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Mutants of a lovastatin-hyperproducing *Aspergillus terreus* deficient in the production of sulochrin

V.A. Vinci [1], T.D. Hoerner [1], A.D. Coffman [1], T.G. Schimmel [1], R.L. Dabora [1], A.C. Kirpekar [1], C.L. Ruby [2] and R.W. Stieber [1]

[1] *MCMD, Merck & Co., Elkton, Virginia and* [2] *MSDRL, Merck & Co., Rahway, New Jersey, U.S.A.*

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SUMMARY

A lovastatin-hyperproducing culture of *Aspergillus terreus* was shown to produce several co-metabolites extracted from whole broth. The predominant co-metabolite was the benzophenone, sulochrin, reported to arise from a polyketide biosynthetic pathway. This compound was targeted for elimination by classical mutagenesis and screening. A surface culture method employing microtiter plates was used to ferment mutants for the primary screen. Qualitative determinations of lovastatin and sulochrin production were achieved by high-performance thin-layer chromatography. A mutant, strain AH6, which produced lovastatin titers equivalent to the parent culture and no detectable sulochrin was isolated. In addition, a lovastatin-hyperproducing mutant designated CB4 was capable of producing 16% more lovastatin and 30% less sulochrin than the parent culture in shake flask fermentations. In a pilot-scale 250-gallon fermentation, strain CB4 gave a 20% increase in lovastatin titer while producing 83% less sulochrin than the parent culture.

INTRODUCTION

Continuous improvement of an industrial microorganism is an essential component in the commercial success of a fermentation process. Improvement in the yield of a desired product, maintenance of desired morphological characteristics, and the elimination of unwanted co-metabolites are goals of an industrial strain improvement program. A classical strain improvement program, also known as random screening, offers an advantage for short-term strain development as it can be implemented in the absence of detailed genetic information on the microorganism. The utility of this approach is best exemplified by the increases in penicillin yields from *Penicillium* achieved over a period of years [9,13]. Various *Aspergillus* species were also successfully manipulated to give increased titers of penicillin [16] as well as biologics such as asperlicin [10]. Enhanced production of citric acid and industrial enzymes such as glucoamylase [15] and glucose oxidase [4] were achieved by classical strain improvement. Classical means can also be applied to achieve the elimination of unwanted co-metabolites in a fermentation broth. Elimination of these undesireable co-metabolites may be required due to their inherent toxicity [9] or to difficulties in their removal during downstream processing.

Aspergillus terreus produces a spectrum of secondary metabolites. Some compounds which have been isolated from various strains of *A. terreus* include asterric acid [4], butyrolactone [12], citrinin [14], emodin [5,8], itaconic acid [18], geodin [5,8], sulochrin [7,8], terretonin [17], and lovastatin [1,2]. A thorough study of the putative biosynthetic routes to lovastatin, also known as mevinolin and Mevacor^{R}, indicated that biosynthesis proceeds via a polyketide pathway [11]. The production of sulochrin and asterric acid are related by a common polyketide precursor, emodin [5,6,8]. An industrial strain of *A. terreus* which overproduces lovastatin was shown to produce several of the above polyketides. Because sulochrin was an unwanted co-metabolite in the industrial fermentation of this culture, a classical mutagenesis and screening program was begun to eliminate its production. The research related to the development of a strain improvement program for this. *A. terreus* culture is presented.

MATERIALS AND METHODS

Microorganism

The lovastatin-producing strain, designated 6R9, was previously derived from *A. terreus* ATCC 20542 following

Correspondence: V.A. Vinci, Merck Chemical Manufacturing Division, Merck & Co., P.O. Box 7, Elkton, VA 22827, U.S.A.

several rounds of natural re-isolation and chemical mutagenesis [2].

Growth medium

The ingredients used for P-9 and P-7 media are complex and were selected on the basis of their ability to support high lovastatin productivity in shake flasks or stirred tanks. These media were developed for production based on previous medium optimization studies [2]. A yeast–malt extract medium (YME) was used for inoculum flasks and supplemented with agar for plates and slants. P-9 medium with agar was used for microtiter fermentations.

