Development of a Large-Scale Continuous Substrate Feed Process for the Biotransformation of Simvastatin by *Nocardia* s.p.

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Received May 16, 1990/Accepted August 15, 1990

This article describes a process for microbial hydroxylation of simvastatin by a *Nocardia* sp. Simvastatin (Zocor) belongs to the family of HMGCoA reductase inhibitors used as cholesterol-lowering drugs. Studies at 14 L scale showed that high substrate (simvastatin) concentrations inhibited product formation; consequently, continuous slow feeding of the substrate was introduced to maintain low residual simvastatin concentrations. Dissolved oxygen levels above 50% air saturation were desirable for the biotransformation. The process was scaled up to 19,000-L fermentors using an on-line filter sterilization system for substrate feeding. The feed rate was regulated by off-line high-pressure liquid chromatography (HPLC) assays to keep the substrate concentration below 20 mg/L. Intermittent addition of nutrients helped to boost the bioconversion rate to give final titers of 400 mg/L 6-β-hydroxymethyl simvastatin. Enrichment of the nutrient medium led to bioconversion titers of 800 mg/L 6-β-hydroxymethyl simvastatin. Bioconversion efficiencies (desired product/substrate) of 22–25% with a ratio of desired product/side products of 0.7 were obtained by this process.
Key words: simvastatin • microbial • hydroxylation • fermentation • biotransformation • scale-up • dissolved oxygen

INTRODUCTION

Microbial transformations are of great interest to the pharmaceutical industry because they allow the regioselective and stereoselective modification of complex molecules. Their main application traditionally has been for the hydroxylation of steroids[7] and more recently the partial degradation of sterol side chains.[6] In those cases where the biotransformations have involved a hydrolytic reaction as in the deacylation of benzylpenicillin or phenoxypenicillin and cephalosporins, they are normally done with enzymes rather than intact cells.[8] Further chemical modification of the enzymic products has led to new antibiotics with different properties. In the same way it is now common practice to examine the properties of a wide range of derivatives synthesized chemically or biologically from other parent molecules with pharmacological activity. The biochemical complexity of many of these modification reactions requires the use of intact metabolically active microorganisms.[5] Here we describe the development and scale-up of a continuous substrate feed microbial transformation to convert simvastatin to the 6-β-hydroxymethyl derivative.

The lovastatin family of HMGCoA reductase inhibitors, with potent cholesterol-lowering therapeutic activity, are made through a challenging combination of biosynthetic and chemical synthetic routes. The parent compound, lovastatin, is produced as a secondary metabolite from the fermentation of *Aspergillus terreus*.[1] This compound is chemically methylated at the 2′ sidechain carbon to make simvastatin (MK733, Zocor) (Fig. 1). Further microbial hydroxylation of simvastatin by *Nocardia autotropica* leads to 6-β-hydroxymethyl-simvastatin. This reaction is accompanied by the formation of other hydroxy derivatives, including 6-hydroxy-simvastatin and to a lesser extent 3-hydroxy-iso-simvastatin and 3-desmethyl-3-carboxy-simvastatin.

MATERIALS AND METHODS

Organisms

Two strains of a *Nocardia* sp., designated 1 and 2, obtained from Dr. J. Williamson (Merck culture collection) were used. Strain 2 was a later isolate giving higher yields of the hydroxymethyl derivative of simvastatin.

Culture Media

For strain 1 the medium used for seed cultures consisted of (g/L) Cerelose [Corn Products Corp. (CPC)], 10; Hycase (Sheffield Products), 2; beef extract (Difco Laboratories), 1; and corn steep liquor, 3. The original seed medium for strain 2 consisted of (g/L) glucose, 4; malt extract (Difco Laboratories), 10; yeast extract (Difco Laboratories), 4; and nutrient broth (Difco Laboratories), 4. For later experiments a modified seed medium was used (g/L) Cerelose (CPC), 4; Fidco yeast extract (Food Ingredients Development Co.), 10; Ar-

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Biotechnology and Bioengineering, Vol. 37, Pp. 1101–1107 (1991)

