The effect of feedback regulation and in situ product removal on the conversion of sugar to cycloheximide by *Streptomyces griseus*

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Abstract. An addition of cycloheximide to cycloheximide-producing *Streptomyces griseus* cultures resulted in reductions in the production rate and in the conversion of sugar into cycloheximide. In situ cycloheximide adsorption was observed to enhance: total cycloheximide titers; productivities; and the conversion of sugar to cycloheximide. During the secondary metabolite-producing phase, sugar consumption was observed to be linearly dependent on cycloheximide productivity. From this analysis a true product yield and maintenance coefficient were estimated to be 0.08 g cycloheximide/g glucose and 0.028 g glucose/g cell-h, respectively. The sixfold difference between this true product yield and a theoretical value obtained from knowledge of the biosynthetic pathway is discussed. Since the maintenance sugar requirement for cycloheximide production is large, stimulation of biosynthesis through in situ adsorption significantly increases the overall efficiency of sugar conversion to this secondary metabolite.

Key words: In situ removal — In situ adsorption — Cycloheximide — Secondary metabolism — *Streptomyces griseus*

In situ product removal has been proposed for improving biological processes for the production of microbial secondary metabolites. Potential advantages include increases in titers, productivities and substrate conversions resulting from reductions in the effects of feedback regulation (Kominek 1975b; Wang et al. 1981), product toxicity (Marshall et al. 1987), and/or product degradation (Wang et al. 1981; Marshall et al. 1987). In the present study, the effects of in situ adsorption on the biosynthesis of the glutarimide antibiotic, cycloheximide, is examined.

There have been several preliminary studies conducted to characterize the metabolism and feedback regulation of the biosynthesis of glutarimide antibiotics (Spizek et al. 1965; Dolezilova et al. 1965; Vanek and Vondracek 1966; Roszkowski et al. 1972). More recent work by Kominek (1975a) demonstrated that addition of various amounts of cycloheximide to the cycloheximide-producing culture of *Streptomyces griseus* resulted in proportional reductions in

further biosynthesis. Subsequent studies demonstrated that in situ removal of cycloheximide was able to increase cycloheximide production (Kominek 1975b; Wang et al. 1981).

The objective of this study was to characterize the effect of in situ product removal on the efficiency of sugar conversion to cycloheximide. Improved sugar conversion is significant since substrate costs for secondary metabolite production are often considerable. Enhanced conversions would result from the elimination of unnecessary, competing biosynthetic pathways or by reducing the relative consumption of substrate for maintenance energy requirements. This latter effect is particularly important when metabolites such as cycloheximide, are produced by non-growing cells. Cooney and Avecedo (1977) and Heijnen et al. (1979) suggested that 60% – 70% of the sugar consumed in the penicillin fermentation is required for maintenance functions of the cells. Large maintenance requirements were also observed in the present study. Also, the results indicate that in situ cycloheximide adsorption stimulated glucose conversion into cycloheximide, and thus reduced the fractional consumption of sugar for maintenance.

Materials and methods

Streptomyces griseus UC-2132 was cultivated in a complex medium containing cerelose, 60 g; defatted soybean flour, 15 g; yeast, 2.5 g; $(NH_4)_2SO_4$, 5 g; $CaCO_3$, 8 g; NaCl, 4 g; and KH_2PO_4, 0.2 g in 1 l of water. A complex medium was used in these studies to ensure that observed effects would be relevant under industrial conditions. A neutral polymeric adsorbing resin (XAD-4, Rohm and Haas) was employed to adsorb cycloheximide for in situ product removal. Details of the experimental procedures have been previously described (Payne and Wang 1988). Reducing sugar concentrations were determined by the Somogyi method (1952). Because glucose is the predominant carbohydrate in the fermentation medium, the results are reported as glucose concentrations. Cycloheximide concentrations were determined by the colorimetric procedure described by Takeshita et al. (1962).

