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Synthesis of hydrolytic enzymes during production of tylosin by *Streptomyces fradiae*

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SUMMARY

The exposure of a wild-type tylosin producing strain of *Streptomyces fradiae* to mutagenic agents resulted in the isolation of several tylosin over-producing strains. Examination of three mutants, T4310, 612 and 3204 showed that improved tylosin production was associated with increased hydrolytic enzyme activity and cell growth. The wild-type strain showed lower levels of hydrolytic activity including, protease, amylase, lipase and esterase activities and attained a lower cell density than the mutants.

INTRODUCTION

The antibiotic tylosin is employed extensively in veterinary medicine for treatment of infections caused by Gram-positive bacteria and mycoplasma [1]. The commercial importance of tylosin has spurred on scientific investigations with a view to improve tylosin yield during fermentation. Tylosin is a macrolide antibiotic composed of a 16-member lactone (tylactone) and three deoxy-sugar moieties [10]. It is produced by fermentation of *Streptomyces fradiae* and may be converted to less biologically active analogs such as relomycin. The pathway for terminal biosynthesis of tylosin has been investigated and a number of enzymes and genes identified [1,7,10]. Tylactone itself appears to be derived from fatty acid and amino acid, i.e., catabolic products of oils and proteins, respectively, while the biosynthesis of tylosin sugars involves carbohydrate metabolism. Limited information is available concerning enzymatic activities of the early stages in the biosynthesis of tylactone or of the tylosin sugars. Optimal tylosin production appears to require a complex medium containing starch, insoluble protein, lipid and mineral salts [10].

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This report studies the fermentation kinetics of hydrolytic enzymes involved in the utilization of the insoluble, complex nutrients for growth and production of tylosin by *S. fradiae*. The amylase, protease, lipase and esterase activities were compared with the levels of production of tylosin.

MATERIALS AND METHODS

Microorganism and culture. S. fradiae CCRC 11172 (ATCC 19609) was obtained from the Culture Collection and Research Center in the Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China. The vegetative inoculum was prepared in medium containing ($g\,l^{-1}$) glucose 5, starch 20, proflo 15, corn steep liquor 30, soybean oil 12, salts and minerals, and incubated for 3 days at 28 °C. One ml of seed culture was added to 25 ml of fermentation medium in 250 ml Erlenmeyer flasks containing ($g\,l^{-1}$) fish meal 30, starch 55, Torula yeast 9, corn steep liquor 3, soybean oil 40, salts and minerals. Cultures were incubated at 28 °C on a rotary shaker (70 mm throw diameter, 175 rpm) for up to 10 days.

Mutants were obtained after successive exposure to UV irradiation and to the mutagen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG). Isolated colonies were extensively screened for tylosin by bioassay and then by high performance liquid chromatography (HPLC) after flask fermentations. Constant care to culture maintenance was observed for culture purity and stability. In this study three

tylosin over-producing mutants were examined: T4310, 612 and 3204. Isolates 612 and 3204 were mutants derived from T4310. Approximate mutation frequencies for these mutants were around 1×10^{-6} or lower.

Starch hydrolysate. Starch hydrolysates were prepared by the addition of 60 mg of α-amylase Type XI-A, *Bacillus* sp. (Sigma) to 30 g of potato starch in 200 ml H_2O. The slurry was stirred gently for 4 h at ambient temperature. The α-amylase activity was subsequently inactivated during autoclaving of the fermentation media. The preparation was examined qualitatively and quantitatively respectively, by TLC and by a spectrophotometric assay for maltose, before and after treatment. TLC plates (silica gel 60, Merck) were run as previously described [20] and the maltose concentration was determined as previously described [3].

Cell growth, water content and fractionation. Growth curves were established using a dilution plate count assay using an aliquot of culture broth homogenized for at least 30 s with glass beads [22]. Results are expressed as the Log_{10} colony forming units (cfu) ml^{-1}.

The dry weight of the fermentation broth was estimated by weight determination before and after drying, to constant weight, of a homogeneous aliquot of whole culture broth.