CCC 0006-3592/91/01101101–07$04.00

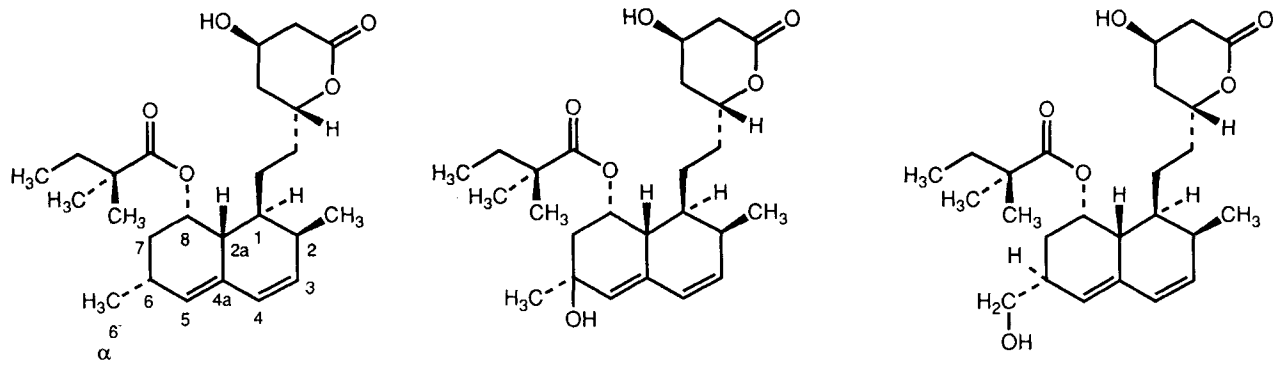

Figure 1. Structures of simvastatin and main derivatives.

damine pH (Champlain Industries), 4; and Primatone HS (Sheffield Products), 4. The production medium for both strains consisted of (g/L) Cerelose (CPC), 10; Hycase (Sheffield Products), 2; beef extract (Difco Laboratories), 1; and corn steep liquor, 3. Polyglycol P-2000 (Dow Chemicals) antifoam was added to media (1 mL/L) before sterilization.

Small-Scale Culture Conditions

The seed train consisted of growth in 20 mL seed medium, which was then transferred to 250 mL seed medium using flasks in a rotary shaker. The contents of two flasks were used to inoculate a fermentor containing 10 L seed medium. All biotransformation experiments were done in 14-L NBS fermentors fitted with turbine impellers. The production medium (8 L) for the biotransformation was inoculated with 800 mL from the seed fermentor. The transfer was normally made after 22–24 h when the exhaust gas CO_2 content from the seed culture had reached 1–1.2% (v/v).

The biotransformations were maintained at 27°C and either pH 6.2 (for strain 1) or 6.8 (for strain 2). During the growth phase the agitation speed was 300 rpm and the aeration rate was 3 L/min. For strain 2 these were reduced to 200 rpm and 2 L/min, respectively, at the time when feeding of simvastatin was started. This decreased the amount of foaming and eliminated the need for any further addition of antifoam.

The concentration of the simvastatin feed was 15.6 g/L. Additions were made using a peristaltic pump the speed of which was adjusted during the biotransformation. The feeding was normally started about 24 h after inoculation of the production medium, at which time the oxygen uptake rate was 6–7 or 9–10 mmol/L h depending on whether the seed medium had been the original or modified medium.

Large-Scale Culture Conditions

Large-scale biotransformations were done in a 19,000-L fermentor interfaced to a Honeywell TDC-2000 computer. An 800-L fermentor was used for intermediate-scale runs. The tank configuration, instrumentation, and computer interface have been described elsewhere.[2,4] The vessel contents were agitated by three turbine impellers rotating at 90 rpm and aerated at 4000 L/min with a head pressure of 10 psig. The broth volume was measured by differential pressure. The culture was maintained at 27°C.

The inoculum was developed through a four-stage seed buildup (Table I). Following inoculation and cell growth for 24 h, simvastatin feeding was initiated. The substrate was fed through an on-line filter sterilization system (Fig. 2). A 1000-gal feed tank was batched with 1500 L of substrate at a concentration of 20 g/L, which was maintained at 45°C to prevent crystallization of substrate out of the feed solution. An overpressure of 20 psig was maintained on the feed vessel to enable flow through the filtration system, which consisted of a porous stainless steel prefilter (1–2 μm nominal pore size, Pall) followed by 0.22 μm depth and absolute cellulose acetate filters (Sartorius) in series. A spare prefilter was installed in parallel to avoid interruption of flow if the first one became blocked. Fermentor broth was sampled hourly and assayed for simvastatin concentration. The feed rate was manually regulated in the range 0–2 L/min to keep the simvastatin level in the fermentor below 20 mg/L.

