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Application of affinity adsorption in thienamycin fermentation

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Summary. Many antibiotic fermentations are sensitive to high concentrations of their own product possibly due to product regulation and toxicity mechanisms. In this paper we discuss the feasibility of using affinity adsorption with biospecific ligands for in situ product removal to alleviate this problem. The concept of using whole cells containing the biospecific ligands is demonstrated in the case of thienamycin fermentation using whole cells of *Bacillus stearothermophilus* and immobilized β-lactamase. It is observed that thienamycin production continues for an extended period of time.

Introduction

Most industrial fermentations are limited by end-products that are formed by the producing microorganisms. The product may inhibit the activity or repress the synthesis of enzymes that are responsible for product formation. Similarly, product degradation can be quite significant if the end-product is unstable. Optimization of culture media and environmental conditions may yield only nominal increases in volumetric productivity if product limitation and instability are significant. One option for circumventing product limitation and product instability is to simultaneously remove the product from the fermentation broth as it is formed. Methods proposed for in situ product removal include solvent extraction, dialysis/ultrafiltration coupled fermentations, and addition of solid adsorbents (Abbot and Gerhardt 1970; Raymond et al. 1969; Tone et al. 1969). In our laboratory, we have successfully utilized hydrogel immobilized non-ionic polymeric adsorbents, XAD-4 (Rohm and Haas, Philadelphia, PA) in a *Streptomyces griseus* fermentation for enhancing the production of cycloheximide by facilitating in situ product removal (Payne 1984; Wang 1983; Nigam and Wang 1986; Dykstra et al. 1988).

The use of non-specific adsorption or extraction methods often results in the concentration of desired products together with other contaminants having similar physico-chemical characteristics. One way to minimize this problem is to use affinity adsorption during fermentation processes. Not many articles have addressed the application of affinity adsorption for the purpose of primary separation. The major reason presumably is the prohibitive cost and limited variety of affinity adsorbents available.

The goal of this work is to examine the effect of specific in situ product removal by using whole cells as adsorbents on the production of thienamycin by *Streptomyces cattleya*. Thienamycin is one of the most potent broad spectrum β-lactam antibiotics discovered in recent years (Kahan et al. 1978, 1979). Unfortunately, thienamycin is extremely sensitive to pH, metallic ions, nucleophilic reagents, and even high concentrations of thienamycin itself (Kahan et al. 1979). Thienamycin, like the other β-lactam antibiotics, acts by binding very strongly to a class of bacterial cell wall proteins referred to as penicillin binding proteins (PBP) (Abraham 1981; Spratt 1983). It is also a strong inhibitor of β-lactamases (Kahan et al. 1979). Thus in principle both PBP and β-lactamases can be used effectively as affinity ligands for selectivity removing thienamycin.

Unfortunately, the mechanism of binding β-lactam antibiotics to PBP and β-lactamases involves the formation of covalent linkage through the carbonyl moiety on the β-lactam ring. The elu-

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tion of the antibiotic by using nucleophilic reagents results in the cleavage of the active β-lactam ring of the molecule. Protein engineering studies are required to change key amino acid residues in the active site of the protein such that it loses its covalent binding and degradative ability while retaining high affinity for reversibly binding β-lactam antibiotics. Initial experimental results have demonstrated the feasibility of this concept using recombinant β-lactamase for antibiotic separation (Imanaka et al. 1989).

This paper presents the preliminary results investigating the influence of specific in situ removal of thienamycin using affinity adsorbents based on PBP and β-lactamases on the overall production of the antibiotic. The focus of this study is to illustrate that in thienamycin fermentation, which is sensitive to high concentrations of thienamycin, the product can be extracted in situ using whole cells containing the biospecific ligands leading to improved productivity. The problem of non-degradative elution of active thienamycin will be addressed elsewhere in conjunction with protein engineering studies.

Materials and methods

Microorganism and culture media

Streptomyces cattleya NRRL-5057 was used in this study. Inocula for the fermentations were prepared in a seed medium consisting of glucose, 10 g; yeast extract, 10 g; KH_2PO_4, 182 mg; Na_2HPO_4, 190 mg; $MgSO_4$, 50 mg; distilled water, 1 liter. The pH of the medium was adjusted to 6.5. The fermentation medium consisted of: corn meal, 20 g; yeast extract, 10 g; soy flour, 15 g; sodium citrate, 4 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $MgSO_4 7H_2O$, 0.1 g; $CaCl_2 \cdot 16H_2O$, 0.1 g; $FeSO_4 7H_2O$, 0.01 g; distilled water, 1 liter.

