

The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat

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Streptomyces thermoviolaceus was grown in a chemostat under conditions of glutamate limitation. The effects of growth rate on production of the antibiotic granaticin, extracellular protein and protease activity as components of secondary metabolism were studied at 37, 45 and 50 °C. The amount of each secondary metabolite synthesized was highly dependent on growth rate and temperature. Granaticin yields were highest at growth rates of 0.1 to 0.15 h⁻¹ at 37 °C, 0.175 h⁻¹ at 45 °C and 0.045 h⁻¹ at 50 °C. Protease activity of culture supernatants responded to low nutrient concentration and/or low growth rate. Measurements of extracellular protein revealed complex changes in amount which were dependent on growth rate and temperature. At 45 °C and a growth rate of 0.15 h⁻¹, biomass yield was highest between pH 5.5 to 6.5 whereas granaticin synthesis was low at pH 5.5 and rose to highest values at between pH 6.5 and 7.5.

Introduction

A recent taxonomic survey characterized the properties of a number of thermophilic isolates of *Streptomyces* (Goodfellow *et al.*, 1987), and showed that their biochemical properties resulted in a taxonomic grouping distinct from that found for the mesophilic ones. One thermophilic species, *Streptomyces thermoviolaceus*, produces a coloured antibiotic and has been used by us to study the effects of temperature on growth and secondary metabolism. This organism grows between 25 and 57 °C despite evidence that indicates that some of the reactions of energy production are thermolabile at temperatures upwards of 50 °C (Edwards & Ball, 1987). *S. thermoviolaceus* utilizes a wide range of carbon sources for growth and many of these also support antibiotic synthesis at temperatures between 30 and 55 °C (James & Edwards, 1988), but optimally at 45 °C (James & Edwards, 1989). At 45 °C, growth in batch culture is biphasic, antibiotic synthesis commencing at the point of inflexion and continuing during the second, slower growth phase. These growth kinetics appear to be due to substantial amounts of protein secreted by the mycelium concomitant with granaticin excretion, both of which mark the onset of the phase of secondary metabolism (James & Edwards, 1991). Protease activity has been identified as one enzymic component of the excreted protein. Therefore the major processes associated with the phase of

secondary metabolism in *S. thermoviolaceus* consist of antibiotic synthesis and protein and protease secretion. These occur at a time when nutrients in growth media are still in excess and therefore when growth is still occurring (James & Edwards, 1989). This observation is interesting in the light of the more usual finding that secondary metabolites are synthesized only in 'stationary phase' cultures or in cultures that have extremely low growth rates (e.g. Demain, 1982; Demain *et al.*, 1979) and implies that it should be possible, using a chemostat, to study secondary metabolite production by *S. thermoviolaceus* at different growth rates. Such an approach would allow the processes of secondary metabolism to be studied under constant cultural conditions at defined but varied growth rates as well as investigating the consequences of changing cultural conditions on these processes in cultures grown at fixed growth rates. In this paper we employ glutamate-limited continuous cultures to investigate the effects of cultural factors (pH and temperature) and growth rate on secondary metabolite production in *S. thermoviolaceus*.

Methods

Organism and growth conditions. *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* NCIB 10076 was maintained on a complex solid medium that was also used for the preparation of stock spore

suspensions as described previously (James & Edwards, 1989). Defined salts medium for batch cultures contained (g l^{-1}) MOPS (5.23), $(\text{NH}_4)_2\text{SO}_4$ (2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1), K_2HPO_4 (1), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05), and 1 ml l^{-1} of a trace element solution that contained (g l^{-1}) $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1) and citric acid (5), together with the appropriate carbon source at concentrations as indicated. The sodium salt of glutamate was used. The medium was adjusted to pH 7.0 prior to autoclaving for 15 min at 121 °C and 15 p.s.i. (103.5 kPa).

For studies of nutrient limitation in batch cultures, a range of concentrations of the relevant nutrient was prepared in different flasks (50 ml of salts medium in a 250 ml flask). Each was inoculated with the same inoculum size from a spore suspension and incubated with shaking at 200 r.p.m. at 50 °C for 48 h. Determinations of dry weight and granaticin titre were then made for each.