Gas Analysis

Exit gases from all fermentors were analyzed for oxygen and carbon dioxide composition using a Perkin-Elmer mass spectrometer linked to the computer.[3]

HPLC Analysis

Samples withdrawn from the fermentors were mixed with an equal volume of acetonitrile and the mixture centrifuged. Samples (6 μL) of the clear supernatant were loaded on a PRP column maintained at 40°C and eluted with a gradient at 1.1 mL/min. The gradient was obtained by mixing an aqueous ammonium phosphate solution, pH 6.1, with acetonitrile in an initial ratio of 84:16 which was changed in steps to 30:70 over 15 min.

Table I. Four stage seed train for large-scale cultures.

	Seed stage			
	1	2	3	4
Vessel size	250 mL	2 L	75 gal	500 gal
Working volume	30 mL	500 mL	180 L	1200 L
Inoculum	Lyoph. tube	10 mL	2 L	120 L
Operating conditions				
Temperature (°C)	27	27	27	27
Agitation (rpm)	220	220	200	110
Air flow (L/min)	—	—	70	400
Pressure (kg/cm^2)	—	—	0.7	0.7
Cultivation time (h)	48	24	24	24

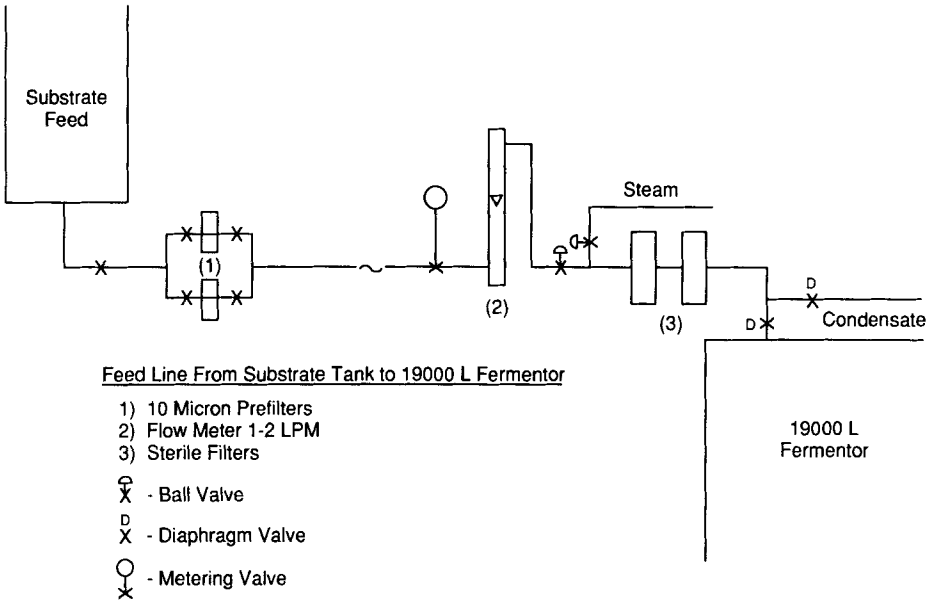

Figure 2. Simvastatin feed system to 19,000-L fermentor.

Both products and simvastatin were detected spectrophotometrically at 238 nm.

RESULTS

Small-Scale Studies

Inhibition of Product Formation by Simvastatin

With strain 1 it had been shown that it would tolerate concentrations of simvastatin up to 200 mg/L. When shots of simvastatin at this concentration were made to biotransformations with strain 2, no products were formed (Fig. 3). As a result, when further shot additions were made, the simvastatin concentration rose further. The deleterious effect of the substrate on the metabolic activities of strain 2 was reflected in a rapid fall in the exit gas CO_2 concentration (Fig. 4). When continuous feeding of substrate at a slow rate was adopted, there was a gradual decline in CO_2 evolution (Fig. 4). When the feeding rate was too fast, the rate of conversion was insufficient to prevent accumulation of simvastatin and cessation of product formation.