Results and discussion

Feedback regulation

To examine the feedback regulation of cycloheximide synthesis, various amounts of this antibiotic were added to

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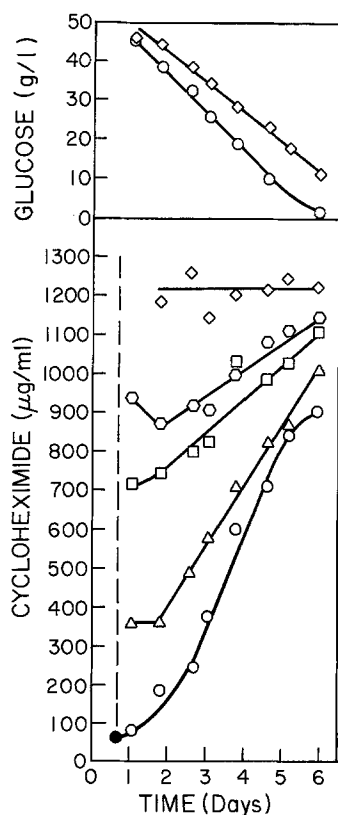
Fig. 1. The effect of cycloheximide additions prior to the onset of antibiotic synthesis. Broth (50 ml) was transferred from a 14 l fermentor at 0.8 days into 250 ml shake flasks containing the following amounts of cycloheximide (g/l): 0 ($\bigcirc$); 0.27 ($\triangle$); 0.67 ($\square$); 0.89 ($\ominus$); and 1.2 ($\diamond$)
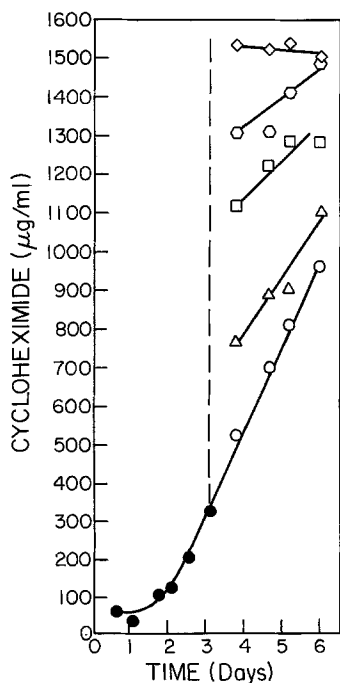

Fig. 2. The effect of cycloheximide additions on a producing culture. Broth (50 ml) was transferred from a 14 l fermentor at 3.1 days into 250 ml shake flasks containing the following amounts of cycloheximide (g/l): 0 ($\bigcirc$); 0.27 ($\triangle$); 0.67 ($\square$); 0.89 ($\ominus$); and 1.2 ($\diamond$)
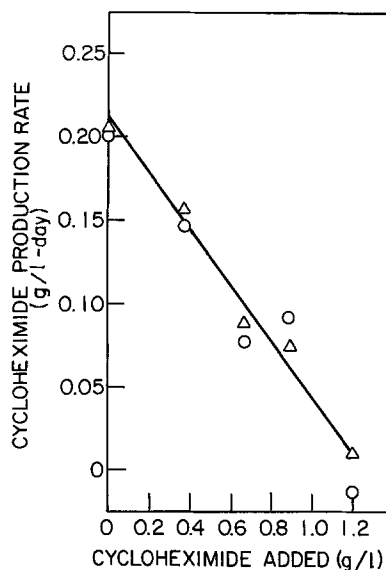

Fig. 3. The production rate vs. amount of cycloheximide added. The production rate reported here is the slope of the cycloheximide concentration vs. time profile for production phase cultures. Cycloheximide additions were made at 0.8 days, prior to the onset of biosynthesis ($\triangle$), or at 3.1 days, after biosynthesis had commenced ($\bigcirc$)

cultures prior to (Fig. 1) and following (Fig. 2) the onset of cycloheximide production. During the secondary metabolite-producing phase, the cycloheximide and glucose concentration vs. time profiles were linear (for clarity, only two of the glucose concentration profiles are shown in Fig. 1), with the slopes dependent on the amount of cycloheximide added. To compare results, the slopes of the concentration vs. time profiles for cycloheximide and glucose were defined as the production (R_p) and glucose consumption ($-R_{st}$) rates respectively. The ratio of the production to consumption rates was defined as the overall product yield. These definitions pertain only to the secondary metabolite production phase. The change in the production rate with the addition of cycloheximide (Fig. 3), is similar to the effect observed by Kominek (1975a). It should be noted that the production rate was reduced similarly for a given cycloheximide addition, independent of the external antibiotic concentration. This is illustrated by the data represented by triangles in Figs. 1 and 2. Although the external cycloheximide concentration of these two cultures varied by nearly 0.3 g/l, the addition of 0.27 g/l of the antibiotic resulted in a similar decrease in the production rate (shown in Fig. 3). Figure 4 shows that the overall conversion of glucose to cycloheximide was also reduced by cycloheximide additions.

