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Bioconversion of the sodium salt of Simvastatin (MK-733) to 6-desmethyl-6-α-hydroxymethyl Simvastatin

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SUMMARY

An actinomycete (MA 6474, ATCC 53828) isolated from a soil sample (Mutare, Zimbabwe) was found to biotransform the sodium salt of Simvastatin (MK-733) to 6-α-hydroxymethyl MK-733, 6-β-hydroxymethyl MK-733, and 6-ring-hydroxy MK-733. The bioconversion efficiency to the desired compound, 6-α-hydroxymethyl MK-733, was enhanced by optimizing the physico-chemical parameters of the process. In shake flask cultures, addition of magnesium (0.125 mg/l Mg $SO_4 \cdot 7H_2O$) to the medium resulted in a five-fold increase in the rate of bioconversion to the α diastereomer. The ratio of bioconversion products (6-α-hydroxymethyl, 6-β-hydroxymethyl, and 6-ring-hydroxy MK-733) was regulated by pH. Process improvements and scale up in 23-l fermentors, which consisted of a controlled addition of substrate (MK-733), resulted in a 2-fold increase in alpha diastereomer production (42 vs. 79 U/ml) and a 23-fold rate increase in the formation of α-diastereomer. A high diastereomeric ratio (α: β = 9 : 1) facilitated downstream processing.

INTRODUCTION

Controlling and reducing the level of cholesterol in the bloodstream is now accepted as a major form of arteriosclerosis prevention [1]. In the past 3 years, the secondary metabolite of *Aspergillus terreus*, Lovastatin, and its semisynthetic derivative Simvastatin (Fig. 1) have been widely used in the therapeutic treatment of hypercholesterolemy [1]. Both compounds limit cholesterol synthesis by inhibiting 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis in humans and animals [1]. Continued efforts have focused on the search for new microbial metabolites that inhibit HMG-CoA reductase and ultimately possess hypocholesteremic activities [4,5,11]. In addition, existing HMG-CoA reductase inhibitors have been modified by microbial transformations or chemical reactions to find analogs with increased activity and better pharmacokinetics [17,19].

Many microorganisms and their enzyme preparations are able to perform site directed and stereospecific modifications of organic molecules that may be difficult or tedious to obtain through ordinary chemical methods [6,13,15]. The first microbial bioconversion of importance in the pharmaceutical industry was certainly the 11-α-hydroxylation of progesterone by *Rhizopus nigricans* [18]. Today a number of commercially important products (steroids, antibiotics and vitamins) are produced by microbial transformations [9,10].

The goals of the following studies were first to isolate a microorganism capable of hydroxylating the C6-methyl of MK-733 (sodium salt) preferentially in the α position. The second phase of our studies focused on optimizing the physico-chemical parameters of the bioconversion process to maximize production of the α-diastereomer and achieve a high diastereomeric (α/β) ratio. The final phase of the studies focused on developing a scalable bioconversion utilizing process improvements from both shake flask and fermentor studies.

MATERIALS AND METHODS

Chemicals

The complex media nutrients were: peptone, yeast extract and beef extract (Difco, Detroit, MI); NZ Amine Type E and hycase (Sheffield Products, Norwich, NY); soluble starch, calcium carbonate and 3-[*N*-Morpholino]propanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, MO); Cerelose (CPC, Chicago, IL); P2000 antifoam (Dow Chemical Co., Midland, MI); Sodium salt of MK-733 (Merck and Co., Rahway, NJ). All other chemicals were of reagent grade and were purchased from Fisher Scientific (Springfield, NJ).

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Media composition

ASM-2 agar medium containing (per liter of 5 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (PIPES)): $NaNO_3$, 0.17 g; $MgCl_2 \cdot 6H_2O$, 0.041 g; $MgSO_4 \cdot 7H_2O$, 0.049 g; $CaCl_2 \cdot 2H_2O$, 0.029 g; K_2HPO_4, 0.017 g; Na_2HPO_4, 0.012 g; $FeCl_3 \cdot 6H_2O$, 0.0011 g; disodium ethylenediaminetetraacetic acid, 0.0075 g; H_3BO_3, 0.0025 g; $MnCl_2 \cdot 4H_2O$, 0.0014 g; $ZnCl_2$, 0.0005 g; $CuCl_2$, 0.11 μg; $CoCl_2 \cdot 6H_2O$, 19 μg; $NaMoO_4 \cdot 2H_2O$, 0.0002 g; and Bacto agar, 15 g. The pH of the medium was adjusted to 7.3–7.5 with a 50% solution of tetramethyl ammonium hydroxide. After autoclaving, cycloheximide (sterilized by filtration through a 0.22-μm porosity membrane) was added to the medium to a final concentration of 50 μg/ml.