Mutagenesis

Strain 6R9 was grown for 7 days at 27 °C on slants at which time slants were removed to a 4 °C cold room. The conidia were harvested by adding 10 ml of sterile 0.1 M Tris buffer (pH 7.0) and rubbing the slant surface with a pipette to release the spores. The suspension was transferred to a sterile glass screw-cap tube containing glass beads and vortexed to disrupt clumps of conidia. Mycelial fragments were removed by filtration. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was selected as the mutagen based on prior work with the culture [2]. NTG was dissolved in acetone to 100 mg/ml before addition to 1 ml of spores in an Eppendorf tube. The range of NTG concentrations used was 2.5–10 mg/ml. Following exposure to the mutagen for 5–10 min, 250 µl of a filter-sterilized solution of 12% thiosulfate was added and the tubes were placed on ice for 30 min. The suspension was microfuged for 5 min and resuspended in 1 ml of 6% thiosulfate/0.1 M Tris buffer, pH 7.0. Following a second pelleting step, the spores were suspended in Tris and plated on YME solid medium at the appropriate dilutions. Conditions were selected to achieve a 99% kill. Plates were incubated 4–5 days at 27 °C at which time colonies were transferred with sterile toothpicks to master or patch plates. A total of 25 square patches (1 cm × 1 cm) were grown for 5 days at 27 °C on the master plates (15 × 15 × 100 mm) containing YME agar.

Primary screen

Sterile P-9 agar medium was added aseptically to 48 wells of a sterile 96-well microtiter plate (Fig. 1). Additions were made such that alternate wells in each row were filled and in adjacent rows wells were filled on a staggered basis. This pattern was necessary to prevent cross-contamination of fungal colonies from adjacent wells. Filter paper discs (Schleicher & Schuell, Keene, NH) were wetted in liquid seed medium and placed on the surface of the solid P-9 medium in each well. A cotton swab moistened in seed medium was used to rub one half of the sporulated surface of a colony from the master plate and subsequently used to inoculate a filter disc on the microtiter plate. The microtiter plates were incubated for 9 days at 27 °C at which time they were harvested.

High performance thin layer chromatography (HPTLC) was utilized to analyse extracts of the solid fermentation step. Following growth on the microtiter plates, a filter with attached mycelium was removed from a well and placed in an Eppendorf tube. 50 µl of methanol was added and the filter was allowed to soak for about 15 min. From this extract, 5 µl was removed with a TLC capillary pipette and loaded onto an HPTLC plate (E. Merck Silica Gel60 F-254 plates, 10 × 20 cm, with a concentration zone). Each HPTLC plate optimally accomodated 12 spottings in the concentration zone. The solvent system used to develop plates was acetonitrile : methanol : ammonium acetate, pH 5.0 (110 : 5 : 10 v/v/v). A Shimadzu CS9000U densitometer was used for ultraviolet and visible scans of LTC lanes or individual bands. Scans of individual bands were performed at 235 nm for lovastatin and 285 nm for sulochrin. For routine screening, the plates were examined visually under a hand-held, short-wave ultraviolet light.

Secondary screen

The secondary screen (Fig. 1) utilized 16-day shake flask fermentations followed by extraction with methanol. Seed flasks containing 40 ml of seed medium were inoculated from a washed YME slant of the appropriate culture and grown for 28–30 h at 220 rpm, 27 °C and 85% relative humidity. 2 ml of inoculum was transferred to the production flasks containing 30 ml of P-9 medium which were incubated under the same conditions. Methanol extracts were submitted to HPLC assay of lovastatin, sulochrin, and asterric acid. For routine quantitative assays, an isocratic elution with a mobile phase of 0.1% H_3PO_4/acetonitrile (40 : 60, v/v) was used to assay for lovastatin on a Whatman Partisil 5 C8 column (4.6 × 250 mm). Absorbance was monitored at 235 nm and compared to standard concentrations of the above compounds. For profiles of extracts from the mutants, a gradient elution on a Rainin Dynamax C18 column (4.6 × 250 mm) was performed with the following mobile phase: Buffer A, 0.1% trifluoroacetic acid (TFA)/acetonitrile (80 : 20, v/v); Buffer B, water/0.05% TFA in acetonitrile (15 : 85, v/v); 0 to 15 min, 70% A/30% B; 15 to 25 min, 0% A/100% B; 25 to 35 min, 70% A/30% B. A Beckman System Gold HPLC equipped with diode array detector was used for diode array spectroscopy and identification of individual peaks.

