streptomycins in idiophase.
7) Ratio of SM to (SM+SMP) at the end of idiophase ($=1-\text{SMP}/P_I$).
8) Streptomycins produced in trophophase were degraded in idiophase (see Fig. 2-d).

mycins in the idiophase, $(P_I-P_T)/X_m$, was remarkably higher (31.0 mg/g) at pH 6.5 than at the other pHs. This suggested that pH had a critical effect on the formation of streptomycins, especially in the idiophase.

The values of SM/(SM+SMP) at acidic pHs were only slightly lower than unity on this defined medium. This is in contrast with the sharp decrease to 0.2 on the complex medium,[6] which was due to inhibition of alkaline phosphatase.

Effect of initial ratio of glucose to phosphate on streptomycin formation To examine the effects of glucose and phosphate on the formation of streptomycins at a constant pH of 6.5, initial glucose concentration in the defined medium was varied between 3.6 and 19.9 g/*l* in the presence of a fixed concentration of initial phosphate (ca. 0.3 g/*l*). The results observed in four batch cultures are shown in Fig. 3.

With the highest glucose supply (19.9 g/*l*, Fig. 3–a), phosphate was completely consumed after 50 h of cultivation, when glucose and asparagine remained. Cellular growth ceased with phosphate exhaustion, and the stationary phase continued until glucose had been completely consumed. Thereafter, significant cellular lysis began.

With the lowest supply of glucose (3.6 g/*l*, Fig. 3–d), phosphate remained at a high level (0.12 g/*l*) when glucose had been completely consumed. Although asparagine was not determined in this run, the results of other runs indicate that asparagine might also remain up to 60 h of cultivation. After glucose exhaustion, gradual cellular lysis occurred, in contrast to the sharp decrease observed in Fig. 3–a. Formation of streptomycins was very low in the idiophase.

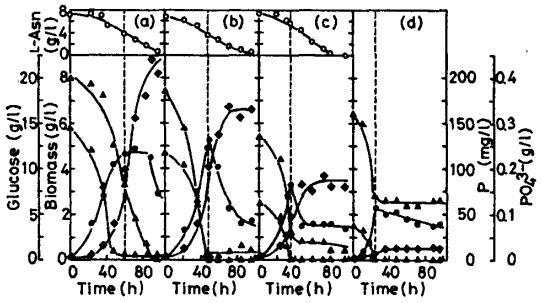

Fig. 3. Effect of initial ratio of glucose to phosphate on formation of streptomycins by *Streptomyces griseus* on a defined medium at pH 6.5. The values of glucose/PO_4^{3-} were 68.1 (a), 31.6 (b), 22.6 (c) and 11.2 (d). (a) is the same as Fig. 2-b. The transition from trophophase to idiophase was assumed to be at the time indicated by broken line.
●, biomass; ▲, glucose; ◆, P[=streptomycin (SM) +phosphorylated streptomycin(SMP)]; △, PO_4^{3-}; ○, L-asparagine (L-asn)

With intermediate levels of glucose (11.6 and 6.1 g/*l*, Fig. 3–b and –c), glucose was completely consumed in trophophase, while phosphate remained at very low levels (ca. 0.02 g/*l* in Fig. 3–b and ca. 0.05 in Fig. 3–c) into the idiophase. The trends of cellular growth and lysis, and of formation of streptomycins in the idiophase were similar in both cases, although maximum biomass yield and formation of streptomycins depended on the initial level of glucose.

To further clarify the effect of phosphate on formation of streptomycins, the cellular potentials for streptomycin formation in the trophophase and the idiophase were calculated from Fig. 3 (Table 2). The formation of streptomycins associated with growth in the trophophase, $P_T/(X_m-X_i)$, decreased with the increase of the initial ratio of glucose to phosphate, while the biomass yield from glucose increased. The formation of streptomycins in the idiophase, $(P_I-P_T)/X_m$, was more greatly affected by the initial ratio of glucose to phosphate. The maximum formation of streptomycins in the idiophase occurred in the absence of phosphate when a large amount of glucose remained in the idiophase (Fig. 3–a), but little formation occurred in the presence of phosphate when little glucose remained in the idiophase (Fig. 3–d). It is concluded that the balance of initial phosphate level to glucose level in the trophophase plays an important role in the formation of streptomycins, particularly in the idiophase. Accordingly, in order to give the cells a high potential for the formation of streptomycins in the idiophase, phosphate should be completely consumed while glucose should remain in the trophophase.