For continuous culture studies $(\text{NH}_4)_2\text{SO}_4$ was omitted, K_2HPO_4 lowered to 0.5 g l^{-1} and glutamate supplied at 3.8 g l^{-1} . These changes were determined experimentally to be sufficient to maintain all nutrients except the carbon source in excess. Due to the large volumes used for medium reservoirs (20–30 litre) and the consequent longer autoclaving times (1 to 1.5 h at 15 p.s.i.) it was necessary to lower the pH of the media to 4.0 with a small quantity of sulphuric acid before autoclaving in order to avoid precipitation of the salts, particularly the phosphate and trace elements.

Nutrient-limited continuous culture. This was carried out in a LH 500 series fermenter using a 2 litre vessel with a working volume of 1200 ml preset by adjusting the height of an overflow weir inside the vessel prior to autoclaving. Agitation was at 1200 r.p.m. and air was supplied through the vessel head-space at a rate of 2.41 min^{-1} . The pH was controlled to 7.0 by using 1 M-NaOH as the titrant. Preparation of the inoculum was as described previously (James & Edwards, 1989). Wall-growth by attached mycelium became a problem with longer running times. A number of measures were adopted to minimize this and included the following: (1) the baffles were removed from the vessel to reduce the surface area available for attachment; (2) the internal vessel surfaces, agitator, probes, heater and thermometer were coated with dichloromethylsilane; (3) a magnetic follower was included inside the vessel and periodically moved around the internal walls by means of a powerful horseshoe magnet held on the external surface to scrape off any attached mycelia; (4) biomass was restricted to a maximum value of around 1 g l^{-1} . These precautions were sufficient to keep wall growth to a minimal and acceptable level. Culture volume was kept constant by an internal weir of internal diameter 3 mm which was more than sufficient to allow egress of mycelial fragments. In addition, negative pressure was applied to the exit line by peristaltic pumping counter to that applied to the inflowing medium. These precautions ensured that the culture effluent was representative of the vessel contents.

Analytical methods. Biomass, granaticin and glutamate were all quantified as described previously (James & Edwards, 1989). Protease activity was assayed in cell-free culture supernatants at 40 °C by the method described by Jones *et al.* (1988), whereby 1 unit (U) is taken as that amount of enzyme sufficient to produce an A_{440} of 0.01 after 40 min at 40 °C using an azocasein assay. Extracellular protein was estimated by the Lowry method.

Results

Continuous culture studies

Glutamate was selected as the carbon source for continuous culture studies because previous work

showed that it supported high yields of biomass and antibiotic (James & Edwards, 1988, 1989). Preliminary studies on the yield of biomass obtained at different glutamate concentrations showed a linear response between 5 and 25 mM-glutamate. A concentration of 12 mM-glutamate was selected for chemostat studies because it limited biomass to approximately 0.8 g l^{-1} , a culture density that also reduced wall growth but still allowed for the production of appreciable amounts of granaticin. Glutamate can serve as both sole carbon and nitrogen source with no diminution in the yield of either biomass or antibiotic – a reflection of the low C:N ratio of 5:1 for this substrate. A concentration of 12 mM was found to limit carbon alone (and not nitrogen) because ammoniacal nitrogen could be measured in culture supernatants taken from chemostat cultures irrespective of growth rate. In fact, antibiotic biosynthesis in *S. thermoviolaceus* appears to be largely indifferent to the presence of ammoniacal nitrogen in cultures utilizing glutamate as the sole carbon source (James & Edwards, 1989).

The effects of different temperatures on growth of *S. thermoviolaceus* in glutamate-limited chemostats revealed that 30 and 55 °C were unsuitable due to very slow growth rates which in turn resulted in significant wall growth and at 55 °C frequent washouts (probably due to temperature instability). Accordingly, continuous culture studies were confined to 37, 45 and 50 °C and at least three separate chemostat runs were set up for each temperature. The data in Figs 1 to 3 are typical of these replicates. To ensure that cultures were in steady state, at least three culture volumes of growth medium were passed through the culture vessel prior to making a measurement. Finally, measurements were made for a range of D values in both ascending and descending order.

