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Biochemical and physiological characterization of the efrotomycin fermentation

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SUMMARY

An efrotomycin fermentation was characterized through physical, chemical and biochemical studies. Growth of the actinomycete, *Nocardia lactamdurans* occurred during the first 50 h of the fermentation cycle at the expense of glucose, protein, and triglycerides. The initiation of efrotomycin biosynthesis was observed when glucose dropped to a low concentration. Upon glucose depletion, cell growth ceased and a switch in the respiratory quotient occurred. Efrotomycin biosynthesis was supported by the utilization of soybean oil and starch. Analysis of triglyceride metabolism showed that no diglycerides or monoglycerides accumulated during the fermentation. The activity of extracellular enzymes (lipase, protease, and amylase) increased during the cell growth phase and decreased significantly after 150 h. The concentrations of DNA, tetrahydro-vitamin K_2 (a membrane component), and free amino acids in the supernatant increased dramatically late in the fermentation cycle (225 h), indicating massive cell lysis. During this same time period, a reduction in cellular respiratory activity and efrotomycin biosynthesis were observed.

INTRODUCTION

Efrotomycin, a modified polyketide of the elfamycin class of antibiotics (Fig. 1) is a secondary metabolite produced by the actinomycete *Nocardia lactamdurans* [28]. This antibiotic is targeted for use as a swine productivity improver [7,10]. Efrotomycin is the disaccharide of aurodox and presents a similar labeling pattern by acetate, pyruvate, butyrate and the methyl groups of methionine [2,19]. The pyridone ring is formed by the condensation of β-alanine, derived from the reductive degradation of uracil, with an acetate moiety [8]. Recently, the peptide bond adjacent to the carbonyl residue was traced as originating from glycine [25]. The two sugar residues, probably originating from glucose units, bear O-methyl groups derived from methionine [24]. No intermediates in the biosynthesis of the backbone of efrotomycin have ever been isolated, and only the accumulation of aurodox and monosaccharide have been reported in certain classes of mutants [24].

Like other secondary metabolite fermentations, efrotomycin synthesis is highly influenced by the physicochemical environment [1,5,9,21,29]. When supported by glutamate or glycerol feeding in a synthetic medium, efrotomycin biosynthesis is sensitive to the feed rate and to the accumulation of ammonia and uracil [16,24]. In complex medium, efrotomycin synthesis was found to be sensitive to ingredients such as calcium [6] and to sterilization conditions [15].

Little information is available regarding the biochemical chain of events and interactions associated with efrotomycin synthesis in complex medium. An example is given here of a detailed physical, chemical and biochemical analysis of a 40 000-l fermentation. The data reported here suggest that the efrotomycin fermentation can be improved by nutrient feeding strategy.

MATERIALS AND METHODS

Chemicals for analyses

Deoxyribonucleic acid (DNA), protease, amylase, lipase, monoglycerides, diglycerides, and triglycerides were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were obtained from Fisher Scientific (Springfield, NJ).

Efrotomycin fermentation

The efrotomycin fermentation process consisted of a three-stage inoculum and a 40 000-l production stage

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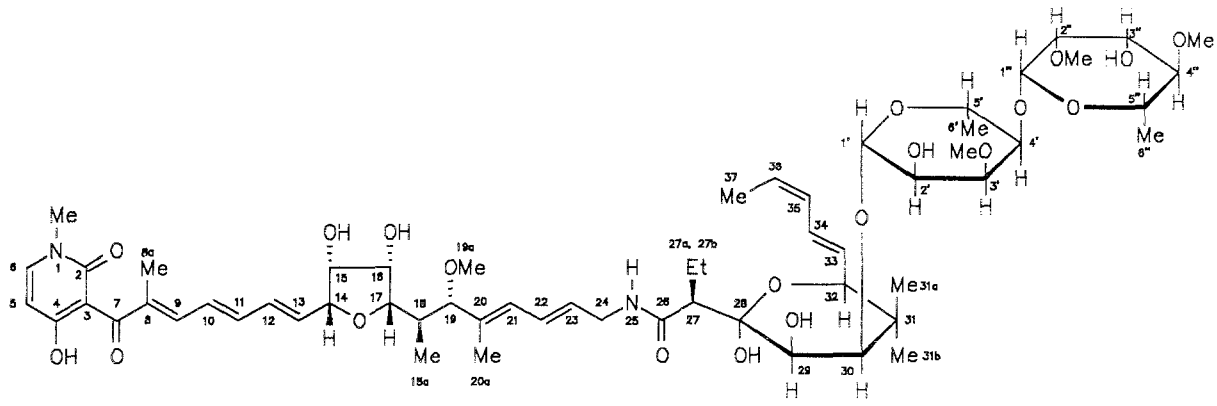