Intracellular fractions were prepared from centrifuged (3000 rpm, 10 min) pellets of the whole culture broth. Pellets were homogenized (Waring blendor) in 20 ml dH_2O and passaged through a microfluidizer (Microfluids Corporation, Massachusetts). Cell lysis was verified by microscopic examination (Nikon, Optiphot). Aliquots of lysed samples were centrifuged (10000 rpm, 10 min) and the supernatants assayed for enzyme activities.

Amylase activity. Total amylase activity was detected as previously described by Bernfeld [3] employing soluble starch (5 $g\,l^{-1}$) (Sigma) in 0.1 M sodium phosphate buffer, pH 6.7, containing 20 mM NaCl and 0.5 ml of enzyme containing solution (10000 rpm, 5 min, culture supernatants). Tubes were incubated at 28 °C for 0, 5, 10, 15, 20, 30 and 40 min and the reaction stopped with 0.5 ml of 2 M NaOH. The activity is expressed as units ml^{-1} and defined as the amount (mg) of reducing sugars liberated $min^{-1}\,ml^{-1}$ of culture supernatant. Maltose was used as standard. The assay was tested with the α-amylase from Sigma.

Protease activity. Protease activity was detected using azocoll (Sigma) as substrate as previously described [19] and the procedure modified as follows. One ml of a 0.2% azocoll suspension in 0.1 M sodium phosphate buffer, pH 7.4, was added to 1.5-ml Eppendorf tubes. Enzyme containing solution, i.e., 50–100 μl of whole culture broth, were added to the tubes. To control tubes, 100 μl of 50% trichloroacetic acid (TCA) was added immediately. Tubes were incubated at 28 °C for 30 min with sufficient shaking to keep the azocoll in suspension. After incubation, 100 μl of 50% TCA was added to the test tubes. Tubes were vortexed and then centrifuged at 10000 rpm for 5 min. Without disturbing the pellets supernatants were removed and their absorbances read at 520 nm. One unit of activity was defined as the amount of enzyme which catalyzes the release of azo dye causing a $\Delta A/\Delta t = 0.001\ min^{-1}$ [19]. The reliability and reproducibility of the assay procedure was tested with proteinase K (Sigma).

Lipase activity. Lipase activity was detected titrametrically using an olive oil emulsion (Sigma) and the end point determined with thymolphthalein indicator (Sigma). Activity was expressed as mmol triglyceride hydrolysed $h^{-1}\,ml^{-1}$ at pH 8 and at 23 °C. Plate assays for lipase activity were performed on Bacto peptone agar containing 0.1 $g\,l^{-1}$ $CaCl_2 \cdot H_2O$ and 1% Tween-80 [12]. Positive cultures showed a zone of precipitate around colonies. Non-specific esterase activity was determined using *p*-nitrophenyl acetate in 0.1 M sodium phosphate buffer, pH 7.4, as described previously [6]. Activity was expressed as the amount of enzyme which hydrolyses 1 μmol of *p*-nitrophenyl acetate per min at 25 °C and pH 7.4. Soluble protein was determined by the Bradford method [5] and polysaccharide by the orcinol method [20].

Tylosin assay. Tylosin levels in fermentation cultures were monitored by HPLC using a method modified from literature [4]. Tylosin tartrate (Sigma, purity 88.3%) was used as assay standard. A Waters QA1 liquid chromatography system equipped with a Waters model 745 Integrator, or a Beckman liquid chromatography system equipped with a model 163 variable wavelength detector were employed.

RESULTS AND DISCUSSION

The wild-type strain of *S. fradiae* produced almost undetectable levels of tylosin during fermentation. Exposure of this strain to UV irradiation and to the potent mutagen NTG followed by massive screening for tylosin lead to the isolation of several mutants which produced levels of tylosin higher than previously reported [10] for a 10-day fermentation. The profile of tylosin production was examined for three of these mutants (Fig. 1). Strain 3204 consistently showed the highest level of tylosin production. The induction of mutations and selection for improved antibiotic producing isolates have long been shown to be a useful approach to improvement in antibiotic producing strains [16]. After examination of the medium composition and the level of tylosin production, the complex medium described in this study was found to give optimal levels of tylosin (data not shown).