Thienamycin fermentation

A 250 ml erlenmeyer flask containing 50 ml of the seed medium was inoculated with *Streptomyces cattleya* from an agar slant and incubated for 48 hours on a Shaker (250 RPM) at 25° C. After this incubation, 5 ml of this seed culture was used to inoculate fermentation flasks containing 50 ml of the fermentation medium. All media were sterilized at 121° C for 20 min. The fermentation flasks were incubated at 25° C on a shaker until harvested. Aliquots were removed from the shake flasks for pH and cell measurements. 10 ml of well-mixed broth was placed into graduated conical centrifuge tubes and spun at 2400 RPM for 20 min. The packed cell volume (PCV) thus obtained was used as the index of growth. From each fermentation flask, only one sample was taken. Thus, each data point was obtained from a different flask. Even though there could be some variability in each flask, the general trend of the cell mass and product formation is not likely to change.

The supernatant was saved for antibiotic assay.

Thienamycin assay procedure

Thienamycin content of the samples was determined by a microbiological assay procedure using *Streptococcus aureus* NRRL-B313 as the indicator organism (Kahan et al. 1979). For each assay, 100 µl of the supernatant sample after proper dilution, was pipetted onto a 0.5 inch diameter sterile paper disc, which was then placed onto the surface of the seeded agar. These plates were then inverted and incubated for 24 h at 37° C Any resulting inhibition zone size was measured with a Fischer-Lilly zone reader. The zone sizes were correlated with a standard curve produced with various ampicillin standards. The thienamycin concentration was expressed as equivalent units of ampicillin. Since the potency of thienamycin is more than three orders of magnitude higher than ampicillin, the value obtained in terms of equivalent ampicillin units is significantly higher than the actual concentration of thienamycin. For example, under these assay conditions 0.1 µg/ml of pure thienamycin solution produces an inhibition zone of approximately 25 mm diameter (Kahan et al. 1979). Our experiments indicate that approximately 0.75 µg/ml of ampicillin is required to produce the same size of the inhibition zone. Therefore, ampicillin equivalent units should be utilized for the purpose of comparison only.

Choice of affinity ligands

In general, gram-positive microorganisms containing PBP have been shown to bind upto 1–5 µg of penicillin per gram of dry cells (Blumberg and Strominger 1974). Affinity adsorption using whole bacterial cells as bioadsorbents represents a cost effective alternative since this approach bypasses the need for purifying and immobilizing affinity ligands on solid supports. Late growth phase cells of *Bacillus stearothermophilus*, a thermophilic bacterium, were utilized as one of the affinity adsorbents in this study. The reason we chose thermophilic bacteria was because they generally do not grow a lower fermentation temperatures, and therefore do not seem to take up significant amount of nutrient when added directly to the fermentation as active bioadsorbents.

Thienamycin is a β-lactamase inhibitor (Georgopapadakou and Liu 1980). Therefore, immobilized β-lactamase can be used as an affinity adsorbent for thienamycin. β-lactamase (Sigma, St. Louis, Mo) was first immobilized onto cyanogen bromide activated sepharose 4B beads (Pharmacia, Uppsala, Sweden). Estimated immobilized β-lactamase activity was about 0.5 mole Penicillin/minute/ml of beads.

Conditions of β-lactamase immobilization and assay

β-Lactamase (penicillinase, Sigma) was immobilized on cyanogen bromide activated Sepharose 4B (Pharmacia) by covalent bonding. Throughout the immobilization process, the β-lactamase and Sepharose were maintained at 4° C whenever possible.

Two grams of freeze-dried Sepharose was washed on a sintered glass filter and reswollen with 400 ml/g of 1.0 M HCl. While the Sepharose was swelling, 37.5 mg of protein containing 7.5 mg of β-lactamase was dissolved in 10 ml of sodium bicarbonate buffer at pH 8.3. The Sepharose was rinced with small amounts of sodium bicarbonate buffer, vacuum filtered and brought to semidryness. The Sepharose was then transferred to the enzyme solution. To block any unoccupied binding sites remaining on the Sepharose beads, 8 ml of 0.2 M glutamic acid solution was added to the Sepharose solution. The

resulting mixture was shaken for 2 h. A final sequence of washes was necessary to remove any unbound β-lactamase or glutamic acid. The washes consisted of 5–50 ml bicarbonate buffer washes, 5–50 ml 0.1 M acetic acid buffer, and 2–50 ml phosphate buffer at pH 6.8. Each wash was performed while under vacuum. The final moist Sepharose was resuspended in 12.0 ml of phosphate buffer, well mixed and stored at 4° C until ready for use.

β-Lactamase was assayed by the method described by Sargent (1968). One unit of β-lactamase activity is defined as the amount of enzyme that hydrolyzes one μmol Penicillin G per minute at 25° C and pH 7.

Determination of binding characteristics

Binding capacity and kinetics for different affinity adsorbents were determined by carrying out batch adsorption experiments. 1–6 wt% adsorbents was added to 250 ml flasks containing 50 ml thienamycin solution. Supernatant samples were withdrawn periodically for thienamycin assay.