Fig. 1. Efrotomycin structure.

[6,15]. The major ingredients of the production medium were, glucose, soybean oil, and Pharmamedia (a cotton seed flour commonly used as a source of protein, starch, and trace-elements [23]). During the fermentation cycle, the temperature was maintained at 28 °C, and the dissolved oxygen tension was kept above 20% by controlled ramping of aeration and agitation, employing a Fisher-Provox control system (Fisher-Provox, Marshalltown, IO). The same unit was used to control pH and back pressure. The off-gas analyses were performed using a Perkin-Elmer mass spectrometer model MGA-1200 (Perkin-Elmer, Emeryville, CA) and a Hewlett Packard computer model HP 1000 as previously described [3,15]. Whole broth viscosity was assayed with a Brookfield viscometer (Brookfield Labs, Stoughton, MA) using a spindle No. 2 at 20 rpm.

Sample preparation

Samples collected during the fermentation cycle were immediately cooled in an ice bath and diluted in an osmoprotectant solution (per liter: NaCl 8.5 g, KH_2PO_4 0.3 g, Na_2HPO_4 0.6 g, and gelatin 0.1 g [11]).

Chemical and biochemical analyses

Cellular and extracellular DNA analyses were performed on samples diluted 1 : 5 followed by centrifugation at 16000 rpm in an Eppendorf microcentrifuge (Brinkman, Westbury, NY). The supernatants were collected, and the tubes containing the pellets were blotted dry. Supernatants (for extracellular DNA analysis) and pellets (for cellular DNA analysis) were stored at − 70 °C. The concentration of DNA was assayed by the diphenylamine reaction according to the method described by Burton [4].

Toward the end of the fermentation (250 h), as broth viscosity increased, pipeting became difficult and affected DNA measurements.

Enzymatic activity determinations: Fermentation samples for the assay of extracellular enzymatic activities (proteolytic, lipolytic and amylolytic activities) were diluted 1 : 2 followed by centrifugation for 20 min at 10000 rpm (12000 × g) in a Sorvall model RC 5B centrifuge (DuPont, Wilmington, DE). The supernatants were frozen at − 70 °C until processing. Proteolytic activity was assayed by the method described by Ginther [12] using azocasein as substrate. Lipolytic activity was assayed by monitoring the release of fatty acids from the hydrolysis of olive oil according to Tiez and Fiereck [27] using a kit manufactured by Sigma. Amylolytic activity was determined by a plate assay developed by Smibert and Krieg [26].

Substrate and metabolite assays: Samples for the analysis of sugars, glycerol, and organic acids were prepared as described in the previous section and the supernatants were frozen at − 70 °C until processing. Upon thawing, the samples were further diluted 1 : 10 in 0.1 N sulfuric acid. These samples were analyzed using a high performance liquid chromatographic system equipped with a SP 4270 integrator (Spectra physics, San Jose CA). The samples (20 µl) were chromatographed on an Aminex HPX87H column (Biorad, Richmond, CA) employing 0.0089 N sulfuric acid as the eluant (flow rate of 0.6 ml/min). Sugars and glycerol were detected with a refractive index detector (SP 8430, Spectraphysics), and organic acids were detected with an ultra-violet detector (SP 8490, Spectra physics) operated at 210 nm. Dextrins were detected on a similar system using an Aminex HPX-42 A column (Biorad) with water as solvent at a flow rate of 0.6 ml/min. For amino acid determinations,

a protease cocktail inhibitor (PMSF, pepstatin, and leupeptin) was added to supernatants obtained as described in the previous section. The supernatants were frozen at −70 °C until processing.