Pilot-scale fermentation

Two 250-gallon, pilot-scale fermentors each received 5% (v/v) inoculum from a seed culture when its glucose

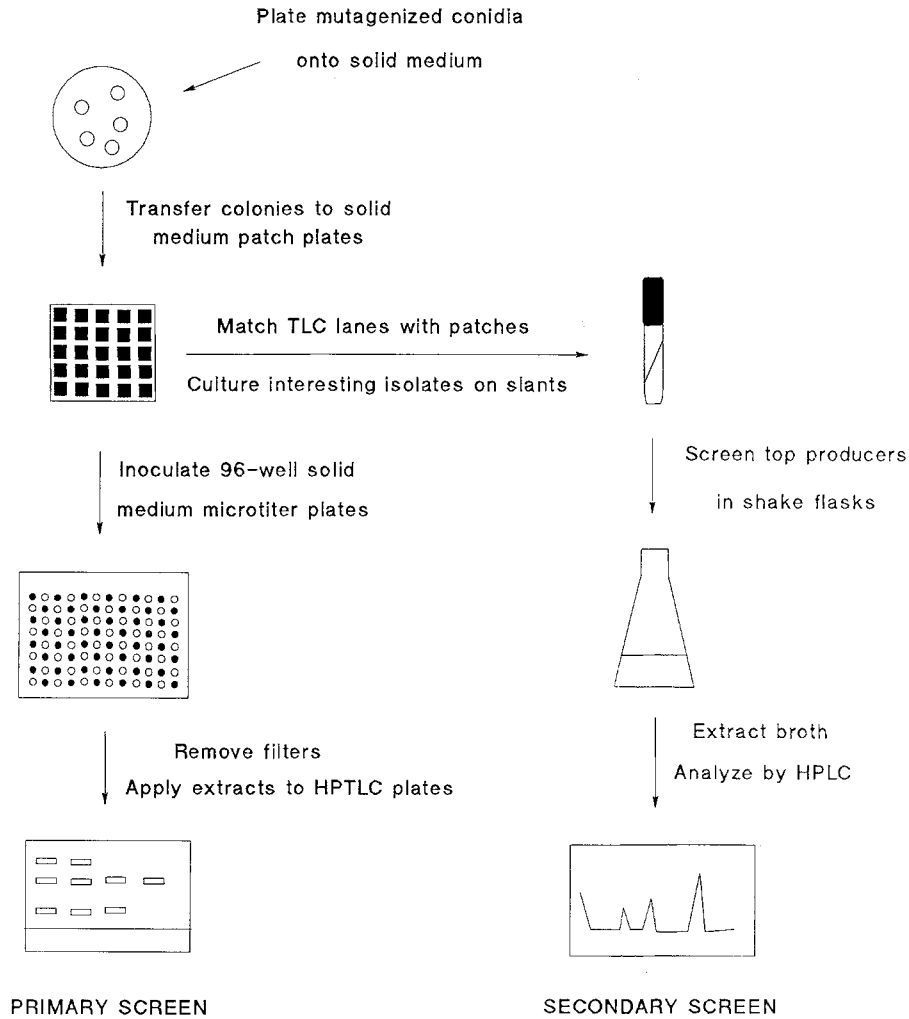

Fig. 1. Schematic representation of the major steps of the screening program.

concentration fell below 0.5 g/l. The pilot-scale fermentors were charged with continuously sterilized P-9 or P-7 medium. During the fermentation, the temperature was maintained at 27 °C while air flow, backpressure, and agitation speed were controlled so as to maintain dissolved oxygen concentration above 20% of air saturation during the period of peak oxygen demand. As oxygen demand decreased, these parameters were controlled to minimize foaming and evaporation. Samples were taken aseptically at various times as indicated and titers were corrected for volume loss.

RESULTS AND DISCUSSION

Chromatographic analyses

A desire to reduce the fermentation impurities feeding into downstream processing led to studies designed to characterize whole broth extracts from large-scale fermentations of *A. terreus*. Five significant peaks in addition to the predominant product, lovastatin, were identified in whole-broth extracts of culture 6R9 from 16-day shake flasks (Fig. 2a) and production-scale fermentors. Reference standards of each compound were spiked individually into extracts to confirm retention times following gradient elution. In addition, diode array spectroscopy was used to identify the peaks based on the characteristic UV spectra (not shown). HPLC peaks from the parent production culture were identified as triol acid (6.5 min), sulochrin (8.2 min), asterric acid (10.8 min), and butyrolactone (12.5 min) (Fig. 2a). Lovastatin is detected as two components in an extract of fermentation broth; the predominant peak with a retention time of 17 min represents the open ring salt, a small amount of the closed lactone form elutes at 20.9 min. The maximum sulochrin produc-

