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Enhanced production of heterologous macrolide aglycones by fed-batch cultivation of *Streptomyces coelicolor*

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A media development program for the enhanced production of macrolide aglycones by *Streptomyces coelicolor* is described. Shake flask studies utilizing a yeast extract and a bakers' yeast increased production by 200% and 80%, respectively. However, ammonia generation and high pH were identified as potential problems in these enriched media. Studies in pH-controlled fermentors revealed that production stage pH significantly affects macrolide titers, with low pH (5.5) being more productive than high pH (6.5). Implementation of glucose feeding in shake flask cultures reduced ammonia generation and controlled production stage pH, resulting in significantly enhanced productivities. The combined effects of media supplementation and glucose feeding resulted in a three to five-fold overall improvement in total macrolide aglycone titers, and is the first reported high-level (>1 g/l) production of recombinant polyketides in a heterologous host.
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Introduction

Polyketides are a broad class of naturally occurring compounds with wide-ranging biological activities. These compounds have generated much interest because of their properties as antibiotics, anticancer agents, and immunosuppressants, and have been a rich source of pharmaceuticals. Current interest in polyketides has emphasized the development of molecules with either enhanced or novel activities. A route to such novel polyketides is the modification of existing molecules by genetic or chemical means. The development of clarithromycin by chemical modification of the polyketide erythromycin has demonstrated the feasibility of this rationale [16].

Erythromycin, a naturally occurring polyketide synthesized by the actinomycete *Saccharopolyspora erythraea*, is commonly used for its antibiotic activity and also has been known to affect both gastric motility [2] and cytokine production [13]. Erythromycin is synthesized in a multistep process *via* the macrolactone intermediate, 6-deoxyerythronolide B (6-dEB), followed by several tailoring steps that include the addition of deoxysugar moieties [12]. 6-dEB synthase (DEBS), the polyketide synthase responsible for 6-dEB production, has been studied extensively and has been successfully expressed in *Streptomyces coelicolor*, resulting in the formation of the macrolide aglycones, 6-dEB and 8-8a-deoxyoleandolide [9].

The production of 6-dEB analogs through manipulation of the polyketide synthase genes in *S. coelicolor* can serve as a useful step in the development of novel macrolides. The resulting molecules can be converted into the respective erythromycin analogs [5,8] and examined for enhanced or novel biological activity.

This work describes media development for the improved production of 6-dEB and 8-8a-deoxyoleandolide by *S. coelicolor*. Useful medium constituents were identified in shake flask studies. The importance of pH on production was elucidated in media and pH-controlled fermentation studies. Fed-batch shake flask cultures were designed to provide additional carbon while controlling pH, thereby improving productivity. Our results suggest a broader utility for the use of fed-batch shake flask cultivations in any media development program.

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Materials and methods

Strains and media

S. coelicolor CH999/p11–26* (1126*) was used throughout this work. Strain 1126* is an isolate of *S. coelicolor* CH999/p11–26. Plasmid p11–26 is a derivative of pCK7 carrying the *eryA*I, *eryA*II, and *eryA*III genes encoding DEBS [9]. The *eryA*III gene on plasmid p11–26 contains new restriction sites in the module 6 domain as engineered by McDaniel *et al* [11].

Cultures were grown at 30°C and 150–250 rpm in FKA basal medium (45 g/l starch, 10 g/l corn steep liquor, 10 g/l dried debittered brewer's yeast, 1 g/l calcium carbonate, and 23.8 g/l HEPES-free acid), unless otherwise noted. Yeast extract (Tastone 310; Universal Flavors, Indianapolis, IN) and bakers' yeast (Basic Yeast 60 MHF; Red Star Yeast and Products, Milwaukee, WI) were incorporated into media formulations as described in the studies detailed below. Shake flask medium pH was adjusted to pH 7.0 prior to sterilization by autoclaving (90 min at 121°C). Bioreactor fermentation medium was prepared without HEPES buffer and autoclaved for 90 min at 121°C. After sterilization and cooling, the medium was adjusted to pH 6.5. All media were supplemented with 50 mg/l thiostrepton (Calbiochem, La Jolla, CA) in (50 mg/ml) DMSO and 10 ml/l 50% (vol/vol) antifoam (Antifoam B; JT Baker, Phillipsburg, NJ) as poststerile additions. Strains were maintained as frozen cell banks prepared by adding glycerol (30% vol/vol final) to an exponentially growing culture (in FKA medium) and freezing 1 ml aliquots at −85°C.