Upon thawing, the samples were dried in a SpeedVac concentrator (Savant Instruments, Farmingdale, NY), and resuspended in Beckman Na-S dilution buffer (Beckman). These samples (50 µl) were injected in a Beckman 6300 amino acid analyzer equipped with an integrator and a high performance column for sodium methodologies. Samples for the analyses of triglycerides were prepared as described in the previous section and the supernatants were frozen at −70 °C until processing. The triglycerides were hydrolyzed and the glycerol liberated was quantified by enzymatic analysis using a Boehringer enzymatic kit (Boehringer, Indianapolis, IN). Samples for the measurement of fatty acids were processed as described in the previous section and the supernatants were frozen at −70 °C. Upon thawing, the supernatant samples were assayed for fatty acid content by a procedure adapted from Lepage and Roy [17]. A Hewlett-Packard model 5890A capillary gas chromatograph equipped with an autoinjector, a capillary column (model DB-23, 0.32 mm × 30 m, J&W Associates), and a FID detector was employed to measure the fatty acid methyl esters. An internal standard of C17 : 0 was utilized for quantification. Tetrahydro-vitamin K_2 content was determined by HPLC using a DuPont Zorbax RX column and methanol/tetrahydrofuran (95 : 5) as solvent at a flow rate of 1.5 ml/min at 65 °C. Tetrahydro-vitamin K_2 was detected at 270 nm and had a retention time of 10.2 min. Samples for the detection of triglycerides, diglycerides, monoglycerides, and tetrahydro-vitamin K_2 were obtained as previously stated. Upon thawing, the samples were extracted with chloroform (1 vol/1 vol). Samples (3 µl) from the chloroform layer were spotted on thin layer chromatography plates (Silicagel GF, Analtech, Newark, DE) and separated at room temperature by a mixture of hexane (70 parts), diethyl ether (30 parts) and acetic acid (1 part). Detection was performed by spraying the plate with a 10% sulfuric acid solution, followed by charring with a heat gun. Efrotomycin was assayed by HPLC and ammonia detected as previously described [15]. All values presented are the average of two determinations.

RESULTS AND DISCUSSION

Biomass production

Measurement of cell growth by dry weight or optical density determinations was not possible in this fermentation because of the particulates present in the production medium. Monitoring cell growth by packed cell volume was also complicated for the same reasons. Furthermore, *N. lactamdurans* present typical growth characteristics of *Nocardia*, a filamentous stage followed by fragmentation [13], thus invalidating viable plate count. Therefore, growth of *N. lactamdurans* was studied by measuring cellular DNA content, and by monitoring respiratory activity through oxygen uptake rate and carbon dioxide evolution rate analyses (Figs. 2 and 3). The kinetics of cell growth measured by cellular DNA content were found to follow cellular respiration (Figs. 2 and 3). During active cell growth, DNA content of the pelleted samples doubled every 8 h. A peak oxygen consumption rate of 62 mmole/l/h was reached at 48 h and correlated to maximum biomass concentration. Based on a cellular DNA content of 3% for bacteria [20], an estimated maximum dry cell weight of 62.5 g/l was achieved in this fermentation. The close correlation between respiration activity and biomass formation validates mass-spectrometry analyses for routine determination of biomass formation kinetics in efrotomycin fermentations.