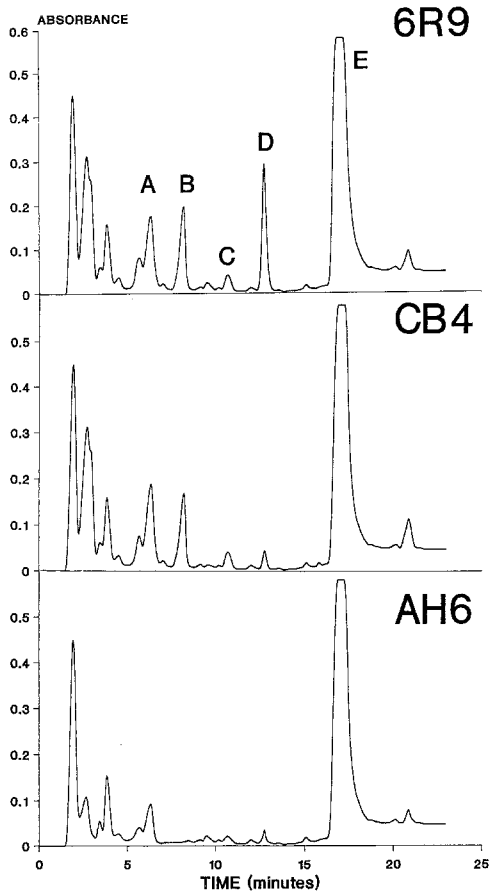

Fig. 2. HPLC profiles of extracts from culture broths of the parent and mutant cultures of *A. terreus*. Conditions are described in the METHODS section. A, triol acid; B, sulochrin; C, asterric acid; D, butyrolactone; and E, lovastatin.

tion was 1.0 g/l achieved at 16 days in shake flask fermentations which corresponded with the cessation of lovastatin production. Precursors to sulochrin were not detected in the whole-broth extracts.

As sulochrin was the primary impurity implicated in downstream processing problems, strain improvement was targeted toward decreasing its production while maintaining high lovastatin titers. In order to reduce the time and equipment constraints imposed by the HPLC assays, a TLC separation was developed as an alternative approach for a primary screen. As shown in Table 1, a thin-layer separation was developed which effectively separated the major co-metabolites. The visible limit of detection on HPTLC plates under UV light was 0.05 g/l sulochrin which was sufficient to detect mutants deficient in production of sulochrin. Under short-wave UV light, extracts could be examined for the absence of a rose-colored band with an R_f equivalent to sulochrin and a strong rose-colored band with an R_f equivalent to lovastatin. The HPTLC plates with concentrating zones were chosen over standard plates due to the sharpness of the bands which allow more accurate interpretations as compared to traditional TLC plates. The ability to load 12 samples per HPTLC plate and develop several plates within 15 min offered significant advantages in minimizing time and expense for the primary assay. However, routine quantitation of metabolites on plates via the densitometer was found to be less satisfactory for reproducible results.

TABLE 1

Separation of various metabolites of *A. terreus* by high-performance thin-layer chromatography

Compound	R_f[a]	Comments[b]
Emodin	0.87	Orange
Sulochrin	0.84	Yellow
Asterric	0.77	
Lovastatin[c]	0.71	Faint Yellow
Triol acid[c]	0.62	
Unknown	0.56	

[a] The solvent system used to develop HPTLC plates was acetonitrile : methanol : ammonium acetate, pH 5.0 (110 : 5 : 10, v/v/v).

[b] Color of the bands under visible light; under UV light all bands exhibited a rose color.

[c] Represents the open ring ammonium salt form of these compounds.

Microtiter fermentation

A 16-day shake flask fermentation was felt to be inefficient for a short-term strain improvement program. A less labor-intensive and inexpensive means of fermenting large numbers of isolates was needed in conjunction with the TLC assay. Accordingly, several complex media were tested as solid media to decrease the time required for fermentation of isolates. P-9 medium with agar was found to optimally produce sulochrin and lovastatin within 9 days as determined by TLC and HPLC methods described.