At 37 °C, the yield of biomass was maximal at a growth rate of 0.15 h^{-1} whilst that of granaticin was optimal at between 0.1 and 0.15 h^{-1} and fell towards zero as D approached 0.3 h^{-1} (Fig. 1*a*) which correlated with the μ_{max} value calculated for batch cultures grown at 37 °C. Glutamate concentrations measured in culture supernatants remained undetectable up to $D = 0.2 \text{ h}^{-1}$ but above this value became measurable as the dilution rate approached μ_{max} (Fig. 1*b*). Protease activity determined in cell-free supernatants decreased with increasing growth rate suggesting that its production and/or secretion responded to low growth rate. Extracellular protein also tended to decrease in amount with increasing growth rate. The concentrations ranged from approximately 95 to 30 $\mu\text{g ml}^{-1}$ but exhibited distinct maxima at $D = 0.1$ and 0.2 h^{-1} (Fig. 1*b*).

Fig. 2 shows a continuous culture at 45 °C. Biomass was highest at $D = 0.225 \text{ h}^{-1}$ and granaticin yield

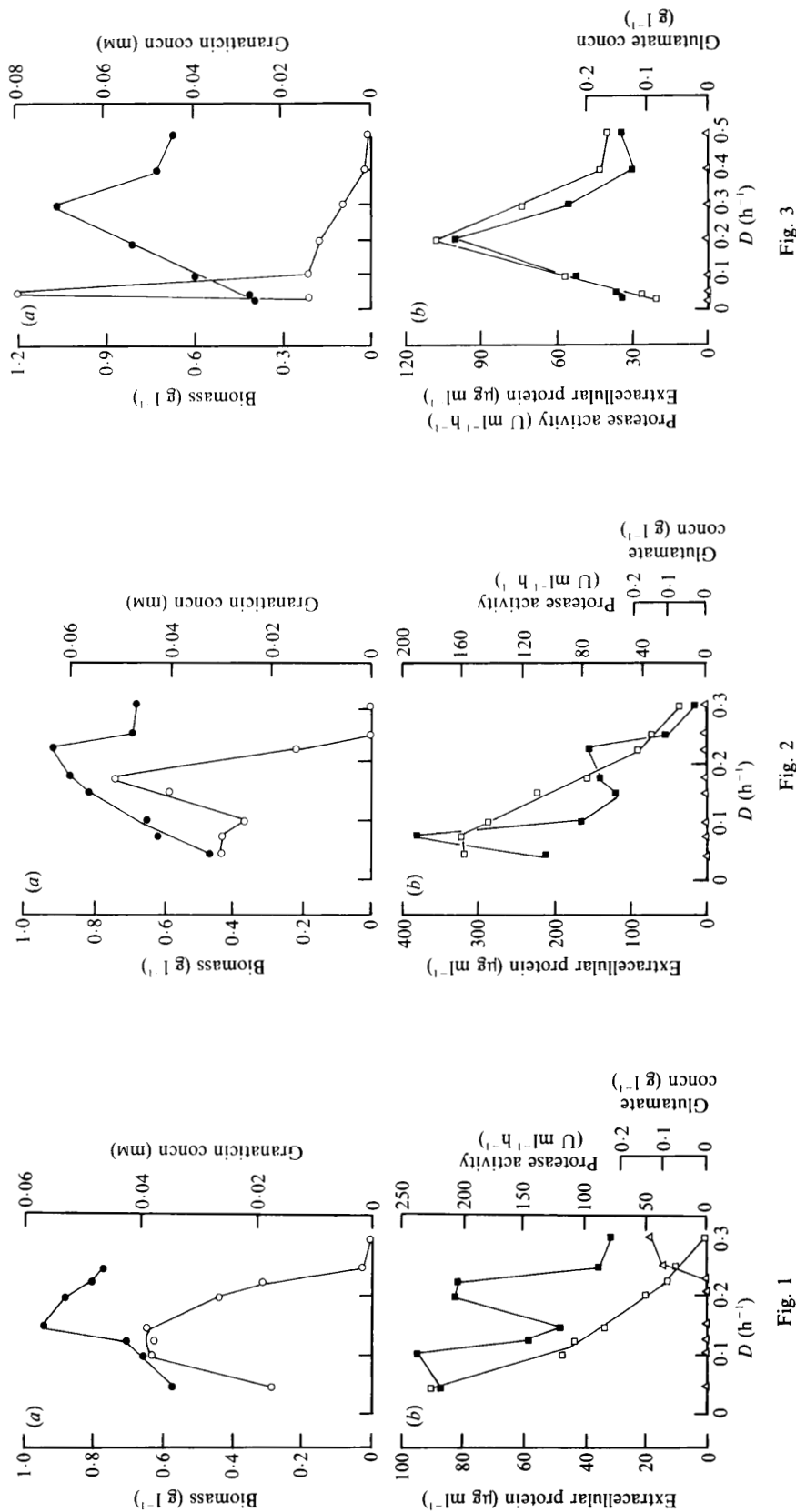


Fig. 1. Growth of *S. thermoviolaceus* in a chemostat at 37 °C at different dilution rates with glutamate as sole carbon and nitrogen source. (a) Biomass (●) and granaticin concentration (○); (b) protease activity (■), extracellular protein concentration (□) and glutamate concentration (△). The data are representative of three different experiments.

Fig. 2. Growth of *S. thermoviolaceus* in a glutamate-limited chemostat at 45 °C. Symbols and experimental conditions were as in Fig. 1.