During cell growth, glucose was consumed at a maxi-

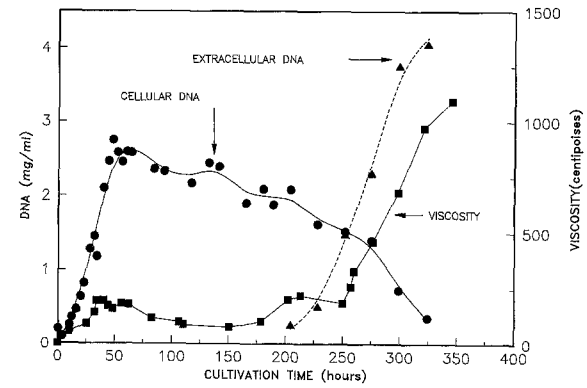

Fig. 2. Cellular and extracellular DNA content and viscosity evolution during efrotomycin fermentation.

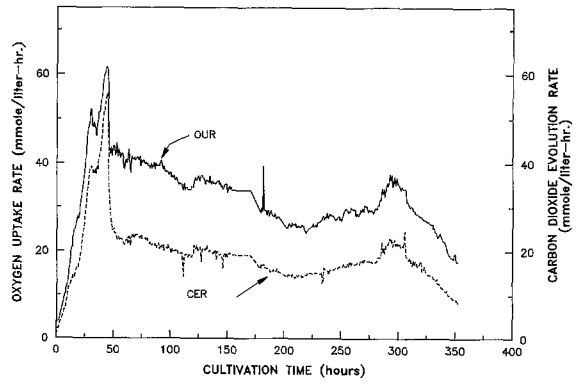

Fig. 3. Respiration activity during efrotomycin biosynthesis by *Nocardia lactamdurans*. Carbon dioxide evolution rate (CER) and oxygen uptake rate (OUR) were measured by mass-spectrometry.

mum rate of 1.75 g/l/h (Fig. 4). After depletion of ammonia (at 20 h), proteolytic activity was detected (Fig. 5). Protease induction has been reported to be controlled by either ammonia [1] or amino acids [22]. In the efrotomycin fermentation, protease expression appears to be controlled by ammonia. After protease activity was detected, the concentration of amino acids in the broth began to increase, reaching a maximum of 6.4 g/l at 36 h (Fig. 5). As cell growth continued, amino acids were consumed with their concentration falling bellow 2 g/l before the end of the growth phase.

Lipolytic activity was produced late during the growth phase, only when the glucose concentration had fallen below 10 g/l (Fig. 6). Consequently, little triglyceride consumption was observed during growth (Fig. 7). Also, no amylolytic activity was detected during the growth phase.

Active cell growth stopped after glucose depletion, and was accompanied by a reduction in respiratory activity at 50 h (Fig. 3). Mass balance calculations indicated that biomass formation occurred at the expense of glucose,

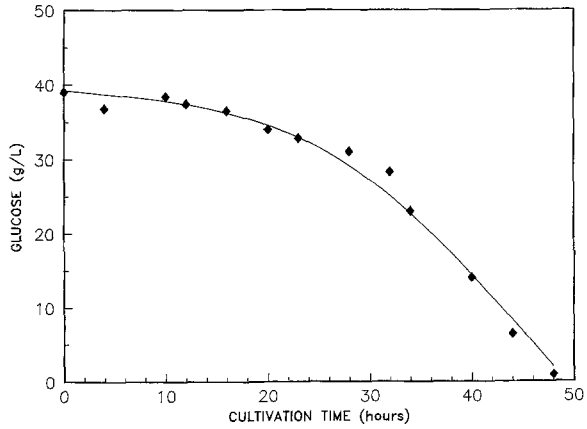

Fig. 4. Glucose consumption kinetics by *Nocardia lactamdurans* during efrotomycin fermentation.