Cultivation

Primary seed cultures were established by inoculating 50 ml of FKA with a cell bank vial and cultivating for 3 days. For shake flask studies, replicate flasks containing 35 ml of production medium were inoculated with 1.75 ml (5% vol/vol) of the primary seed culture. Flasks were incubated for 6–10 days with 1-ml samples withdrawn as necessary and stored at −20°C until analysis. Glucose feeding was performed by the addition of a sterile filtered 50% (wt/vol) glucose solution as one bolus each day to give the desired feed rate.

Bioreactor studies were performed in B. Braun MD 5-l fermentors with 3 l of production medium operated at 30°C, 0.3 VVM airflow, and 600 rpm agitation. Dissolved oxygen concentration and pH were monitored using autoclaveable electrodes (Mettler Toledo, Wilmington, MA). Under these operating conditions, dissolved oxygen was maintained above 50% at all times. Foaming was controlled by automatic addition of 50% (vol/vol) Antifoam B solution. The pH was controlled by automatic addition of 2.5 N sodium hydroxide or sulfuric acid. Bioreactors were inoculated with 5% (vol/vol) secondary seed culture prepared by subculturing 25 ml of primary seed into 500 ml of FKA and cultivation for 2 days. Samples were withdrawn as necessary and stored at −20°C for later analysis.

Analysis

Culture samples were centrifuged at 14,000×*g* for 10–15 min and the supernatant was transferred to a new tube. Quantitation of the macrolide aglycones, 6-dEB and 8-8a-deoxyoleandolide, was performed using a Hewlett-Packard 1090 HPLC equipped with an Alltech 500 evaporative light scattering detector as described previously [10]. Titers are reported as total macrolide aglycone concentration (R-dEB=6-dEB+8-8a-deoxyoleandolide). Glucose was measured using a Biochemistry 27 analyzer (Yellow Springs Instruments, Yellow Springs, OH). Ammonia concentration was determined by the use of an ammonia assay kit (Sigma-Aldrich, St. Louis, MO). The presence of insolubles (starch, yeast cells) precluded the measurement of biomass by typical means (optical density, dry cell weight, total protein, *etc.*) and is not reported for the experiments described below.

Results and discussion

Nitrogen source supplementation in shake flask cultivation

More than 50 complex and defined nitrogen sources were screened in an effort to identify the best medium components for production of the macrolide aglycones. The average impact of nitrogen source supplementation was −13%, suggesting that most of the components tested were detrimental to macrolide production. While various yeast sources and extracts were tested in the screen, the two best components were found to be Tastone 310 (a yeast extract) and Basic Yeast 60 MHF (a bakers' yeast product). Figure 1 depicts the product titers, pH, and ammonia profiles from a series of flasks with Tastone 310 supplementation. Tastone 310 supplementation at 2.5, 5.0, and 10 g/l resulted in 200%, 125%, and 0% higher R-dEB titers compared to the basal medium. Tastone 310 supplementation can enhance productivity but has deleterious effects that reduce titers at higher concentrations. In addition to productivity, Tastone 310 supplementation also affected pH