BAM-2 agar medium contained (per liter of distilled water): glucose, 10 g; hycase, 2 g; yeast extract, 1 g; beef extract, 1 g; and Bacto Agar, 15 g. The pH of the medium was adjusted to 7.0 to 7.2 with 5 N NaOH. After autoclaving, cycloheximide (sterilized by filtration through a 0.22-μm porosity membrane) was added to the medium to a final concentration of 50 μg/ml.

ATCC medium contained (per liter of distilled water): glucose, 10 g; soluble starch, 20 g; yeast extract, 5 g; NZ Amine Type E, 5 g; beef extract, 3 g; and peptone, 5 g. Calcium carbonate was added (1 g/l) after the pH of the medium was adjusted to 7.0 with 2 N sodium hydroxide.

BAM-2 modified (BAM-2 MOD) (contained per liter of distilled water): cerelose, 12 g; hycase, 3.5 g; yeast extract, 1.75 g; beef extract, 1.75 g; and 3-*N*-morpholino propanesulfonic acid (MOPS), 11.6 g. The pH of the transformation media was adjusted to 7.2 with 5 N sodium hydroxide.

BAM-2A MOD contained (per liter of distilled water): cerelose, 48 g; hycase, 14 g; yeast extract, 7 g; beef extract, 7 g; P2000, 37.5 ml; and $MgSO_4 \cdot 7H_2O$, 0.5 g. Glucose was sterilized seperately from the remaining medium components.

All media were dispensed into 250-ml and 2-l unbaffled Erlenmeyer flasks (50 ml and 500 ml, respectively), capped with metal closures and sterilized by autoclaving at 121 °C for 20 min.

Culture isolation

The isolation procedure for the bioconverting microorganism consisted of suspending 10–20 mg of soil in 1.0 ml of sterile distilled water, followed by soil dispersion for 2–3 min with a Branson model B-220 ultrasonic cleaning bath (Smith-Kline, Shelton, CT). A volume of 0.01 ml of the soil suspension and 0.1 ml of sterile distilled water were spread over the surface of ASM-2 agar medium. Colonies that developed on the ASM-2 agar medium were picked, transferred to individual plates of BAM-2 agar medium (one colony per plate), and incubated at 28 °C. After cultivation for 3–4 days, the isolate was spread over the surface of the BAM-2 plate and incubated for an additional 2–3 days.

Detection of bioconversion activity

The ability of the isolates to biotransform the sodium salt of MK-733 was detected by transferring colonies from the BAM-2 agar plates to 250-ml baffled flasks containing 50 ml of sterile BAM-2 broth. Approximately 500 cultures were screened. Following 6 days incubation at 28 °C on a rotary shaker at 220 rpm (2-inch throw), the substrate (MK-733) was added to the cultures to a final concentration of 100 U/ml. The cultures were incubated at 28 °C for an additional 24 h, after which the culture broth was analysed for the presence of residual substrate and substrate derivatives by High Performance Liquid Chromatography (HPLC) as described below.

Inoculum development and bioconversion

Cell suspensions were prepared by mixing equal volumes of the culture (MA 6474) and a 10% glycerol solution. This mixture was dispensed (1.5 ml) into cryogenic vials and stored frozen at − 70 °C. The microorganism has been deposited in the American Type Culture Collection (ATCC 53828).

A frozen cell suspension of MA 6474 was thawed and added to a 250-ml unbaffled Erlenmeyer flask containing 50 ml of ATCC medium. The culture was incubated for 3 days at 28 °C on a rotary shaker at 220 rpm (2-inch throw). A second stage seed was prepared by transferring 25 ml of the 3-day-old culture to a 2-l Erlenmyer flask containing 500 ml of ATCC medium and incubating it for 3 days at 28 °C at 180 rpm (2-inch throw). The bioconversion medium, 500 ml of BAM-2 MOD, was inoculated with 25 ml of a 3-day-old second-stage seed. The cultures were incubated at 28 °C on a rotary shaker at 180 rpm (2-inch throw) for a minimum of 3 days or until maximum growth, as estimated by packed cell volume. After 3 days, the sodium salt of MK-733 (sterilized by filtration using a 0.22-μm porosity membrane) was added to the culture in the indicated amount. The addition of substrate prior to 3 days resulted in cell death. Flasks were incubated under the same conditions described above for an additional 72 h.