The observations that cycloheximide additions prior to, or following the onset of antibiotic synthesis suppressed production rates and reduced net glucose conversion into cycloheximide supports the contention that feedback regulation limits cycloheximide production (Kominek 1975a). Similar results were observed in the chloramphenicol fermentation (Malik and Vining 1970). Although cycloheximide permeabilities were not studied here, changes in cellular permeabilities during the course of cultivation could explain the observation that cycloheximide feedback regulation could not be strictly correlated to the extracellular cyclo-
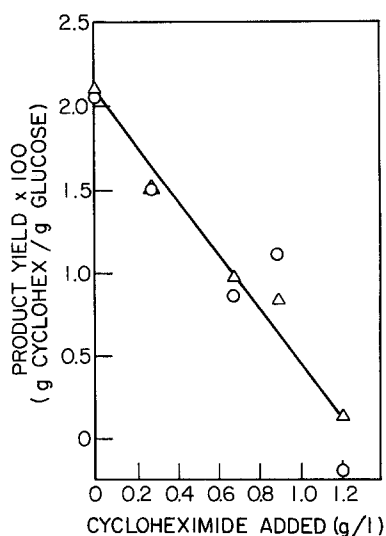
Fig. 4. The overall product yield vs. amount of cycloheximide added. This overall product yield is defined as the ratio of the cycloheximide production to glucose consumption rates for production phase cultures. Cycloheximide additions were made at 0.8 days, prior to the onset of biosynthesis ($\triangle$), or at 3.1 days, after biosynthesis had commenced ($\bigcirc$)

Fig. 5. The cycloheximide production profile in the presence of adsorbing resin. Broth (50 ml) was transferred from a 3 l fermentor to 250 ml shake flasks at 0.7 days. The control (*circles*) flask contained no resin while the experimental flask (*triangles*) contained 4% w/v resin. The aqueous (extracellular) and total cycloheximide concentrations for the experimental culture are represented by the *closed* and *open triangles* respectively

heximide concentration. If the permeability for cycloheximide is reduced during the fermentation, then higher extracellular cycloheximide concentrations would be required in the later stages of cultivation for an equivalent feedback effect to be observed. It has been suggested that chloramphenicol is excreted by the cells; and during production, these cells become less permeable to the chloramphenicol product (Malik and Vining 1972). Jones and Westlake (1974) even proposed that chloramphenicol was not the direct repressor of chloramphenicol biosynthesis. Also, streptomycin-producing cells have reduced streptomycin permeabilities during production (Cella and Vining 1975), and streptomycin uptake has been observed to be associated with inactivating (e.g. phosphorylation) reactions (Miller and Walker 1969).

In situ product removal

Wang et al. (1981) showed that a neutral polystyrene resin (XAD-4, Rohm and Haas) is capable of adsorbing cycloheximide from the fermentation broth. When added during the fermentation, this resin was observed to stimulate cycloheximide production (Wang et al. 1981). To further examine this effect, resin (4% w/v) was added to 0.7 day old cultures. Figure 5 shows that this resin is capable of maintaining low aqueous (extracellular) cycloheximide concentrations, while the total amount and rate of cycloheximide produced were enhanced by resin additions. Glucose utilization was also stimulated in the resin-supplemented culture (data not shown).