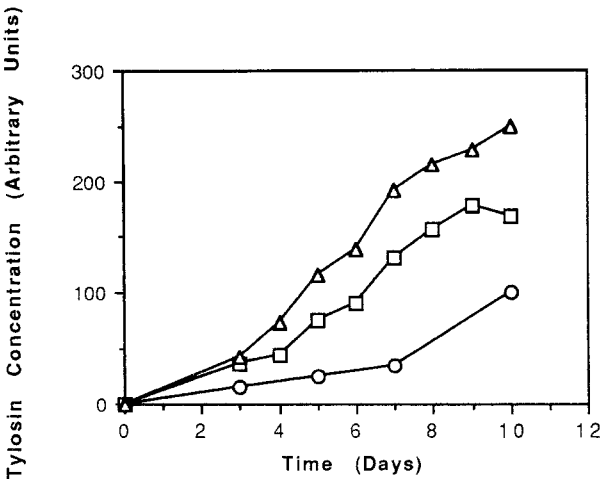

Fig. 1. Tylosin levels produced by mutants of *S. fradiae*: T4310 (○), 612 (□), and 3204 (△). The tylosin concentration at 10 days for T4310 was taken as 100 arbitrary units.

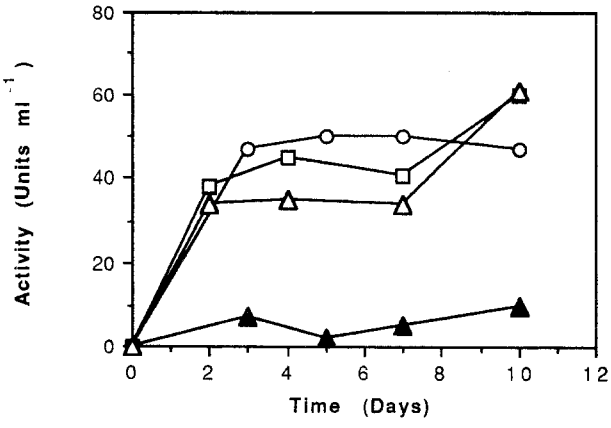

Fig. 2. Extracellular protease activity of *S. fradiae* wild-type (▲) and mutant strains T4310 (○), 612 (□) and 3204 (△) during tylosin fermentation.

The proteolytic activity produced by these mutants increased rapidly during the first 2 days in fermentation medium (Fig. 2). The activity remained high for at least 10 days. In comparison, the wild-type strain showed a 10-fold lower level of proteolytic activity (Fig. 2). The rapid rise in proteolytic activity corresponded to a drop in the amount of soluble protein in the broth from approximately 2.0 to 0.5 g l^{-1} in 3 days. This was followed by a subsequent increase in soluble protein for the mutant strains but not for the wild-type (data not shown). This may represent partial hydrolysis of the insoluble, complex protein substrates in the fermentation medium after 3 days. The presence of hydrolytic activity was also reflected in a rapid decrease in the viscosity of the broth which probably resulted from the solubilization of solid nutrients such as fish

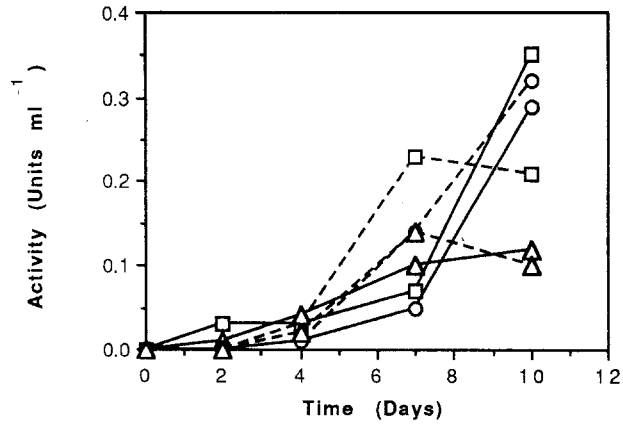

Fig. 3. Amylase activity of *S. fradiae* mutants T4310 (○), 612 (□), and 3204 (△) produced in fermentation medium containing potato starch (——) or hydrolysed potato starch (– – –).

meal. The percent dry weight of the culture broth decreased from approximately 20% to 10% in 3 days, after which it decreased gradually to 5% on day 10. Because of the high concentration of insoluble non-biomass solids, estimation of biomass in the fermentation medium by optical density or packed cell volume or dry weight measurement was not possible. Thus enzyme activities were expressed as units per ml of culture broth rather than per g of cell mass.