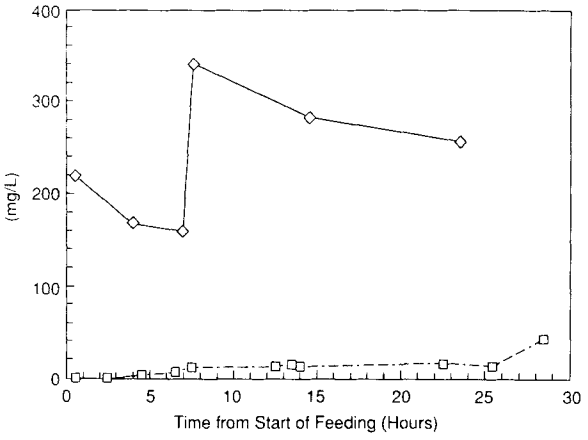

Figure 3. Effect of high initial simvastatin concentration (◇) on the bioconversion by strain 2 to 6-hydroxymethyl-simvastatin (□). Additional substrate was added at 7 h.

Effect of Dissolved Oxygen Tension (DOT) on Product Formation

It seemed likely that the concentration of oxygen in the reaction liquid could influence both the synthesis of

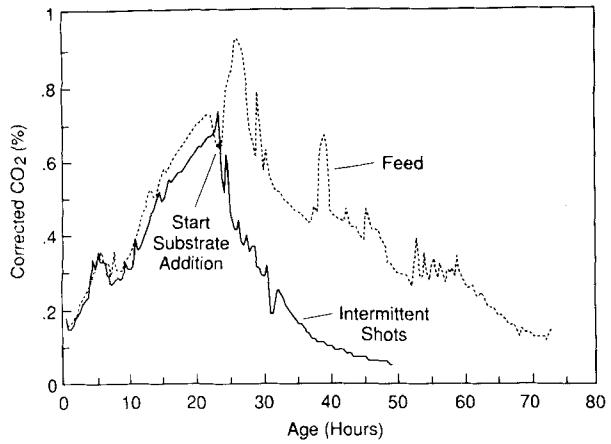

Figure 4. Exhaust gas CO_2 concentrations for two bioconversions by strain 2 with intermittent shot additions and continuous feeding of substrate.

the enzymes involved in the biotransformation and the rates at which these enzymes acted. Figure 5 shows that compared to a batch in which the DOT was maintained at 80% air saturation, the production of the 6-hydroxymethyl and 6-hydroxy derivatives was suppressed in two batches where the DOT was reduced to 10% air saturation just before the start of substrate feeding. When the DOT in one of these batches was raised 2 h after the start of feeding, there was an increase in product formation similar to that observed in the biotransformation where the DOT was maintained at a high level from the start of feeding. This indicates that synthesis of one or more of the enzymes involved is inhibited at low DOT. In a further experiment the rate of 6-hydroxymethyl derivative accumulation was unaffected by a reduction in DOT from 60–75% to 25% for 7 h. In all further experiments the DOT was maintained above 50% air saturation.

Comparison of Strains 1 and 2

The concentration profiles for 6-hydroxymethyl and 6-hydroxy derivatives from the start of substrate feeding for strains 1 and 2 are shown in Figure 6. For the biotransformation with strain 1 the substrate concentration in the fermentor was maintained between 6.2 and 39.6 mg/L by controlling the feed rate, and after 27.5 h the yield of 6-hydroxymethyl derivative was 13%. The ratio of the products (6-hydroxymethyl/6-hydroxy + 3-hydroxy) was 0.26.

Higher levels of products were obtained with strain 2 and the biotransformation was extended to 49.5 h (Fig. 7). Up to 31 h the simvastatin concentration was maintained below 65 mg/L but was then allowed to rise to about 340 mg/L after 49 h. The results for another biotransformation where the substrate concentration was allowed to rise in a similar way (also Fig. 7) indicate the reproducibility of these biotransformations and the inhibition of product formation which occurred as a result of high substrate concentrations. The final yields of 6-hydroxymethyl derivative in the two biotransformations were 22 and 24%, respectively.

Effect of Seed Medium on the Biotransformation

For strain 1 the production stage medium was also satisfactory for previous seed stages including the final seed

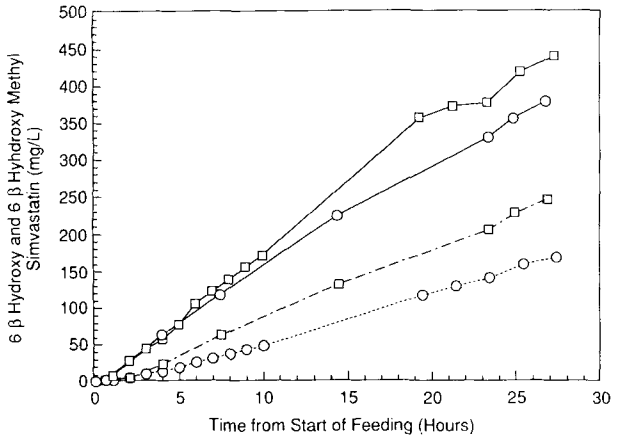

Figure 6. Comparison of the rates of bioconversion for strains 1 (--○--, --□—) and 2 (—○—, —□—); 6-hydroxymethyl-simvastatin, (--○--, —○—); 6-hydroxy-simvastatin (—□—, —□—).