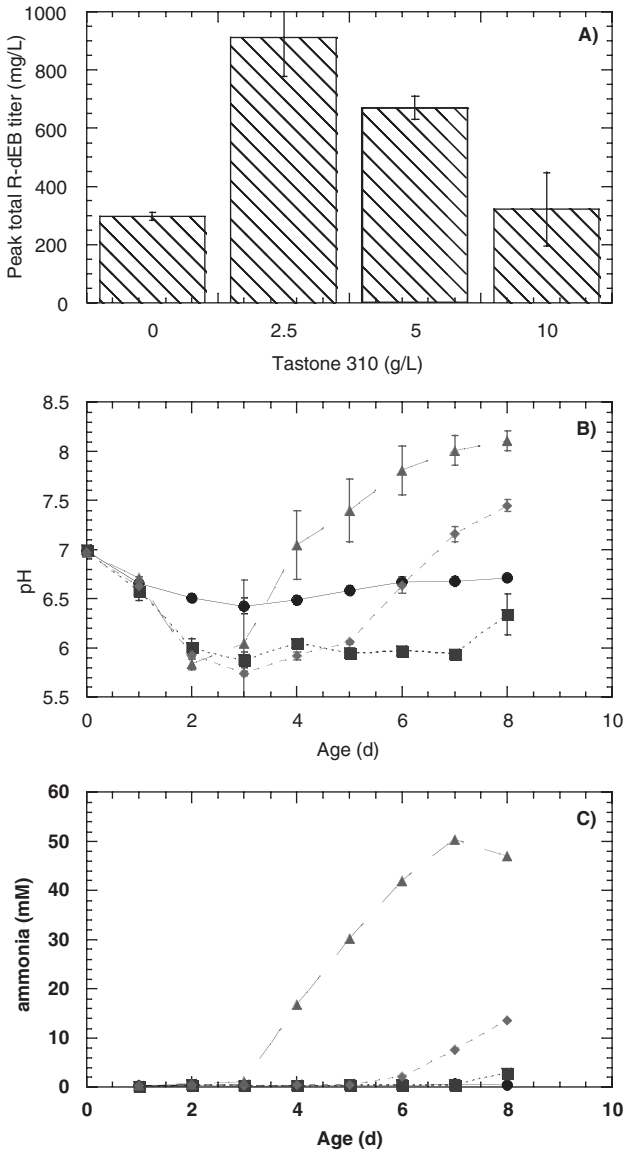

Figure 1 Impact of Tastone 310 supplementation in FKA basal medium on (A) peak total macrolide aglycone production, (B) pH profiles, and (C) ammonia generation. Media supplements: (●) FKA basal medium, (■) 2.5 g/l Tastone 310, (◆) 5.0 g/l Tastone 310, (▲) 10 g/l Tastone 310.

profiles. Initially during growth, the culture reached lower pH values in Tastone 310-supplemented media compared to the basal medium. After the initial drop, culture pH rose in all cases with a more rapid and earlier rise associated with increasing Tastone 310 levels. The rise in pH coincided with ammonia generation and reduced culture productivity. These data are consistent with the notion that cells are utilizing peptides and amino acids as carbon (and nitrogen) sources in a nitrogen-enriched environment with a side effect of ammonia generation. While nitrogen repression of secondary metabolite production is a known phenomenon [3], it is not clear whether the increased ammonia and/or higher pH caused reduced production of R-dEB.

Figure 2 depicts similar results from a direct replacement of the debittered brewers' yeast from the basal medium with Basic Yeast 60 MHF. As in the case of Tastone 310 supplementation, the Basic Yeast 60 MHF medium exhibited ~80% higher final titers, a lower