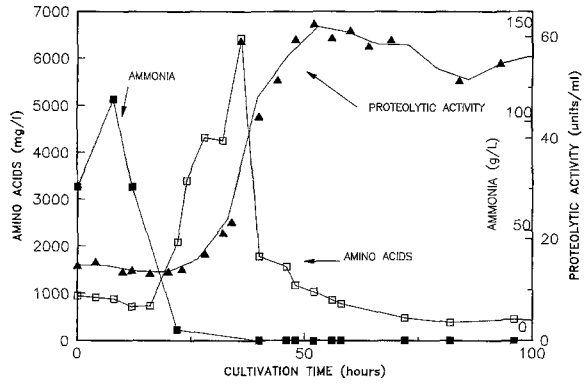

Fig. 5. Extracellular ammonia and amino acid concentrations, and proteolytic activity during efrotomycin biosynthesis.

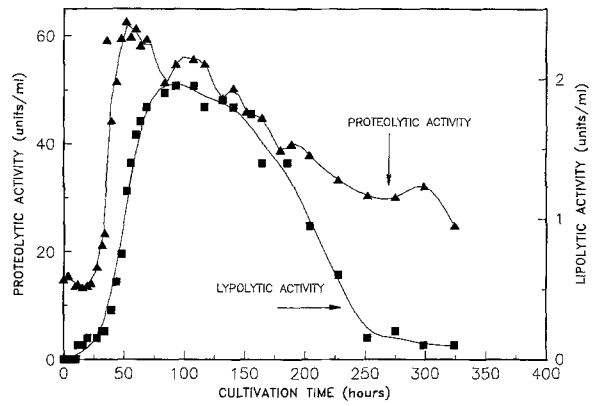

Fig. 6. Extracellular proteolytic and lipolytic activities during the efrotomycin fermentation cycle.

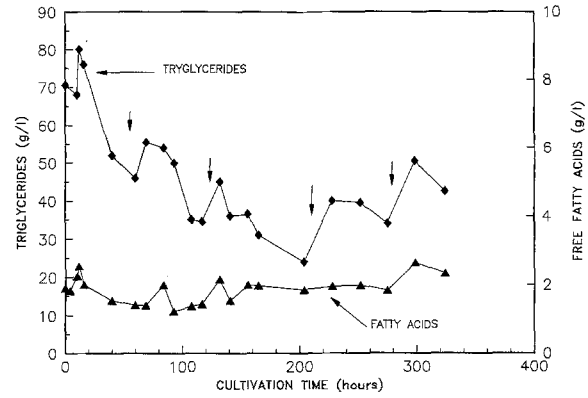

Fig. 7. Extracellular triglyceride and fatty acid concentrations during efrotomycin fermentation. Arrows indicate oil additions.

proteins, and soybean oil (Table 1). Most of the protein present in the Pharmamedia was consumed during the growth phase.

Efrotomycin production

The biosynthesis of efrotomycin was initiated during the latter part of the growth phase (Fig. 8). Biosynthesis continued after cell growth ceased at a linear rate (between 50 and 300 h). During this phase, oxygen uptake rate was between 30 and 40 mmol/l/h while the respiratory quotient fell to 0.6 to 0.7, a value indicative of triglyceride oxidation. The temporary reductions in efrotomycin biosynthesis rate observed at 120, 200, and 280 h were the result of dilutions caused by soybean oil additions (Figs. 7 and 8).

Triglyceride hydrolysis continued after cell growth had ceased (Fig. 7), and several oil additions were necessary to maintain a concentration above 40 g/l. Most of the lipase was produced after cell growth, reaching a maximum activity at 100 h and then slowly declining to about

TABLE 1

Efrotomycin fermentation carbon and nitrogen mass balance
Cell production phase (0–50 h)

Input				Output		
Substrates (g/l)		Carbon (%)	Carbon (g/l)	Products (g/l)	Carbon (%)	Carbon (g/l)
Glucose	(45)	40	18	Cells[1] (62.5)	45[2]	28
Proteins	(33)	32	10.6	CO_2[3] (55)	27	15
Soybean oil	(25)	75	18.8			
Total			47.4			43
		Nitrogen				Nitrogen[2]
Proteins	(33)	6.2	2.04	Cells (62.5)		1.9
Carbon recovery 90%						
Nitrogen recovery 95%						
Efrotomycin production phase (50–300 h)						
Soybean oil	(90)[4]	75	67.5	CO_2[5] (246)	27	66
Maltose	(0.45)	42	0.2	Metabolites[6]	–	–
Starch	(10)	44	4.4			
Total			72.1			66
Carbon recovery[7] 91%						

[1] The dry cell weight was calculated using a 3% DNA content for bacteria [20].