Analytical methods

Acetonitrile (2 ml) was added to each whole broth sample (2 ml), mixed in a glass tube for 30 s, and centrifuged for 5 min in a Beckman model TJ-6 bench top centrifuge (Beckman Instruments, Fullerton, CA) at 3000 rpm. The supernatant was assayed by HPLC to quantitatively determine the concentrations of Simvasta-

tin and bioconversion products in culture broth. Approximately 80% of the substrate can be accounted for. The additional 20% is probably cell associated and is lost in the extraction. The analytical system employed a HPLC (Waters, Milford, MA), equipped with an autosampler (WISP 71013), and a UV detector (Model 490). The sample (10 μl) was injected into a Halmiton PRP-1 (5-μm, 4.1 × 150 mm) column (Ranin, Woburn, MA) maintained at 40 °C. The following gradient was used at a flow rate of 1.1 ml/min: 10–70% B in 3 min, hold 2 min; 70–100% B in 6 min; where solvent A was 20 mM ammonium phosphate (pH 6.1) in water : acetonitrile (9 : 1); and solvent B was water : acetonitrile (3 : 7). The eluant was monitored at 237 nm and 0.1 AUFS. The retention times in this system were 9.4, 9.9, 10.3, and 13 min for 6-ring hydroxy, α-diastereomer, β-diastereomer, and MK-733, respectively.

Biomass was measured by cell dry weight and packed cell volume (PCV) estimations. Cell dry weight determinations were performed using a 0.22-μm filter (Millipore Corporation, Bedford, MA). Packed cell volume determinations were performed by centrifuging 10 ml of whole broth at 3000 rpm for 10 min. Glucose concentrations in the supernatant were analyzed with a glucose analyser (Model 2, Beckman Instruments, Fullerton, CA).

Bioconversion scale-up

The pH experiments were conducted in 2-l Biolaffite vessels (LSL Biolaffite, Inc., Princeton, NJ). A volume of 1.5 l of BAM-2 MOD and 3.8 ml of P2000 antifoam were autoclaved at 121 °C for 40 min. The polygylcol antifoam P2000 was added to the medium to reduce the foaming of the proteinaceous medium. A second stage seed volume of 75 ml was used to inoculate the fermentor. The fermentations were performed under the following conditions: temperature, 28 °C; agitation, 400 rpm; aeration, 1 l air/min. After maximum growth was achieved (3 days) the sodium salt of MK-733 (the indicated amount) was added and the bioconversion monitored.

Scale-up studies of the shake-flask bioconversion process were performed in 23-l fermentors (Chemap Inc., South Plainfield, NJ) using all the previously demonstrated process improvements. A volume of 15-l of BAM-2A MOD and 37.5 ml of P2000 antifoam were sterilized in situ at 121 °C for 20 min. Glucose was autoclaved separately and added post sterilization to the fermentor. Two 2-l Erlenmeyer flasks of second-stage seed were pooled (1000 ml) and used to inoculate the fermentor. The operating conditions of the fermentor were: temperature, 28 °C; aeration, 5 l air/min; and agitation, 500 rpm. The operating conditions of the fermentor were such that the dissolved oxygen was always greater than 80% of saturation. After maximum growth was achieved, the sodium salt of MK-733 was added and the bioconversion monitored.