Table 2. Effect of initial ratio of glucose to phosphate on formation of streptomycins and growth of *Streptomyces griseus* on a defined medium at pH 6.5 (see Fig. 3).

Run[1]	glucose[2] / phosphate (g/g)	S_t[3] (g/l)	S_T (g/l)	X_m (g/l)	P_T (mg/l)	P_I (mg/l)	SMP (mg/l)	$(Y_{X/S})_T$[4] (g/g)	$\frac{P_T}{X_m-X_i}$ (mg/g)	$\frac{P_I-P_T}{X_m}$ (mg/g)	$\frac{SM}{SM+SMP}$ (—)
(a)	68.1	19.9	8.2	4.6	82	225	32	0.39	18	31	0.86
(b)	31.6	11.6	0	5.3	95	167	8	0.45	18	14	0.95
(c)	22.6	6.1	0	3.3	37	83	6	0.53	11	14	0.93
(d)	11.2	3.1	0	2.3	7	14	1	0.61	3	3	0.93

1) (a), (b), (c) and (d) correspond to Fig. 3-a, -b, -c and -d.
2) Initial ratio of glucose to phosphate ($=S_t/PO_4^{3-}$). PO_4^{3-} was 0.29 (a), 0.37 (b), 0.27 (c) and 0.32 g/l (d).
3) Initial concentration of glucose.
4) Cellular growth yield for glucose at the end of trophophase.
Other terms are the same as in Table 1 and initial concentration of biomass, X_i, was fixed at 0.1 g/l.

The fraction of SM in total streptomycins, i.e., SM/(SM+SMP), was not appreciably affected by the initial ratio of phosphate/glucose. This fraction was between 0.86 and 0.95, in contrast to the marked variation on the complex medium.[5]

Effect of asparagine on streptomycin formation As observed in Fig. 3–b and –c, the formation of streptomycins continued after depletion of glucose at the expense of asparagine. These results suggested that asparagine played a role in the formation of streptomycins in this phase. To confirm the role of asparagine, two batch cultures with initial asparagine concentration of 3 g/l were conducted either with or without an additional asparagine supplement just before glucose was completely consumed. The results are depicted in Fig. 4, in which Fig. 3–b is reused as the control (Fig. 4–c). In Fig. 4–c, ca. 3.5 g/l of asparagine remained when glucose was almost completely consumed, and during the idiophase cellular lysis started and the residual asparagine was gradually consumed with a little further formation of streptomycins.

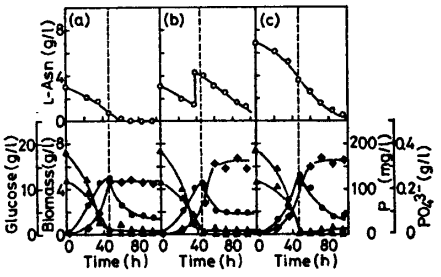

Fig. 4. Effect of L-asparagine on formation of streptomycins by *Streptomyces griseus* on a defined medium at pH 6.5. Initial L-asparagine was 3.0 g/l (a and b) or 6.8 g/l (c). In (b) 5 g L-asparagine was added at 38 h. (c) is the same as Fig. 3-b.
●, biomass; ▲, glucose; ◆, P[=streptomycin (SM) +phosphorylated streptomycin (SMP)]; △, PO_4^{3-}; ○, L-asparagine (L-asn)

In Fig. 4–a, a small amount (ca. 0.7 g/l) of asparagine remained when glucose was completely consumed. In the idiophase no more streptomycins were formed. However, in the Fig. 4–b, when 5 g of asparagine was additionally supplied after 38 h of cultivation, just before complete glucose consumption, it is noteworthy that formation of streptomycins continued similarly as in Fig. 4–c.

This suggests that asparagine in the culture medium might play a role in the formation of streptomycins in the idiophase whether glucose remains (Fig. 3–a) or not (Fig. 3–b, –c and Fig. 4–b). This observation might be related to the role of glutamine, which enhanced the formation of streptomycins as amino-group donor for the aminotransfer from glutamine to streptomycin.[12] Asparagine did not prevent cellular lysis after glucose had been completely consumed.

It is concluded that the efficient formation of streptomycins by *S. griseus* can be achieved in a defined medium by adjusting pH to 6.5 and selecting a culture medium in which phosphate is consumed first in the tropho-

phase while both glucose and asparagine remain. Glucose is also effective for preventing cellular lysis and consequently prolonging the formation of streptomycins in the idiophase. L-Asparagine is necessary for the formation of streptomycins. In fact, remaining glucose alone did not support the formation of streptomycins without asparagine (data not shown).

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