Results and discussion

Time profile of thienamycin fermentation

The time profile of a thienamycin fermentation using *Streptomyces cattleya* NRRL-8057 is shown in Fig.1. Cell growth occurred within the first 24 h. Antibiotic production started in about 24 h and reached a peak around 72–96 h. Beyond this point a decrease in thienamycin concentration was observed primarily due to a dominant degradation rate of the antibiotic even though the pH of the fermentation was maintained around 6.5. This rapid degradation, so far, cannot be minimized through pH adjustment or other environmental manipulation.

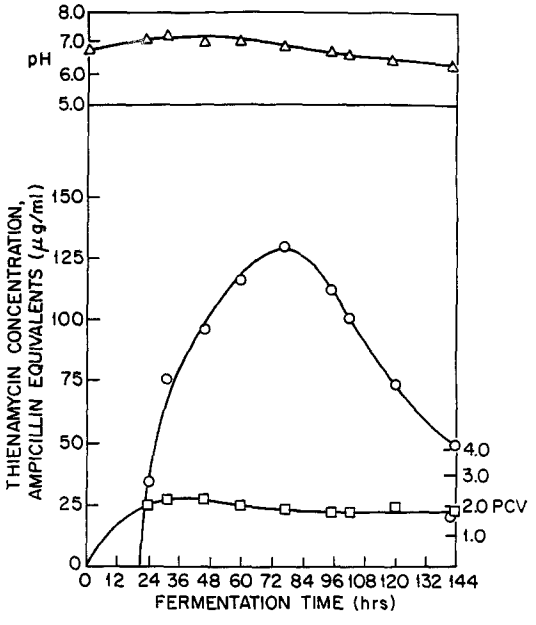

Fig. 1. Time profile of a thienamycin fermentation. △ pH; ○ Thienamycin; □ Packed Cell Volume

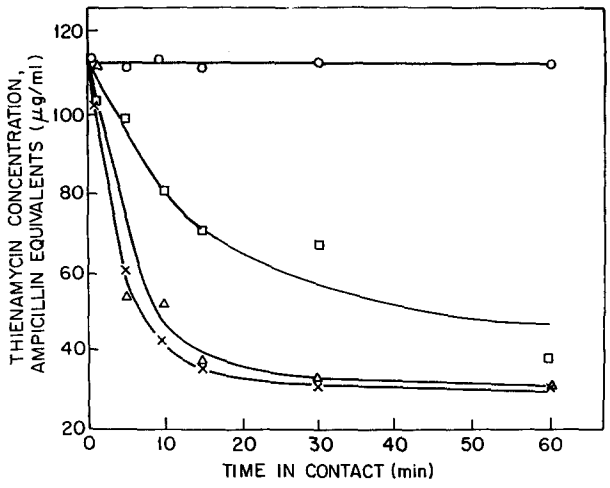

Fig 2. Adsorption of thienamycin by *Bacillus stearothermophilus*. ○ Control, □ 1.1 g dry cell weight/l of fermentation broth; △ 2.2 g dry cell weight/l of fermentation broth; × 3.3 g dry cell weight/l of fermentation broth

Binding capacity of various affinity adsorbents

The adsorption of thienamycin from fermentation broths onto thermophilic bacterial cells and immobilized β-lactamase is shown in Figs. 2 and 3. It is clear that thienamycin can be adsorbed from the fermentation broth fairly rapidly (1–2 h) using these adsorbents. The adsorption was determined

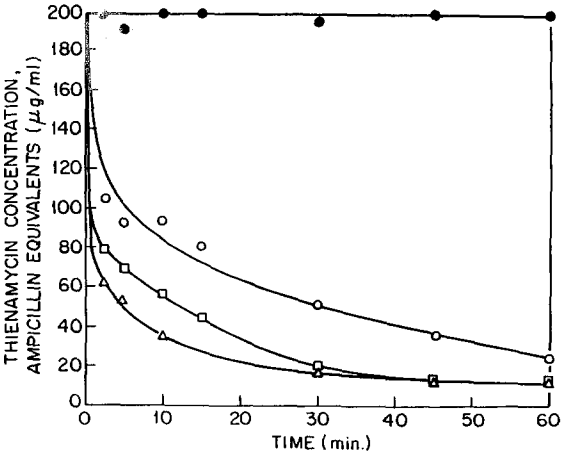

Fig. 3. Adsorption of thienamycin by immobilized β-lactamase. ● Control; ○ 0.062 g Sepharose with β-lactamase; □ 0.142 g Sepharose with β-lactamase; △ 0.250 g Sepharose with β-lactamase