Fig. 3. Growth of *S. thermoviolaceus* in a glutamate-limited chemostat at 50 °C. Symbols and experimental conditions were as in Fig. 1.

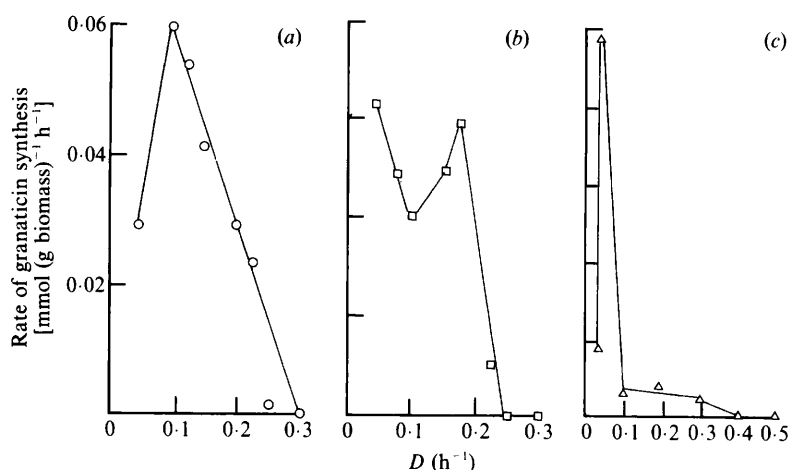


Fig. 4. Rate of granaticin production by *S. thermoviolaceus* grown at different dilution rates at (a) 37 °C (b) 45 °C and (c) 50 °C.

greatest at $D = 0.175 \text{ h}^{-1}$ (Fig. 2a). Glutamate remained undetectable at all growth rates examined. Protein concentrations fell with increased growth rate but again exhibited two maxima at approximate growth rates of 0.1 and 0.2 h^{-1} while doing so. The amounts of extracellular protein were much higher at this temperature compared to 37 °C and ranged between 380 and 10 $\mu\text{g ml}^{-1}$. Protease activity fell with increasing growth rate in much the same way as at 37 °C (Fig. 1b) but the highest activities detected were less at 45 °C (160 $\text{U ml}^{-1} \text{ h}^{-1}$) than at 37 °C (225 $\text{U ml}^{-1} \text{ h}^{-1}$).

At 50 °C the biomass yield was highest at $D = 0.3 \text{ h}^{-1}$ (Fig. 3) but the optimum dilution rate for antibiotic titre was 0.045 h^{-1} and fell sharply away at dilution rates either side of this value (Fig. 3a). This temperature allowed extremely rapid growth rates such that glutamate limitation still occurred at $D = 0.5 \text{ h}^{-1}$ as indicated by the absence of glutamate in the culture medium (Fig. 3b). A correlation between extracellular protein levels and protease activity is apparent with each parameter being highest at $D = 0.2 \text{ h}^{-1}$ (Fig. 3b).