As a means of miniaturizing the solid medium fermentation and thus reducing the materials required, microtiter dishes were used in place of larger plates. A filter disc wetted in seed medium placed on the surface of the solid P-9 medium in a microtiter well was found to support excellent growth following inoculation with conidia from a master plate. The master plate containing 25 patch colonies was more easily utilized and stored than the many slants which are required for shake flask fermentation inoculum. Thus, the selection scheme shown in Fig. 1 was implemented with the microtiter fermentation

used for the primary screen. Shake flask fermentations followed by HPLC analysis were utilized for the secondary screen of useful isolates based on better quantitation of lovastatin and extensive experience in scaling the results of flask fermentations.

Improved mutants

A total of 1623 mutants were examined through the primary screen with 39 strains taken into the secondary stage. Following completion of the secondary screen, useful isolates were subcultured onto additional slants. Cultures were fermented in triplicates for confirmation and titers of lovastatin, sulochrin, and asterric acid confirmed as summarized in Table 2. Gradient HPLC profiles were generated for all strains. Strain AH6 did not produce sulochrin or asterric acid through the primary or secondary screens (Table 2); an HPLC profile (Fig. 2c) reveals the absence of peaks corresponding to these impurities as well as a decrease in butyrolactone production. Interestingly, some other strains exhibited decreased butyrolactone production in addition to decreased sulochrin. The two metabolites are produced by unrelated pathways [5,6,8,12]. However, it is now suspected that a butyrolactone-related metabolite [8,12] ran close to sulochrin on the HPTLC plates used for the primary screen. Because the mutants were selected during the work based on decreased intensity in the sulochrin band, they would also necessarily produce less butyrolactone. Subsequent diode array scans have identified a second butyrolactone compound from shake flask extracts. The highest lovastatin-producing strain, CB4, gave an average titer of 85 U/l, 16% more lovastatin than the parent culture, while producing 30% less sulochrin (Table 2; Fig. 2b). Two other strains, DF12 and BD10, showed more dramatic decreases in sulochrin production while also exhibiting greater lovastatin titers (Table 2).

Lovastatin biosynthesis has been documented to proceed via utilization of acetate units as precursors in polyketide elongation [11,18]. The biosynthesis of emodin and thus sulochrin also proceed through a polyketide pathway [8,18] presumably via a unique polyketide synthase and intermediates. Mutants such as CB4 appear to be capable of shunting more precursors toward lovastatin biosynthesis, thus reducing production of sulochrin-related compounds. Alternatively, the polyketide synthase involved in sulochrin biosynthesis could be rendered less efficient by mutagenesis. Interestingly, isolate AE11 is blocked in the production of all detectable polyketides (Table 2). As this strain grows normally and sporulates well, it appears to be blocked at an early essential step in polyketide biosynthesis.

Pilot-scale fermentation

A. terreus mutant CB4 was selected for scale-up efforts based on the higher lovastatin titers and lower levels of sulochrin production in shake flasks. In P-9 medium and the more concentrated P-7 medium, a 20% improvement in lovastatin titers was observed with culture CB4 when compared to the parent culture 6R9 in the same media (Table 3). In P-9 medium, CB4 produced 83% less sulochrin at the time of peak production confirming the observations at the smaller scale that this strain produced less of the impurity (Table 3). Fermentation characteristics in the 250-gallon fermentors were similar to those of the parent production culture. Culture CB4 fermentations exhibited typical dissolved oxygen profiles. Viscosity measurements indicated low peak values reflecting the

TABLE 2

Production of lovastatin and sulochrin by mutants of strain 6R9

Strain	Lovastatin		Sulochrin (g/l)	Asterric (g/l)
	U/l[b]	% of control		
CB4	85.0	116	0.7	0.1
AH6	61.0	84	0.0	0.0
DF12	76.0	104	0.3	0.1
BD10	79.5	109	0.1	0.0
BB8	61.0	84	0.1	0.0
AE11	ND[c]	0	0.0	0.0
6R9	73.0	–	0.9	0.1

[a] HPLC titers are the results of triplicate 16-day shake flasks in P-9 medium. Conditions are described in the METHODS section.

[b] Lovastatin titers are represented as normalized titers.

[c] ND, not detected.

TABLE 3

Summary of large-scale fermentations with cultures 6R9 and CB4

Culture	Medium	Lovastatin (U/l)[a]	Sulochrin (g/l)	Age (h)
6R9	P-9	48.5	1.2	308
6R9[b]	P-7	49.5	1.6	294
CB4	P-9	58.0	0.2	324
CB4	P-7	59.5	0.2	324

[a] Lovastatin and sulochrin titers shown are the peak values, lovastatin titers are normalized values.