RESULTS

Isolation of the bioconverting microorganism

The selected isolate (MA 6474) is capable of bioconverting the sodium salt of MK-733 into 6-α-hydroxymethyl MK-733 (α-diastereomer), 6-β-hydroxymethyl MK-733 (β-diastereomer) and 6-ring-hydroxy MK-733 (Fig. 1) [8]. The organism grows on solid medium as branching filaments with a diameter of approx. 0.76 μm and produces chains of 2–10 spores. The spores are spherical to ovoid with dimensions of approx. 0.76 × 1.0 μm and the spore chains exhibit coiling. The spores appear to be borne in a sporangium which measures 4–10 μm in diameter. In liquid culture, the orga-

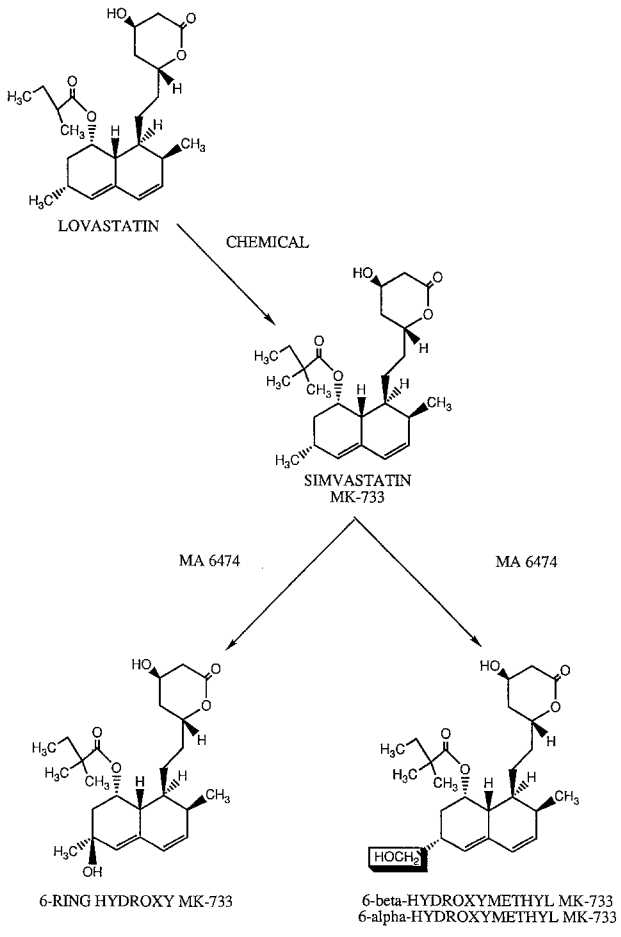

Fig. 1. Bioconversion of MK-733 to 6-α-hydroxymethyl MK-733, 6-β-hydroxymethyl MK-733 and 6-ring-hydroxy MK-733 by the actinomycete MA 6474.

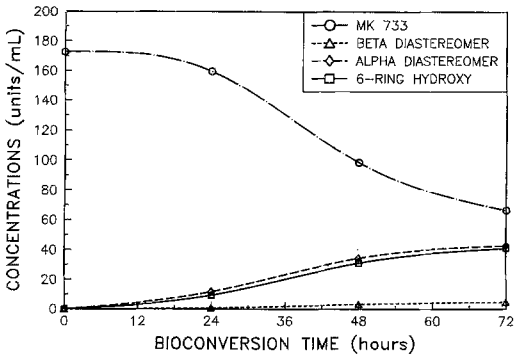

Fig. 2. MK-733 bioconversion kinetics in 2-l shake flask by the actinomycete MA 6474.

nism grows as branching filaments that later fragment, and does not produce spores. Based on the morphological characteristics of the isolate, it appears to be a bacterium which is a member of the order Actinomycetales.

Bioconversion kinetics

Time course experiments were performed in 2-l shake flasks using BAM-2 MOD medium to obtain initial profiles of the bioconversion kinetics. A typical bioconversion time course is presented in Fig. 2. Approximately 180 U/ml of MK-733 were added to a 72-h culture. The majority of the bioconversion occurred between 24 and 72 h. Peak titers of bioconversion products were achieved