Table 1. Loading capacities of various affinity adsorbents

Type of adsorbent	Equilibrium concentration[a](C_{eq}) μg/ml	Loading capacity mg antibiotic/g adsorbent
XAD-2 Resin** (Rohm and Haas Co., PA)	40.0–100.0	01–0.8
Immobilized β-lactamase	10.0–50.0	2.0–8.0
Bacillus stearothermophilus Cells	40.0–90.0	40.0–95.0

[a] Ampicillin Equivalent
[b] non-ionic polymeric adsorbent

by measuring the decrease in bioactivity in broths under test, using the disc-diffusion assay. It appears that bacterial cells are more effective as adsorption agents compared to immobilized β-lactamase or non-specific, non-ionic polymeric adsorbents such as XAD-2 resin (Rohm and Haas, Philadelphia, PA). As shown in Table 1, the bacterial cells have a loading capacity 100 times higher than the resin on a weight basis. Immobilized β-lactamase beads are also more effective than polymeric resins (Table 1). The binding capacity of *Bacillus stearothermophilus* cells translates to about 5–10 μg of thienamycin per gram of dry cells.

On-line extraction of thienamycin fermentation

In this experiments, a sterile preparation of late growth phase *Bacillus stearothermophilus* cells was added to a thienamycin fermentation during its peak synthesis at approximately 84 h (Fig. 4). The final quantity of *Bacillus stearothermophilus* in the fermentation broth was 3.6 grams (dry cell weight)/liter. An immediate drop in thienamycin concentration was observed. The antibiotic synthesis continued to reach a maximum higher than the control run. This result indicates that by specifically removing thienamycin from the fermentation broth it is possible to sustain antibiotic production for a longer duration by alleviating the feedback regulation. Figure 4 also indicates that at the end of 144 h almost twice as much antibiotic was produced in the on-line extracted fermentation compared to that in the control fermentations.

A similar experiment was done using immobilized β-lactamase beads (Fig. 5). Beads were added at a concentration of 5.0 g/l to the fermentation during peak synthesis. An immediate drop in

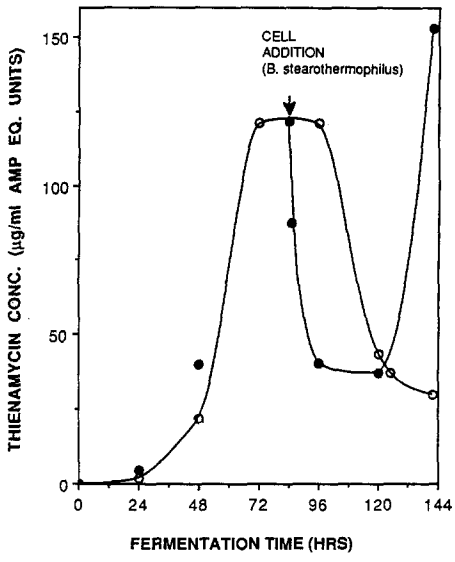

Fig. 4. In situ addition of *Bacillus stearothermophilus* into a thienamycin fermentation. ○ Control; ● With cell addition

thienamycin concentration indicating specific adsorption was observed in this case also. Antibiotic synthesis continued for an extended period of time due to the alleviation of feedback regulation. In this experiment also, the total amount of antibiotic produced using in situ extraction was higher than the control run.

Our preliminary results for thienamycin fermentation indicate that whole cells containing affinity adsorbents can be used for in situ product

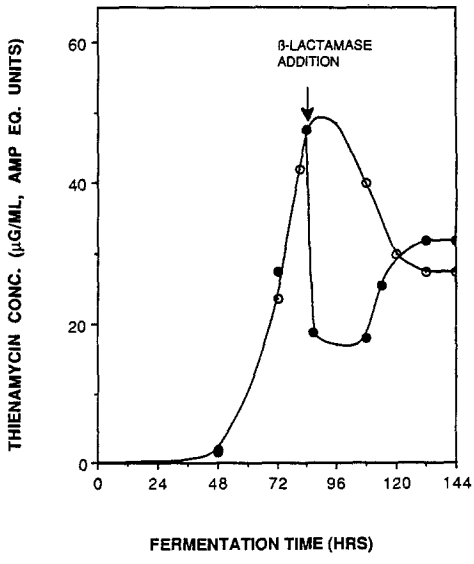

Fig. 5. In situ addition of immobilized β-lactamase into a thienamycin fermentation. ○ Control; ● With immobilized β-lactamase addition

removal. Both *Bacillus stearothermophilus* cells and immobilized β-lactamase, were effective in removing thienamycin from the fermentation broth. However, *Bacillus stearothermophilus* cells have a significantly higher binding capacity on weight of adsorbent basis. This may be the reason for greater enhancement of antibiotic production observed in the fermentations with in situ extraction using *Bacillus stearothermophilus* cells. Complementary research involving protein engineering of PBP and β-lactamases can provide more effective affinity ligands for specific in situ extraction of thienamycin and other β-lactam antibiotics in active form.

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