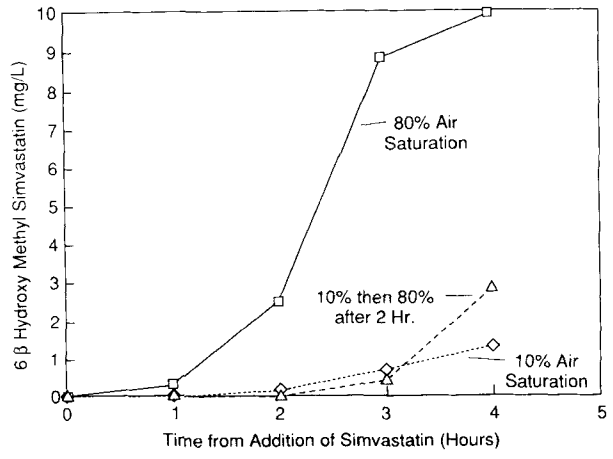

Figure 5. Effect of dissolved oxygen tension on the initiation of the bioconversion by strain 2.

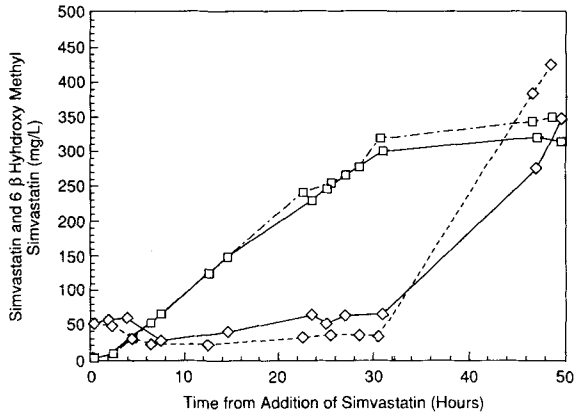

Figure 7. Two bioconversions with strain 2 with controlled feeding of simvastatin (--◇—, —◇—); 6-hydroxymethyl-simvastatin concentration (--□—, —□—).

stage. The original seed medium for strain 2 contained several components too expensive for large-scale work. When the culture was grown on production medium in the final seed stage, growth was much slower (Fig. 8) and the rate of product formation in the biotransformation was much lower. A modified seed medium was chosen which gave good growth at both the final seed stage (Fig. 8) and production stages (Fig. 9). The maximum packed cell volumes was 11% in both stages.

The results of a biotransformation using the modified seed medium are shown in Figure 10. By 14 h the glucose (Cerelose) had been utilized, and a further amount was added to ensure that the metabolic activity of the culture was maintained. The reduction in production rate around 24 h after feeding started was due to the simvastatin concentration being allowed to rise. The yield of 6-hydroxymethyl derivative was 25%. In contrast to previous runs, the feed rate was reduced or stopped before the end of the biotransformation to ensure that all the simvastatin was utilized. The ratio of 6-hydroxymethyl/6-hydroxy + 3-hydroxy was 0.72.

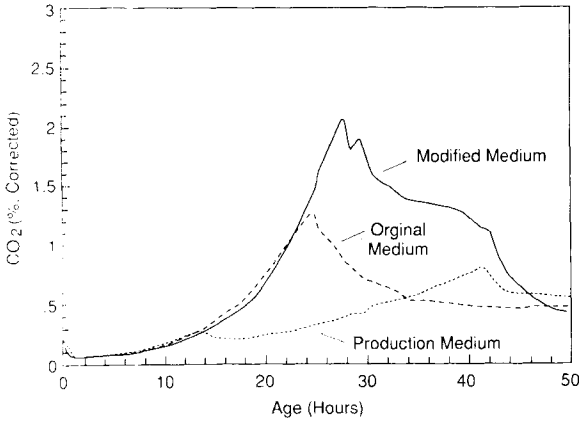

Figure 8. Exhaust gas CO_2 concentrations for seed cultures of strain 2 with the original seed and modified media and production medium.