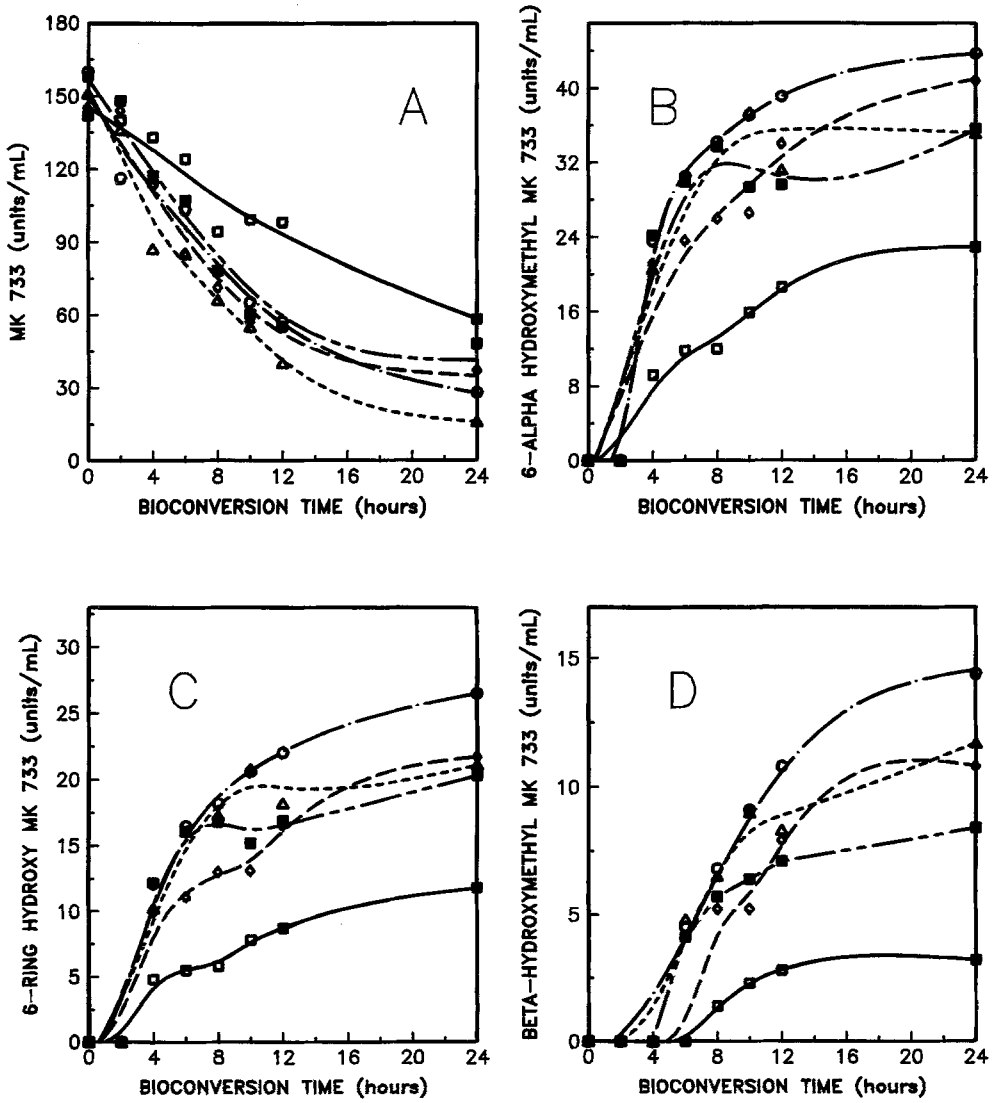

Fig. 3. Effect of magnesium sulfate on the bioconversion kinetics of MK 733 by the actinomycete MA 6474. $MgSO_4 \cdot 7H_2O$ concentrations are: □, control; ●, 0.0625 g/l; ▲, 0.125 g/l; ○, 0.25 g/l; ■, 0.5 g/l. **A.** Substrate bioconversion kinetics. **B.** 6-α-hydroxy-methyl MK-733 production kinetics. **C.** 6-ring-hydroxy MK-733 production kinetics. **D.** 6-β-hydroxymethyl MK-733 production kinetics.

TABLE 1

Effect of magnesium sulfate on the bioconversion of MK-733

Concentration $MgSO_4 \cdot 7H_2O$ (g/l)	6-α-hydroxymethyl MK-733 concentration (% of control)[a]	MK-bioconversion rate (% control)
Control[b]	100	100
0.0625	129	217
0.125	131	192
0.25	137	203
0.50	128	208
1.0	101	142

[a] Maximum titer.

[b] BAM-2 MOD medium without additional magnesium sufate (see MATERIALS AND METHODS for composition).