[b] Data shown are the averages of 18 production-scale 6R9 fermentations in P-7 medium. All other data are from pilot-scale fermentations.

desirable morphological characteristics of CB4 broth, essential at the larger scale. Lower peak viscosity resulted from the use of the less concentrated P-9 medium as compared to P-7 medium (Fig. 3).

Sulochrin was detected in the broth at about 120 h and followed the concentration profile shown in Fig. 4. In P-9 medium, production by 6R9 reached a peak at 240 h and decreased over the final stage of the fermentation. Impurity production by the improved mutant peaked by

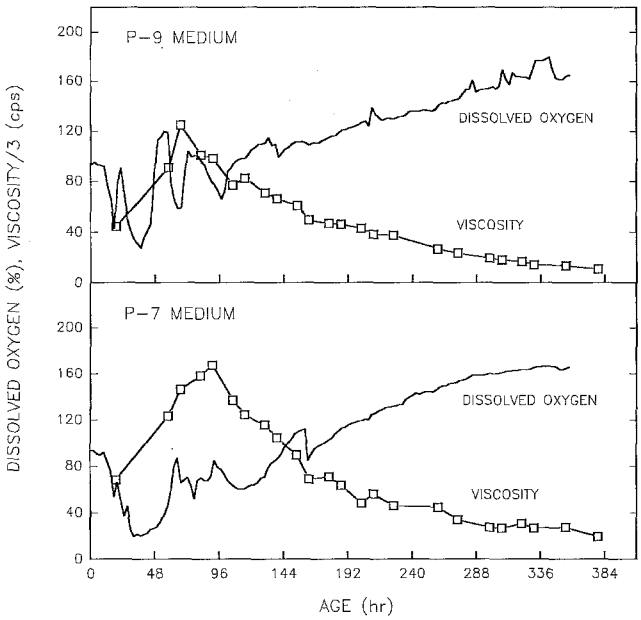

Fig. 3. Dissolved oxygen and viscosity measurements in pilot-scale fermentations of mutant CB4 using two production media.

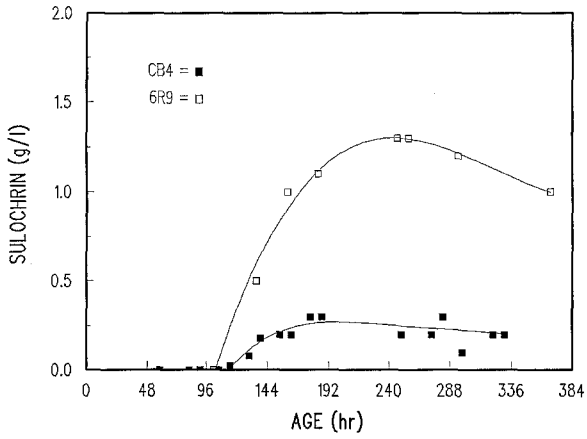

Fig. 4. Comparison of sulochrin production by parent culture 6R9 and mutant CB4 in pilot-scale fermentations using P-9 medium.

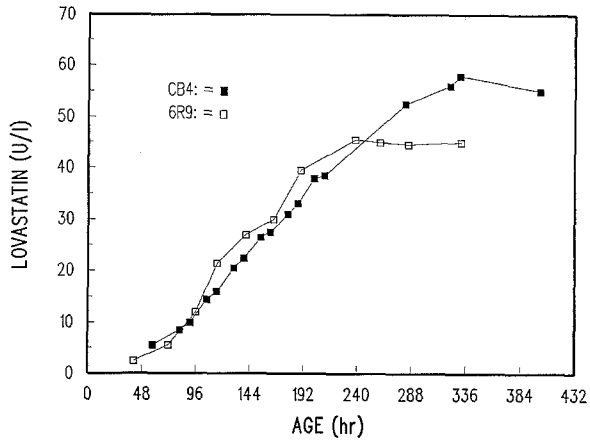

Fig. 5. Lovastatin production by cultures CB4 and 6R9 in pilot-scale fermentations using P-9 medium.

190 h and remained low throughout the run. Titer profiles of CB4 and 6R9 in P-9 medium (Fig. 5) indicate similar linear rates of lovastatin synthesis; however, CB4 sustained synthesis over an extended time.

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