Fig. 4. shows the rates of granaticin production (Q_p) plotted against growth rates at 37, 45 and 50 °C. The rate of antibiotic production at 37 °C was greatest at $D = 0.1 \text{ h}^{-1}$ and thereafter declined with increasing growth rate (Fig. 4a). At 45 °C high production rates were apparent at low growth rates (below 0.1 h^{-1}) followed by a maximum at $D = 0.175 \text{ h}^{-1}$ (Fig. 4b). The latter occurred at a growth rate approaching that which gave the highest yield of biomass (see Fig. 2). The profile of Q_p for granaticin at 50 °C (Fig. 4c) was very similar to that for $Y_{\text{granaticin}}$ described in Fig. 3.

Effects of pH

Earlier work had shown that pH had an effect on antibiotic production by *S. thermoviolaceus* (James &

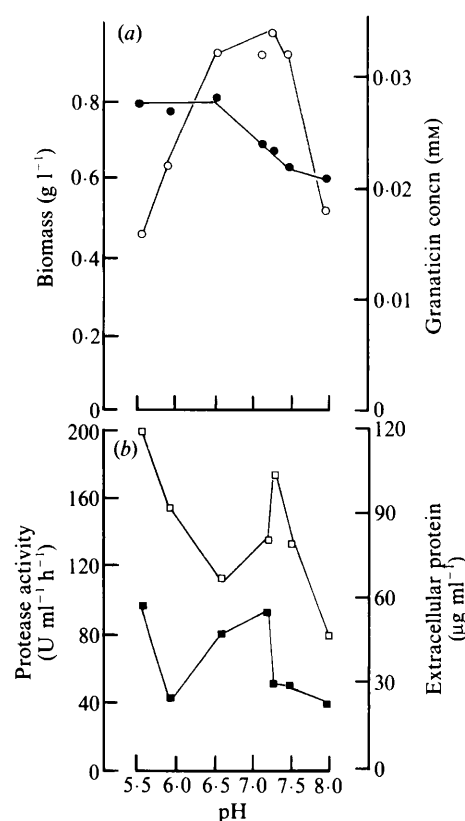


Fig. 5. Effects of different pH values on *S. thermoviolaceus* grown in a chemostat under glutamate-limitation at 45 °C and at a dilution rate of 0.15 h^{-1} . (a) Biomass (●) and granaticin concentration (○); (b) protease activity (□) and extracellular protein concentration (■).

Edwards, 1989). The use of a glutamate-limited chemostat enabled the effects of pH on growth and antibiotic production to be studied under conditions of constant growth rate. Accordingly, *S. thermoviolaceus* was grown at 45 °C and $D = 0.15 \text{ h}^{-1}$ at which growth yield and antibiotic production were both high (see Fig. 2). A pH range from 5.5 to 8.0 was chosen and the results are

presented in Fig. 5. Biomass was approximately 0.8 g l^{-1} between pH 5.5 and 6.5 and thereafter fell with increasing pH to around 0.6 g l^{-1} at pH 8.0. Granaticin yields were low at pH 5.5 but rose steadily with pH to highest values between pH 6.5 to 7.5 and declined markedly at pH 8.0 (Fig. 5a). Glutamate remained undetectable at all pH values indicating that carbon limitation was maintained (results not shown). A plot of Q_p granaticin (not shown) clearly demonstrated that the pH optimum for production of the antibiotic was between 7.3 and 7.5. Extracellular protein levels exhibited a similar profile to granaticin in that values rose to a maximum at pH 6.5 to 7.0 and then fell to a level of around $20 \mu\text{g ml}^{-1}$ at pH 8.0. Protease activity was surprisingly high at pH 5.5 and decreased, apart from a brief rise at pH 7.0, with increasing pH (Fig. 5b).

Discussion

For many streptomycete species, studies using continuous culture have proved difficult because the antibiotic biosynthetic phase is restricted to non-growing stationary phase biomass. Many workers have noted a variety of repressors of secondary metabolism which may be responsible for this. These include the nature of the carbon source (Hu *et al.*, 1984; Payne & Wang, 1989), and levels of ammonia which tends to repress many antibiotic biosynthetic pathways (Vu-trong & Gray, 1987; Dekleva *et al.*, 1985; Tanaka *et al.*, 1985) or the synthesis of some of antibiotic pathway enzymes (Zhang *et al.*, 1989; Cimburkova *et al.*, 1988; Shapiro & Vining, 1983). The levels of growth substrates in the growth medium did not appear to exert any direct catabolite repression or inhibition of granaticin synthesis when *S. thermoviolaceus* was grown in the presence of phosphate or ammonia, as well as in the presence of a number of carbon sources that included glucose, fructose and proline (results not shown).