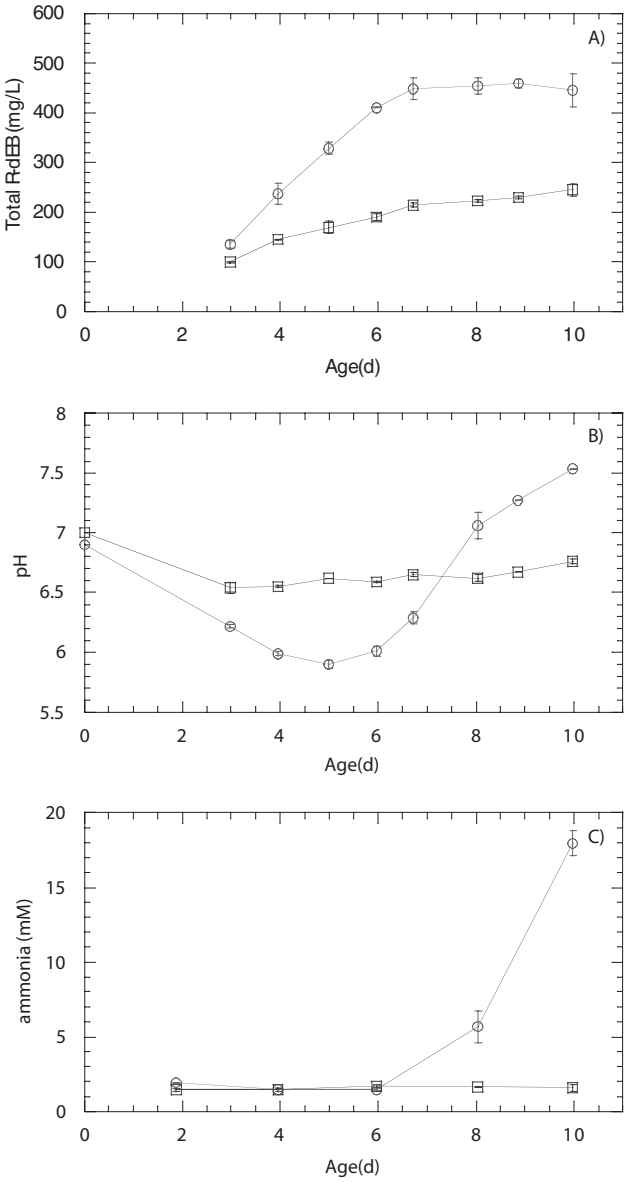

Figure 2 Impact of replacement of debittered brewers' yeast with Basic Yeast 60 MHF on (A) total macrolide aglycone production profiles, (B) pH profiles, and (C) ammonia generation. Media: (□) FKA basal medium and (○) Basic Yeast 60 MHF medium.

initial pH drop followed by a rapid pH rise, and increased ammonia generation. Again, increased ammonia generation coincides with the rapid rise in culture pH. Although the media studies described above indicate some problems in maintaining culture pH and ammonia concentrations, Tastone 310 and Basic Yeast 60 MHF were useful medium constituents for enhancing macrolide aglycone production.

Fermentation under controlled conditions

Fermentations under controlled conditions were also performed to investigate the effects of Tastone 310 supplementation and pH. The fermentations were controlled at pH 6.5 for 40 h after inoculation to maintain a constant pH during the growth stage. Subsequently, the pH was decreased to the production stage pH at a rate of 1 pH unit per 24 h. With this fermentation protocol, growth would be supported under identical conditions, while macrolide aglycone production would be investigated under the desired pH conditions, thereby isolating the effects of pH on production from those of cell density. Figure 3 depicts the impact of 2 g/l Tastone 310 supplementation and production stage pH. In the fermentations controlled at pH 6.5, Tastone 310 supplementation resulted in ~95% increase in final titers relative to the basal medium. However, pH also dramatically affected production. A shift in the production stage pH from 6.5 to 5.5 resulted in a further 95% increase in final titers in the Tastone 310-supplemented fermentations. The overall ~380% increase in final titers correlates well to the titer enhancement observed in shake flasks studies of Tastone 310 supplementation where culture pH remained low (i.e., 2.5 g/l Tastone 310). In these Tastone 310-supplemented fermentations, while ammonia levels varied somewhat, the amount produced was not significant compared to the amounts generated under shake flask cultivations described above.