Amylase activity was also detected in the fermentation broth and was at least 10-fold lower for the wild-type (0.03 U/ml at day 5) compared to the mutant strains when grown in starch-containing medium. The activity showed an initial lag period (Fig. 3) which varied between 3 and 6 days for different batches of cultures. However, the levels of soluble sugar were found to increase initially and decrease after 1–4 days and to stabilize thereafter (data not shown). This decrease suggests that only a limited amount of starch sugar was available during early fermentation and that other sources of carbohydrate present in Torula yeast and corn steep liquor [13] might also be important initially. The hydrolysis of starch involves several enzymes including α-amylases (1,4-a-D-glucan-4-glucanohydrolase, EC 3.2.1.1) which cleaves α (1–4) linkages of glucose residues with the release of maltose [11]. Other enzymes which release glucose such as amyloglucosidase may also be present. The assay for amylase activity would have detected any enzyme activities which brought about the release of reducing sugars. It is most likely that some of these glycolytic enzymes were involved in the hydrolysis of starch during fermentation.

Glucose (2% final) substituting starch in the fermentation medium inhibited the rate of tylosin synthesis (data not shown). Glucose has been shown to depress tylosin

production and to have a repressive effect on carboxylating enzymes involved in tylosin biosyntheses [18]. Since glucose was employed in the broth for vegetative cultures, the inoculum may have required a short period to overcome the repression which may also be enhanced by the small amount of residual glucose which was transferred with the inoculum to fermentation cultures.

The possibility that starch pre-treated with commercial α-amylase, thus partially hydrolysed, was able to facilitate growth and early tylosin production and to thereby increase overall tylosin production was examined. The hydrolysed starch preparation was first examined by TLC and showed spots of similar R_f values to maltose and maltotriose. Thus hydrolysis of starch and release of disaccharides had occurred. The amount of maltose equivalents present was estimated to be 1.3 g l^{-1} of preparation. Comparison of parallel cultures containing hydrolysed or non-hydrolysed starch showed that hydrolysed starch brought about a higher level of tylosin for isolates 612 and T4310, while the level of 3204 remained about the same (Table 1). The levels of amylase, protease and esterase activities did not appear to be very different between these two starch media. Thus one might speculate that the addition of maltose equivalents in the form of α-amylase-treated starch did not repress tylosin levels as did glucose, and did not repress endogenous amylase activity. It might even soften the effect of the lag in amylase activity, i.e., limited supply of readily utilizable carbohydrate in the first few days of fermentation. In addition, α-amylase has been shown to be repressed by glucose in *Bacillus subtilis* [14] and to be induced by maltotriose in *Escherichia coli* [15]. Glucose repression of the α-amylase gene of certain *Streptomyces* spp. has also been reported [17]. Considerable effort is being made to clone the α-amylase genes from Streptomycetes [8,17] which should bring further insight into amylase regulation in Streptomycetes.

Screening by a plate assay for lipase activity showed positive for all tylosin-producing mutants. The level of lipase was many-fold lower for the wild-type (Table 2). The amount of non-specific esterase activity was slightly lower (Fig. 4).

The hydrolytic enzyme activities of the mutant T4310 were predominantly located in the extracellular fraction. Calculation of average percentages extracellular activity, over the entire fermentation, showed that 74%, 78% and 91% of the total protease, amylase and esterase activities, respectively, were extracellular. The wild-type showed a higher proportion of intracellular activity with averages of, respectively, 48%, 27% and 87% of the total protease, amylase and esterase activity being extracellular.

Examination of the filamentous biomass showed a considerable difference in the amount of growth of the *S. fradiae* wild-type and mutants. Comparison of the growth profiles showed that the wild-type attained considerably lower biomass levels (Fig. 5) after similar initial rates of growth. Although the initial growth was similar, the initial enzyme activities were lower for the wild-type. The growth profiles of these mutants were similar to those described

TABLE 1

Tylosin production by *S. fradiae* mutants after 10 days in media containing starch or hydrolysed starch

Strain	Tylosin plus Relomycin (Arbitrary units[a])	
	Starch	Hydrolysed starch
T4310	100	190
612	140	200
3204	320	300

[a] Relative to T4310, taken as 100.