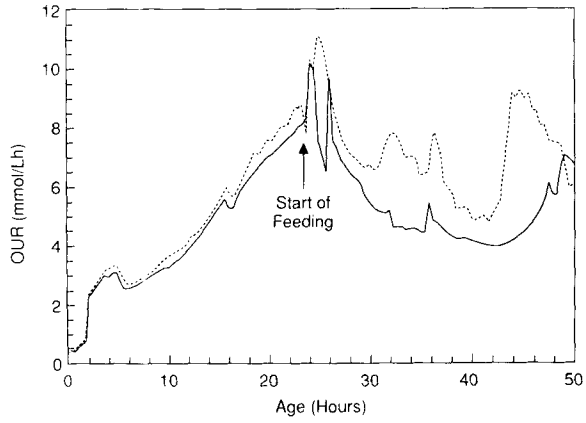

Figure 9. Oxygen uptake rate profiles for two bioconversions by strain 2 with controlled substrate feeding.

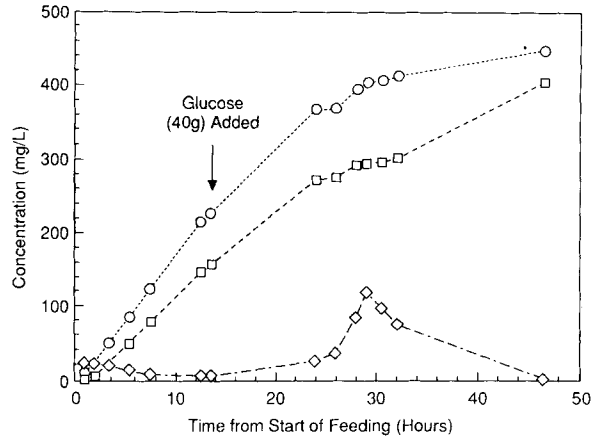

Figure 10. A bioconversion by strain 2 with controlled feeding: simvastatin (◇), 6-hydroxymethyl-simvastatin (□), and 6-hydroxy-simvastatin (○).

Large-Scale Studies

Intermittent Substrate Feeding

Earlier fermentations at the 19,000-L scale were carried out by feeding shots of simvastatin intermittently to a fermentation of strain 1 (Fig. 11). Initially, the shots were added at approximately 5-h intervals to keep the substrate concentration in the fermentor below 200 mg/L. It was observed during the biotransformation that the bioconversion rate tended to increase as the residual substrate concentration fell below 100 mg/L. Consequently, a more frequent substrate feed regime with addition of smaller amounts of substrate was introduced accounting for the improved rate of bioconversion between 30 and 45 h of the run.

These observations suggested a continuous substrate feeding strategy such that the residual simvastatin concentration in the fermentor was kept below the level needed to achieve high rates of conversion. This objective was accomplished by experimentation at the 14-L scale as described earlier.

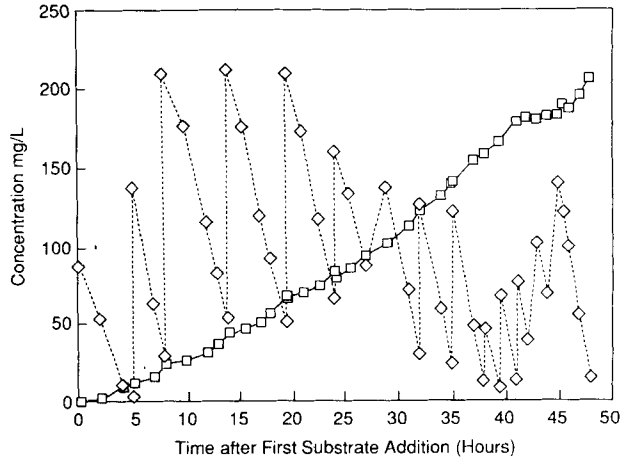

Figure 11. A 19,000-L bioconversion by strain 2 with intermittent shots of simvastatin (◇) to 6-hydroxymethyl-simvastatin (□).

Continuous Substrate Feeding

Based on the results obtained at the 14-L scale, the continuous feeding strategy was adopted at the 19,000-L scale by regulating the substrate feed rate in response to hourly off-line assays of residual substrate in the fermentor. This successfully maintained the residual substrate concentration below 20 mg/L (Fig. 12). The rates of oxygen uptake and carbon dioxide evolution reached a peak within 20 h of the start of the fermentation and then declined during the bioconversion period to below 2 mmol/L h by 40 h (Figs. 13 and 14). It was also noted that the culture pH drifted upward from 7.0 and the bioconversion rate slowed at the same time.