To further investigate the effect of in situ adsorption on the culture's metabolism, fermentations were conducted in the presence of varying amounts of resin. Figure 6 shows that the average aqueous cycloheximide concentration was reduced by increased resin levels. However, increases in the production rate were not observed for resin concentrations exceeding 4% w/v. Figure 7 shows that the overall product
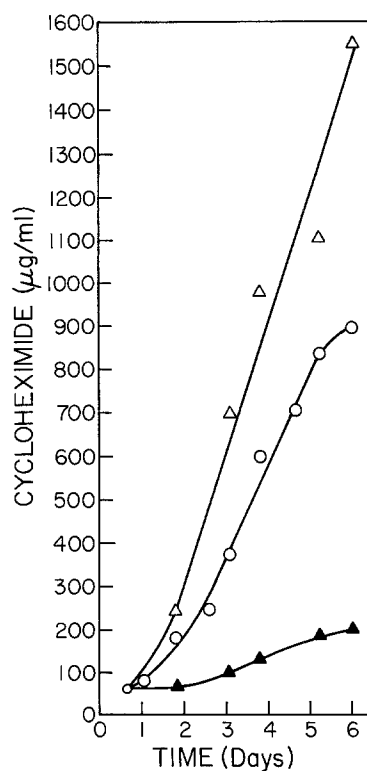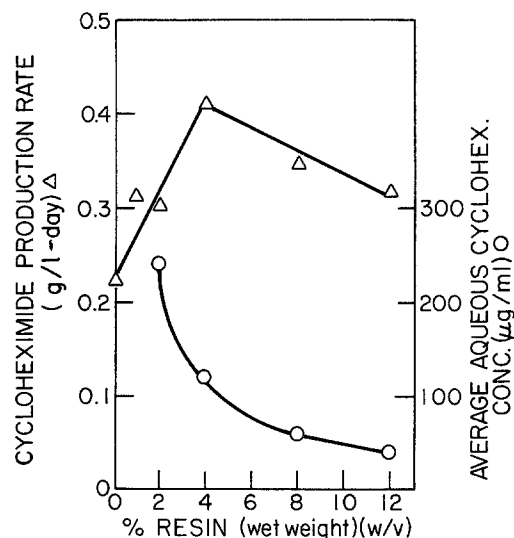

Fig. 6. The production rate ($\triangle$) and average aqueous (extracellular) cycloheximide concentration ($\bigcirc$) for cells cultivated in the presence of various amounts of resin

yield was enhanced by resin additions and this effect also could not be strictly correlated to the aqueous cycloheximide concentration. These results demonstrate that in situ adsorption improved both the cycloheximide productivity and the efficiency for glucose conversion into cycloheximide.
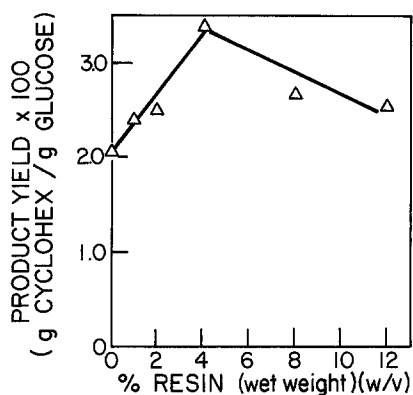
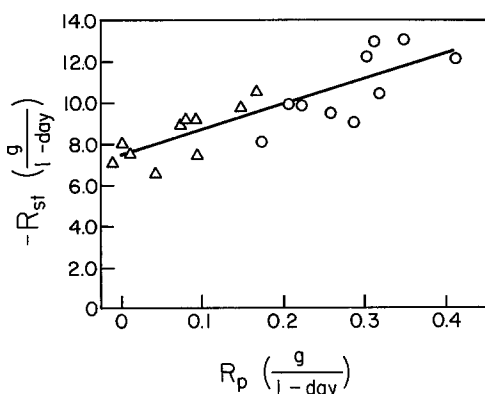**Fig. 7.** The overall product yield for cells cultivated in the presence of various amounts of resin

Fig. 8. The correlation between total glucose consumption and cycloheximide production for production phase cultures. Metabolism was altered by adding cycloheximide (*triangles*) or by in situ cycloheximide adsorption (*circles*)

Quantification of sugar consumption

If sugar is considered to be consumed by three processes: growth, product synthesis and maintenance, then the total rate of sugar consumption $(-R_{st})$ is given by:

$$-R_{st} = 1/Y_{xs} R_x + 1/Y_{ps} R_p + mX \tag{1}$$

where R_x and R_p are growth and production rates, Y_{xs} and Y_{ps} are true growth and true product yields, X is cell concentration and m is the maintenance coefficient. Since cycloheximide is typically produced by non-growing cultures, the growth related term in Eq. (1) can be neglected when analysis is restricted to the production phase. As predicted, Fig. 8 shows a linear dependence between the total sugar consumption and cycloheximide production rates.

The intercept of Fig. 8 indicates that 7.4 g sugar/l-day are consumed by the cells of maintenance. This high maintenance consumption is consistent with the observation that 10 to 12 mmol O_2/l-h are consumed by production phase cultures (Payne 1984). If oxygen is assumed to be used for the complete catabolism of glucose (Righelato et al. 1968; Cooney 1979) and if this catabolism is primarily associated with meeting the culture's maintenance requirements, then the observed oxygen uptake rate corresponds to a maintenance requirement of 7 to 9 g glucose/l-day.