72 h after substrate addition. The average rate of α-diastereomer production was 1.13 $U\,l^{-1}\,h^{-1}$. α- and β-diastereomer maximum concentrations were 42.9 and 4.8 U/ml respectively, and 6-ring-hydroxy MK-733 reached a maximum concentration of 41.2 U/ml. The maximum average rate of substrate consumption (2.55 $U\,l^{-1}\,h^{-1}$) occurred between 24 and 48 h. These data will serve as a reference for future experiments.

Effect of magnesium sulfate

The effect of various inorganic salts (KH_2PO_4, $FeSO_4$, $MgSO_4$, $MnSO_4$) on the bioconversion of MK-733 was studied at the shake flask scale. These salts were added to the medium prior to autoclaving. Under the conditions tested, only magnesium sulfate was found to have a significant stimulatory effect on the bioconversion of MK-733. Faster rates of substrate utilization and product formation were observed when $MgSO_4 \cdot 7H_2O$ was added in the concentration range of 0.0625 to 0.5 g/l (Fig. 3). A two-fold increase in substrate consumption rate (Table 1) and a five-fold increase in the rate of α-diastereomer production (Fig. 3) was obtained when $MgSO_4 \cdot 7H_2O$ was added at a concentration of 0.125 g/l. No significant increase in biomass was observed.

To insure that the increase in substrate consumption and in the rate of bioconversion was caused by magnesium, additional shake flask experiments were performed in which magnesium chloride was used as an alternative source of magnesium. Magnesium sulfate and magnesium chloride additions produced the same stimulatory effects on substrate consumption rate and α-diastereomer production (Table 2). Like $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$ at low concentrations (0.0625–0.5 g/l) produced an increase in the production rate of the α-diastereomer. Concentrations of magnesium chloride above 0.5 g/l were inhibitory to growth and bioconversion.

To determine if magnesium was stimulating growth or the bioconversion reaction, magnesium sulfate (0.125 g/l final concentration) and MK-733 were added simultaneously to production medium containing a 3-day-old culture. The addition of magnesium sulfate after complete growth, resulted in faster rates of product formation.

Effect of pH

Studies to determine the optimal pH for bioconversion were performed in 2-l stirred vessels. The pH of the medium was not regulated during the growth phase (first 72 h). After growth was completed, the substrate (MK-733) was added, and the pH of the broth adjusted to and then controlled at the desired value (6.7, 7.0, 7.3, 7.9, 8.2 and 8.5). Controlling pH during the bioconversion of MK-733 to 6-α-hydroxy methyl MK-733, 6-β-hydroxymethyl MK-733, and 6-ring-hydroxy MK-733 resulted in

TABLE 2

Effect of magnesium on the bioconversion of MK-733

Concentration of magnesium (g/l)	Source of magnesium	MK-733 bioconversion rate ($Ul^{-1}\,h^{-1}$)	6-α-hydromethyl MK-733 (% control)
0	–	3.91, 3.67	100
0.006	$MgSO_4$	7.5	171
0.006	$MgCl_2$	10.8	164
0.012	$MgSO_4$	9.16	209
0.012	$MgCl_2$	8.92	176
0.024	$MgSO_4$	8.58	183
0.024	$MgCl_2$	8.18	137
0.05	$MgSO_4$	8.58	136
0.05	$MgCl_2$	9.25	133

a shift in the final ratio of these products. Poor bioconversion (7%) was observed when the bioconversion was controlled at a pH of 6.7. The maximum yield of α-diastereomer (approx. 70 U/ml) occurred when the pH was controlled at 7.3 (Fig. 4A). As the pH increased, the production of α- and β-diastereomer decreased while the production of 6-ring-hydroxy MK-733 increased (Fig. 4A,B). The highest ratio of α/β diastereomers was obtained when the bioconversion was controlled at 8.0–8.2 (Fig. 4C).