As these observations were indicative of nutrient limitation, 500 L of fresh production medium was added aseptically to the batch at 58 h. The response of the fermentation to the nutrient shot was an instant burst in the rates of oxygen uptake (Fig. 13) and carbon dioxide evolution (Fig. 14) to a peak of 4.5 mmol/L h, more than twice their levels before nutrient addition, and an increase in bioconversion rate (Fig. 12). The rates of oxygen uptake and carbon dioxide evolution gradually subsided, and 240 L of 50% (w/v) glucose solution was added at 102 h. Again the metabolic activity rose but the bioconversion rate remained sluggish. Another 500 L of production medium was added at 115 h, resulting in a faster bioconversion rate. Further nutrient shots of production medium were made at 153 and 182 h (Fig. 15), which sustained the bioconversion rate up to 195 h, by which time the 6-hydroxymethyl simvastatin had reached almost 400 mg/L and the run was terminated.

The impact of the nutrient shots on the bioconversion rate suggested that further process improvements could be made through a strategy of medium enrichment. Accordingly, an 800-L fermentation was run with a medium double the concentration of the normal production medium. With this enriched medium the bioconversion rate was faster than in the normal fermentation and a 6-hydroxymethyl simvastatin concentration of over 800 mg/L was reached in 132 h (Fig. 16).

The bioconversion yields (based on the desired product) obtained at the 19,000- and 800-L scales were 22

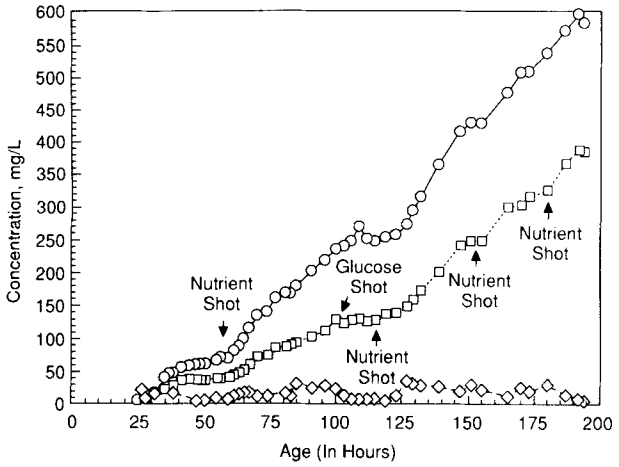

Figure 12. A 19,000-L bioconversion by strain 2 with continuous feeding of simvastatin (◇), 6-hydroxymethyl-simvastatin (□), and 6-hydroxy-simvastatin (○). Sugar and nutrient shots were made as indicated.

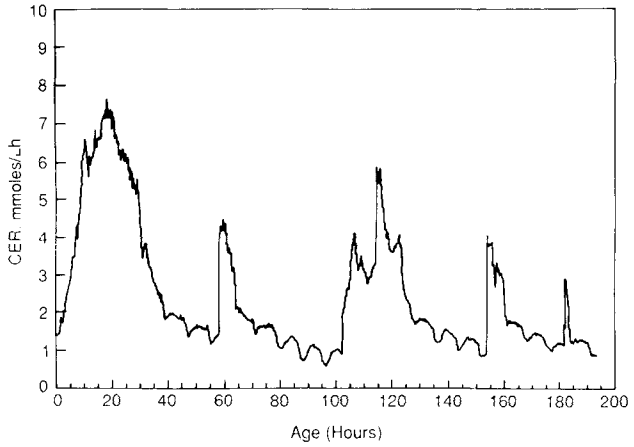

Figure 13. Carbon dioxide evolution rate profile for the bioconversion shown in Figure 12.