Enhanced production by glucose feeding

The detrimental pH rise coincided with ammonia generation in the batch shake flask experiments. In the cultivation of *S. hygroscopicus*, Roth *et al* [15] and Grafe *et al* [6] observed that cells growing on maltose were acidogenic, whereas cells growing on amino acids were alkalinogenic. Furthermore, cells growing in the presence of both maltose and amino acids were alkalinogenic because amino acids were preferentially utilized, resulting in the generation of ammonia *via* deamination reactions. Hence, the presence of a preferred carbon source such as glucose may reduce ammonia generation and the concomitant pH rise by minimizing the

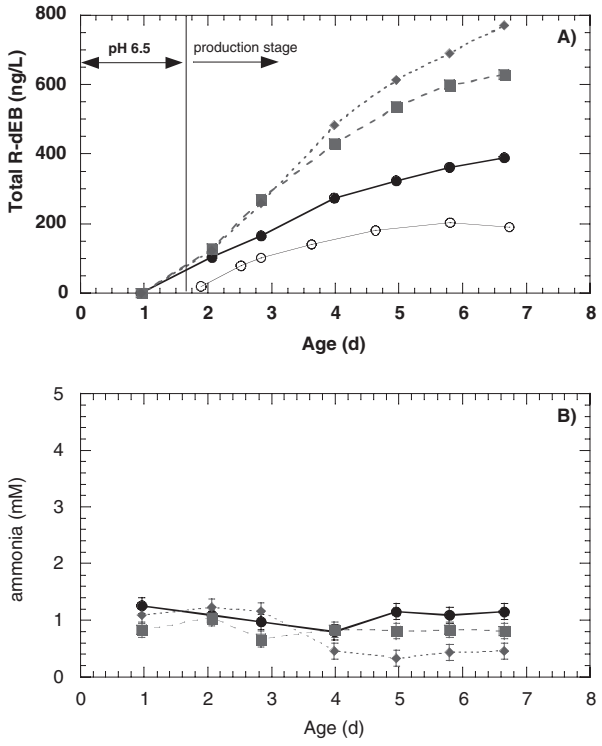

Figure 3 (A) Total macrolide aglycone production and (B) ammonia in pH controlled fermentations. Production stage pH in fermentations (in FKA+2 g/l Tastone 310 unless otherwise noted): (○) pH 6.5 in FKA basal medium, (●) pH 6.5, (■) pH 6.0, and (◆) pH 5.5.