[2] Carbon and nitrogen composition for an average bacterial cell were obtained from [24].

[3] The total CO_2 production during that phase was 1.2 mol.

[4] Calculated from the concentration at 50 h (50 g/l) plus 4 additions of 20 g/l, minus final concentration (40 g/l).

[5] The total CO_2 production during that phase was 5.6 mol.

[6] Unaccounted metabolites include, efrotomycin, extracellular enzymes, etc.

[7] The recovery for this part of the fermentation does not include unaccounted metabolites (see footnote 6).

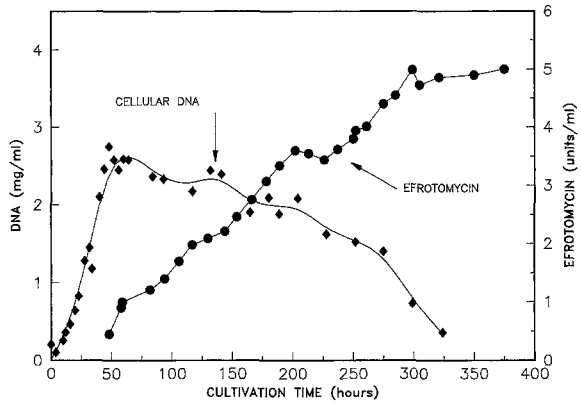

Fig. 8. Efrotomycin biosynthesis rate and cellular DNA content.

10% of its initial value by 250 h. Lipases have been reported to be sensitive to proteolytic activity [18], and it is possible that this phenomenon accounts for the lipolytic activity decline during the fermentation cycle. The rate of triglyceride consumption was directly related to the extracellular lipolytic activity and kept falling as lipolytic activity decreased (Table 2 and Fig. 6). Thin layer chromatography analyses indicated that intermediates of triglycerides hydrolysis, namely diglycerides and monoglycerides, did not accumulate in the fermentation broth (data not shown). Among the end-products of triglycerides hydrolysis, only fatty acids were detected in the fermentation supernatant. The extracellular fatty acid concentration was between 1.5 and 2.5 g/l during the whole fermentation cycle, and reflected an active consumption by the microorganisms.

Nutrients other than soybean oil were consumed during efrotomycin biosynthesis. Maltose was initially present at 0.45 g/l (Fig. 9) and was consumed only after glucose depletion, suggesting regulation of complex carbohydrate utilization by glucose. After 150 h, a slow accumulation of limit dextrins (Fig. 9), correlating with the presence of a weak amylolytic activity in the culture supernatant, indicated that the starch present in Pharmamedia

TABLE 2

Triglycerides hydrolysis rates

Time period (h)	Triclycerides utilization (g/l/h)	Glycerol released[a] (g/l/h)	Fatty acids released[a] (g/l/h)
20– 60	0.87	0.087	0.783
76–110	0.65	0.065	0.585
132–204	0.28	0.028	0.252

[a] Calculations based on a 10% w/w for glycerol.