TABLE 2

Production of tylosin and maximum lipase activity by wild-type and mutant strains of *S. fradiae*

Strain	Tylosin concentration (Arbitrary units[a])	Lipase activity (U ml^{-1})
Wild-type	0 (10)[b]	7 (5)
T4310	100 (10)	75 (5)
612	220 (10)	50 (5)

[a] Relative to T4310, at 10 days, taken as 100.
[b] Fermentation day.

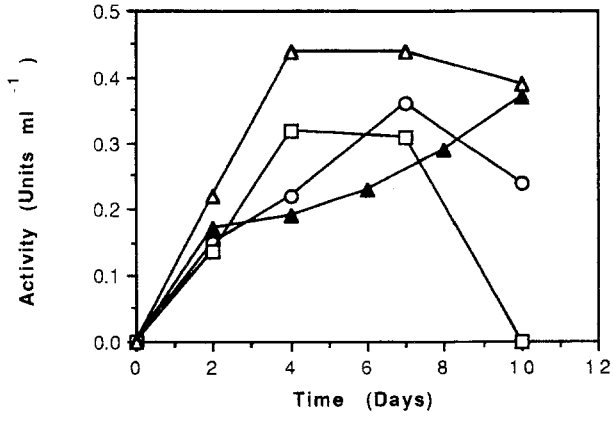

Fig. 4. Esterase activity of *S. fradiae* wild-type (▲) and mutants: T4310 (○), 612 (□) and 3204 (△).

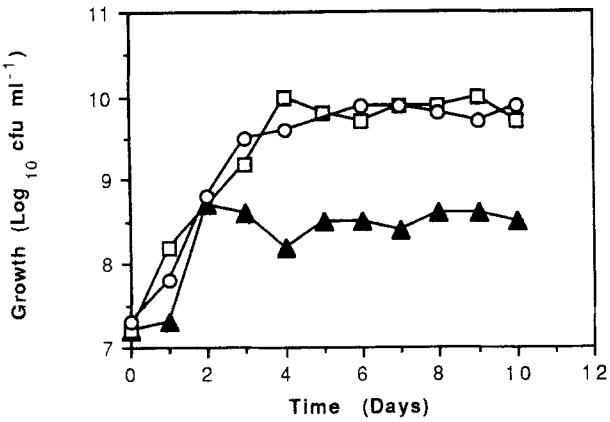

Fig. 5. Growth of *S. fradiae* wild-type (▲) and mutants T4310 (○) and 612 (□) during fermentation.

by Seno and Baltz [16]. Because of the high concentration of insoluble solids in the culture medium, a dilution plate assay was the method of choice for quantitation of biomass. However, in actinomycete fermentation, the cell size and frequency of cross-walls in hyphal strands can lead to underestimation. Despite this drawback, the results support the observations of Gray and Bhuwapathanapun [9] who suggested that tylosin production is growth related. We, in turn, suggest that growth is linked to the activities of key hydrolytic enzymes which supply the carbon building blocks and energy substrates for cell propagation.

In this study mutagens were used to generate higher tylosin producing derivatives of the wild-type strain. Comparison of key hydrolytic enzyme activities and the tylosin levels of the mutants does not indicate a direct correlation between these activities and the levels of tylosin. However, comparison of the wild-type tylosin producing strain with the tylosin over-producing mutants showed that increased tylosin production was associated with higher hydrolytic enzyme activities and cell growth. The wild-type strain showed low protease, amylase, lipase and esterase activities and attained a lower cell density.

Recent studies have concentrated on the cloning of tylosin biosynthetic genes [7] and tylosin resistance genes [21], and on the isolation and characterization of terminal tylosin synthetic enzymes [2]. Our results suggest that strain improvement may also be tied to the enhancement of the cellular catabolic activity. The activity of complex nutrient hydrolysing/solubilizing enzymes may limit the rate of tylosin production.Experimental measurements of these activities during fermentation may also hold important clues for fine tuning more advanced fed-batch protocols. Thus a more classical approach focusing on nutrient effects and the hydrolytic enzymes involved in carbon/energy nutrient supply may provide new insight into improvement of tylosin yield.

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