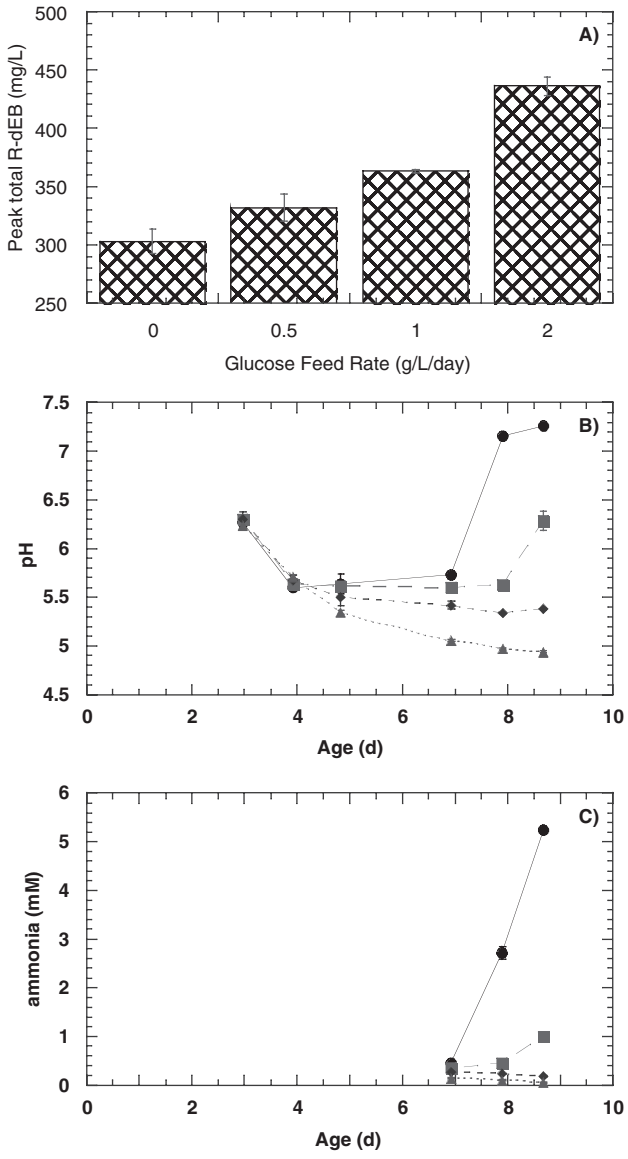

Figure 4 Glucose feed rate effects on (A) peak total macrolide aglycone titers, (B) pH profiles, and (C) ammonia generation in FKA basal medium. Glucose feed rates: (●) 0 g/l/day, (■) 0.5 g/l/day, (◆) 1.0 g/l/day, and (▲) 2.0 g/l/day.

use of amino acids as a carbon source. However, since glucose repression of secondary metabolite production [1,14] is a well-known phenomenon, glucose concentrations should be maintained at a level high enough to sustain optimal production, but low enough to ensure that it does, or its catabolites do not, repress R-dEB synthesis. A daily feed protocol was selected to maintain glucose in the culture at low levels. Figure 4 shows the effect of varying glucose feed rates on cultures in unbuffered FKA basal medium. An unbuffered medium was chosen because it is more responsive to pH changes by ammonia generation. The daily residual glucose concentration (measured prior to daily feeding) was less than 0.1 g/l, indicating complete consumption. As seen in Figure 4, the total R-dEB titers, final culture pH, and ammonia generation were dependent on the glucose feed rate. This dose-dependent behavior for glucose concentrations up to 2 g/l is consistent with the reported glucose transport K_m of 6.1 mM (or 1.1 g/l) in *S. coelicolor* A3(2) [4,7]. At concentrations higher than 2 g/l, glucose utilization is likely to be limited by its transport into the cell. A dose response to glucose feed rates much higher than 2 g/l/day would not be expected and was verified in later experiments (data not shown). These results suggest that glucose feeding at 2 g/l/day serves to prevent ammonia generation and the associated pH rise in FKA basal medium. Furthermore, glucose feeding appears to result in an acidogenic culture that leads to a lower pH during the production stage, which consequently results in an enhanced final titer.

Glucose feeding was also tested in the more enriched media containing either Tastone 310 or Basic Yeast 60 MHF. Figure 5