Assuming a typical cell concentration of 11 g/l (Payne and Wang 1988), m can be estimated to be 0.028 g glucose/

g cell-h. Maintenance coefficients for the penicillin fermentation have been reported to be 0.022 (Righelato et al. 1968; Pirt and Righelato 1967), and 0.025 g/g-h (Heijnen et al. 1979). Mou and Cooney (1976) also reported a maintenance coefficient for the novobiocin-producing *Streptomyces niveus* to be 0.028 g/g-h.

The large intercept in Fig. 8 suggests that a significant amount of sugar is consumed for maintenance functions. This is consistent with the suggestion that maintenance requirements account for 60% – 70% of sugar consumption in the penicillin fermentation (Cooney and Acevedo 1977; Heijnen et al. 1979).

From the slope of Fig. 8, the true product yield for cycloheximide synthesis (Y_{ps}) was determined to be 0.08 g cycloheximide/g sugar. Considering cycloheximide to be produced from 6 malonate units (Vanek and Vondracek 1966) which result from 3 glucose molecules, then 3 mol of glucose are theoretically required per mol of cycloheximide. However, the value obtained for Y_{ps} suggests that 19 mol of glucose are used per mol of cycloheximide actually produced. It is doubtful that the energy requirments for cycloheximide biosynthesis, which are neglected in deriving a theoretical yield, could explain this sixfold difference in the glucose requirement for cycloheximide production. It is possible that other products, not measured in this study, contributed to this increased glucose requirement. However, for consistency with this analysis, biosynthesis of these additional products would have to be regulated in a manner similar to the regulation of cycloheximide biosynthesis. Spizek et al. (1965) reported that cycloheximide additions to a cycloheximide and actiphenol producing culture suppressed both cycloheximide and actiphenol synthesis. Later work by Roszkowski et al. (1972) showed that increased antibiotic production was associated with increased lipogenesis. Also, discrepancies between the true product yield and the theoretical value may be due to cycloheximide degradation which has been reported to occur through both enzymatic (Kominek 1975a) and chemical (Payne 1984) mechanisms. Although it is difficult to estimate the extent of degradation, it is possible that cycloheximide degradation during these studies was negligible. Kominek (1975a) reported that glucose, which was present during these studies, prevents degradation. Also, glucose metabolism by this culture generally leads to slightly acidic conditions which would limit cycloheximide degradation by alkaline hydrolysis (Garrett and Notari 1965).

Similar theoretical yields (0.3 to 0.6 g antibiotic/g glucose) have been derived for the penicillin fermentation (Cooney and Acevedo 1977) while observed values are much less (Cooney 1979).

Conclusions

Cycloheximide additions to a cycloheximide-producing culture led to reductions in both the production rates and conversions from glucose. Although indicative of a cycloheximide feedback regulatory mechanism, the effect of cycloheximide on its own biosynthesis could not be strictly correlated with the extracellular cycloheximide concentration. It is possible that quantitative changes in the effect of extracellular cycloheximide on its own biosynthesis could result from changes in cycloheximide permeabilities during the course of cultivation. Such permeability changes have

been reported for other antibiotic-producing *Streptomyces* (Malik and Vining 1972; Cella and Vining 1975).

In situ adsorption of cycloheximide resulted in increased cycloheximide titers, productivities and product yields. These observations are also indicative of a cycloheximide feedback regulatory mechanism. Analysis of sugar consumption showed that maintenance requirements for this culture were high, while the rate of sugar conversion to cycloheximide was proportional to the cycloheximide production rate. Thus, increased productivities associated with in situ cycloheximide adsorption reduced the fraction of glucose consumed for maintenance requirements. This could be economically important since maintenance sugar requirements can be quite significant in antibiotic producing cultures (Cooney and Acevedo 1977; Heijnen et al. 1979).

The true product yield observed in these studies (0.08 g cycloheximide/g sugar) was six times less than a theoretical value based on stoichiometric requirements for cycloheximide biosynthesis (0.5 g/g). This apparent discrepancy may be due to the biosynthesis of other products associated with the cycloheximide biosynthetic pathway, or due to cycloheximide degradation during the fermentation. When the culture maintenance is included, the overall sugar conversion is reduced even further to 0.02 g cycloheximide/g glucose.

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