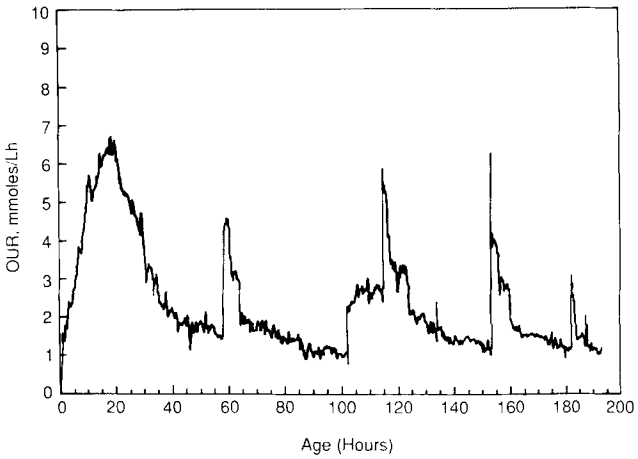

Figure 14. Oxygen uptake rate profile for the bioconversion shown in Figure 12.

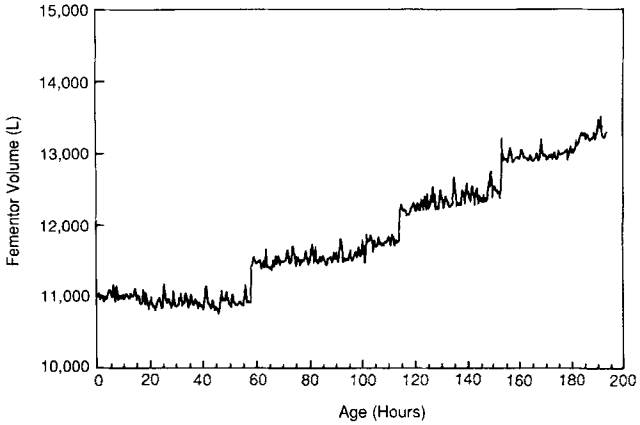

Figure 15. Fermentation volume profile for the bioconversion shown in Figure 12.

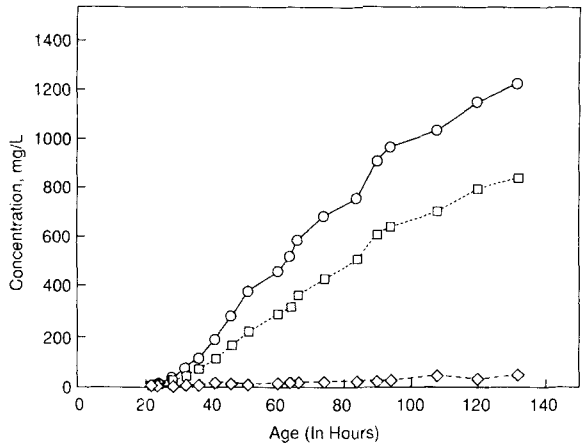

Figure 16. An 800-L bioconversion by strain 2 with enriched production medium: simvastatin (◇), 6-hydroxymethyl-simvastatin (□), and 6-hydroxy-simvastatin (○).

and 24%, respectively. These yields are closely identical with those achieved in the 14-L-scale experiments.

DISCUSSION

In this article describe the successful scale-up of a continuous substrate feed biotransformation from a 14- to a 19,000-L scale. During this project an improved strain became available but illustrated clearly the need for fermentation development that often arises in such cases. A change from intermittent to continuous feeding of the substrate (simvastatin) was necessary to prevent inhibition by the substrate, to which the improved strain was much more susceptible. The seed medium also had to be changed. After making these process alterations, the improved strain gave a doubling in yield and an improvement in the ratio of the desired product to other derivatives. The final titer was now over 400 mg/L after 46 h from the start of feeding. The reproducibility of the biotransformation was clearly demonstrated on a small scale.

On the small scale glucose was added to maintain the metabolic activity of the culture, but on the large scale several nutrient additions were made to achieve this. The rate of product formation in the 19,000-L fermentation was lower partly because the biomass concentration was not as high as on the small scale, probably because of the different medium sterilization conditions. Also the level of substrate in the biotransformation was kept deliberately low to avoid any possibility of a rise in level to a point where inhibition would occur and the biotransformation would have to be aborted. This is particularly important in such biotransformations where the cost of the substrate is high so that it is crucial that batches are not lost and yields are reproducible. By medium enrichment a further improvement was made, raising the titer to 800 mg/L and giving an overall improvement of about fourfold.

The authors wish to thank Dr. Joanne Williamson, who provided the cultures and information on shaker flask conditions for the bioconversion used in this work. They also wish to thank S. Gatchalian, V. Santamarina, and K. Glazomitsky for their invaluable assistance with laboratory and pilot plant operations. One of us (M.D.L) wishes to thank Merck Sharp and Dohme for the provision of a Visiting Engineering Fellowship.

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