Continuous cultures at 37 or 45 °C confirmed that antibiotic synthesis in *S. thermoviolaceus* is growth associated. This observation is supported by the findings of Rhodes (1984) with oxytetracycline production and Trilli *et al.* (1987) with erythromycin production, who independently demonstrated that production of these antibiotics in chemostats increased with growth rate. Protease activity showed a similar response to increased growth rate at both temperatures in that activity fell steadily. The reasons for the fluctuations in amounts of extracellular protein with growth rate at 37 and 45 °C remain obscure. The presence of extracellular enzymes and proteins is a common feature of streptomycetes (Matsue *et al.*, 1987; Gibb *et al.*, 1989; Renko *et al.*, 1989) because of their role in biodegradation and recycling of

carbon in soils and solid substrates such as composts (Amner *et al.*, 1988). Work on batch cultures of *S. thermoviolaceus* (James & Edwards, 1991) shows that protein secretion is invariably linked to that of granaticin. This relationship is independent of temperature or the nature of the carbon source and therefore protein secretion would appear to be linked to, or a component of, secondary metabolism.

When *S. thermoviolaceus* is grown in a glutamate-limited chemostat biomass should reflect the concentration of the limiting nutrient and be independent of growth rate. This obviously does not apply to *S. thermoviolaceus* at any of the growth temperatures examined because biomass levels increased over a range of growth rate values and then declined at higher growth rates. These unusual growth responses may reflect growth as hyphal mycelia. Alternatively, accurate determinations of biomass produced may be affected by the substantial amounts of protein secreted. In fact, a true measurement of extracellular protein may also be impaired by the protease activity that is a component of it.

Highest yields of granaticin were obtained at 50 °C in chemostat cultures at $\mu = 0.45 \text{ h}^{-1}$. This contrasts with previous work with batch cultures which clearly showed that highest titres were obtained at 45 °C (James & Edwards, 1989). The reason for this anomaly lies in the fact that at 50 °C batch cultures are committed to rapid biomass production until substrate exhaustion – low growth rates are never achieved at this temperature. The value of continuous culture is to enforce low growth rates at this temperature which in turn allow production of high yields of granaticin, albeit over an extremely narrow range of growth rates. At 45 °C batch cultures exhibit biphasic growth with antibiotic synthesis confined to the second, slower phase. This second phase in glutamate-grown cultures has a value for μ of approximately 0.13 h^{-1} which approximates to the growth rate of 0.17 h^{-1} at 45 °C under glutamate-limitation that yields highest antibiotic titres. Granaticin synthesis becomes undetectable at high growth rates irrespective of temperature. Antibiotic synthesis appears to be restricted to the lower growth rates that occur for a given temperature. Manipulation of growth rates using different carbon sources also stresses the importance of low growth rate to antibiotic production. We have found that the slower the second phase of growth in batch cultures at 45 °C, the greater the amount of antibiotic that is synthesized (unpublished work).

The use of a glutamate-limited chemostat also demonstrated the important effect of culture pH on the parameters tested. The pH optimum for biomass yield was different to that for antibiotic production and protease activity also exhibited some pH dependence.

The observation that activity was high at pH 5.5 and again rose to a distinct maximum at pH 7 could be explained by the fact that *S. thermoviolaceus* synthesizes at least two different proteases (one serine, the other a metallo-protease). These may have slightly different pH optima for activity and/or secretion (James & Edwards, 1991). Extracellular protein also exhibited a maximum in amount at pH 7.

Because cultures are growing at a constant growth rate and no obvious stress is being imposed on the mycelium, particularly at pH 7.0, our data provide strong support that protein secretion is not occurring through some cultural perturbation and may be linked to antibiotic production as an important element of secondary metabolism in *S. thermoviolaceus*. Further work is now in progress to identify the reasons and regulatory factors that operate secretory functions in *S. thermoviolaceus* during the phase of secondary metabolism.

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