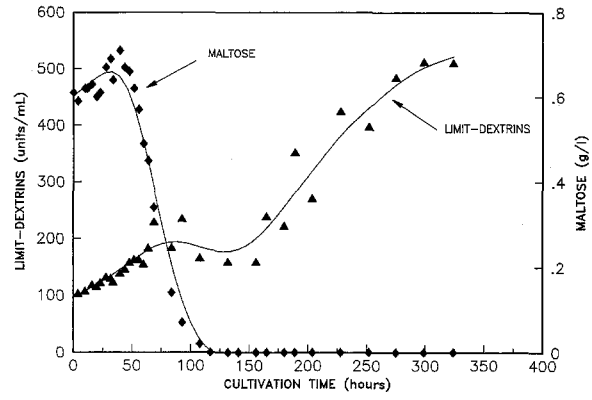

Fig. 9. Maltose consumption kinetics and limit dextrin concentration during the efrotomycin fermentation cycle.

was slowly hydrolysed. Extracellular amino acids remained below 0.5 g/l during the phase of efrotomycin biosynthesis, while proteolytic activity slowly declined to reach only 50% of its maximum activity by 250 h (Fig. 6).

A respiratory quotient value of 0.6–0.7 and carbon mass balance calculations (Table 1), indicated that soybean oil oxidation was the major contributor to *N. lactamdurans* metabolic activity during efrotomycin biosynthesis. Most of the soybean oil was used for dissimilatory metabolism, suggesting that efrotomycin biosynthesis was most probably associated with cell maintenance.

Metabolic decline

After 300 h, efrotomycin synthesis dropped dramatically (Fig. 8). Metabolic activity as measured by respiratory activity and triglyceride consumption was reduced in this final phase of the fermentation cycle (Fig. 2 and Table 2). The activity of extracellular hydrolytic enzymes either decreased dramatically for lipase and protease to 5 and 45% of their respective maximum values (Fig. 6), or totally disappeared, as with amylolytic activity. The detection in the culture supernatant of large amounts of DNA, tetrahydro-vitamin K_2 (a membrane component [30]), and amino acids, was indicative of cell lysis (Fig. 2). Correlating with this lysis an increase in broth viscosity (Fig. 2) thought to be due to DNA release was observed and complicated mixing. Cell lysis may have been the result of starvation caused by the almost total disappearance of lipase activity late in the fermentation cycle.

CONCLUSIONS

These physico-chemical and biochemical studies provided a better understanding of the efrotomycin fermentation and insights for process improvements. Cell growth accompanied by a peak oxygen demand occurred during the first 50 h of the fermentation cycle. Cell growth ceased after glucose depletion followed by a reduction in respiratory activity and a switch in the respiration quotient. Cellular DNA content profiles and mass-spectrometry analyses correlated closely during the cell formation phase, allowing the determination of growth kinetics and an estimate of biomass formation. Carbon and nitrogen mass balance data indicated that cells were synthesized from glucose, proteins, and triglycerides. The two latter substrates were hydrolysed by secreted proteases and lipases respectively. Efrotomycin was produced late in the growth phase and continued for the remainder of the fermentation cycle. After growth had ceased, extracellular amylase was secreted and lipase continued to be produced, resulting in the hydrolysis of starch, and the continued hydrolysis of soybean oil. Triglyceride hydrolysis may be the the rate-limiting step in soybean oil utilization since hydrolytic intermediates and end-products never accumulated. Mass-spectrometry analyses and carbon mass balances indicated that most of the soybean oil was utilized for dissimilatory metabolism, suggesting that the bulk of efrotomycin was synthesized by non-growing cells operating on maintenance energy. Late in the fermentation cycle (200 h), lipase activity was dramatically reduced, resulting in a reduced nutrient availability for the microorganisms. Massive cell lysis accompanied by the release of amino acids, DNA, and membrane components in the fermentation broth occurred at the end of the fermentation cycle (250 h). Concurrently with this lysis, an increase in broth viscosity (thought to be due to DNA release) was observed and resulted in mixing difficulties. These studies have provided insights to the efrotomycin fermentation that were used to improve the process. Based on the hypothesis that nutrient depletion was the major cause for efrotomycin synthesis to cease, an oil-fed and later a glucose-fed processes were developed. Under these conditions, sizeable improvements in efrotomycin production were achieved (A. Kirpekar, W. Cover, G. Hunt, M. Chartrain, R. Stieber and M. Angelo, AiChE Annual Meeting, Chicago, IL, 1990).

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