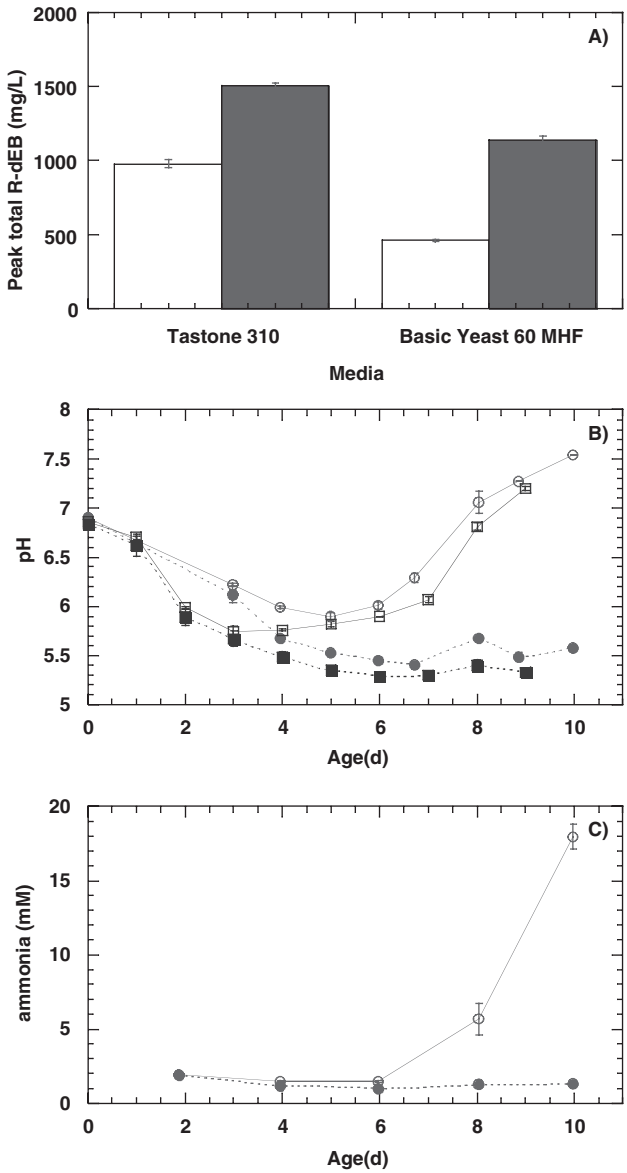

Figure 5 Effect of glucose feeding in shake flask cultures on (A) peak total macrolide aglycone production, (B) pH profiles, and (C) ammonia generation. (Open symbols/bars represent cultures without feeding and filled symbols/bars represent cultures with feeding.) Enriched media containing Basic Yeast 60 MHF: (○) no feed or (●) 2 g/l/day glucose feed; or FKA with 2.5 g/l Tastone 310: (□) no feed or (■) 2 g/l/day glucose feed.

depicts the impact of (2 g/l/day) glucose feeding on total R-dEB production and pH in these enriched media. Again, the daily residual glucose concentration was below 0.1 g/l, indicating complete consumption. In the Tastone 310 and Basic Yeast 60 MHF media, glucose feeding resulted in ~50% and ~145% higher production, respectively, compared to similar cultures without glucose feeding. The enhanced production also correlated with lower pH in glucose-fed cultures compared to batch cultures in which the pH rose rapidly. A final corroboration of the glucose feed effect is indicated in Figure 5C. Ammonia generation was significantly reduced in the Basic Yeast 60 MHF medium with glucose feeding compared to the batch culture without feeding. These experiments clearly show the utility of glucose feeding in improving productivity and controlling pH.

Conclusions

A screen of more than 50 nitrogen sources led to identification of a yeast extract and a whole yeast source for optimum macrolide aglycone production. Incorporation of Tastone 310 and Basic Yeast 60 MHF resulted in a significant improvement in final titers. However, increased ammonia generation and culture pH were also associated with the enriched media. Culture pH plays a major role in macrolide aglycone production. While low pH was ideal for enhanced production, it is not optimal for growth. Simple incorporation of a buffering agent did not resolve the problem. However, glucose feeding in shake flasks reduced ammonia generation, controlled culture pH, and significantly enhanced productivity. The experiments described here represent the first report of production of recombinant polyketides in a heterologous host at levels exceeding 1 g/l. Such high-level production by *S. coelicolor* suggests the viability of producing novel polyketides using recombinant DNA technology [8,10].

More generally, fed-batch cultivation was useful in achieving stable conditions in shake flask cultures. Bioreactor-controlled fermentations, which typically maintain such conditions, are not feasible for screening large numbers of components. Fed-batch cultivation in shake flasks represents a cost-effective tool useful for media development in any process development program.

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