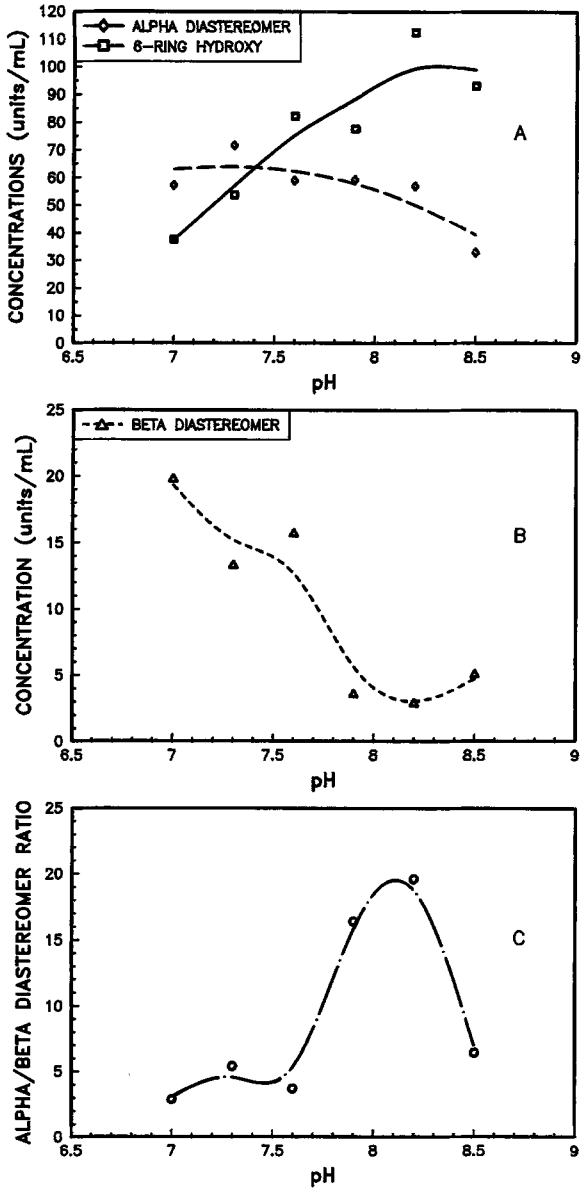

Fig. 4. Effect of pH control, during the bioconversion phase, on the MK-733 bioconversion products. Fermentations were performed in 23-l fermentors. **A.** Effect on α-diastereomer and 6-ring-hydroxy production. **B.** Effect on β-diastereomer production. **C.** Effect on the ratio of α/β diastereomers.

Medium strength

The effect of medium strength on the bioconversion of MK-733 was studied in shake flask cultures. Medium concentrations ranging from 1-fold to 4-fold (1× to 4×) were studied. Glucose was autoclaved separately from the remaining medium ingredients to prevent caramelization. As medium concentration increased (1× to 4×), the length of the growth phase increased from 72 h to 144 h. Increasing medium strength to 4× resulted in a 7.5-fold increase in cell production (measured by cell dry weight) and a 1.25-fold increase in α-diastereomer hydroxylation.

Bioconversion scale-up

The scaled-up bioconversions were performed in 23-l fermentors using the previously demonstrated improvements (addition of magnesium salts, pH control of the bioconversion phase and increased medium strength). The medium used in fermentor studies was BAM-2A MOD. Growth was followed by packed cell volume (PCV), carbon evolution rate (CER), and oxygen uptake rate (OUR). Preliminary bioconversion studies in shake flasks suggested that the concentration of β-diastereomer was greater when the concentration of substrate fell below 100 U/ml. Therefore, a subsequent shot of MK 733 was added to the fermentor 1.5 h after the bioconversion was started in an effort to maintain low β-diastereomer concentrations (less than 12%) and to prevent substrate limitation during the bioconversion.

A maximum PCV of 15% was obtained when less than 50% of glucose (20 g/l) was consumed (Fig. 5B). The substrate, MK-733, was added after maximum CER (11 mmol l^{-1} h^{-1}) was achieved. A subsequent addition of MK-733 (100 U/ml) was made at 1.5 h. To facilitate downstream isolation and product recovery, the bioconversion was stopped when the ratio of α/β diastereomer reached and/or exceeded 12%. Bioconversion broths containing concentrations of α/β greater than 12% were not desirable due to the difficulty in separating the α- and β-diastereomers. The rate of α-diastereomer production was 26 U l^{-1} h^{-1} (Fig. 5A). This represents a 23-fold increase in production rate over shake flasks. A ratio of α/β diastereomer of better than 9 : 1 was obtained 2 h after the bioconversion was initiated.

DISCUSSION

An actinomycete was isolated after extensive microbial screening capable of biotransforming MK-733 into a mixture of 6-α-hydroxymethyl MK-733, 6-β-hydroxymethyl MK-733, and 6-ring-hydroxy MK-733. Process

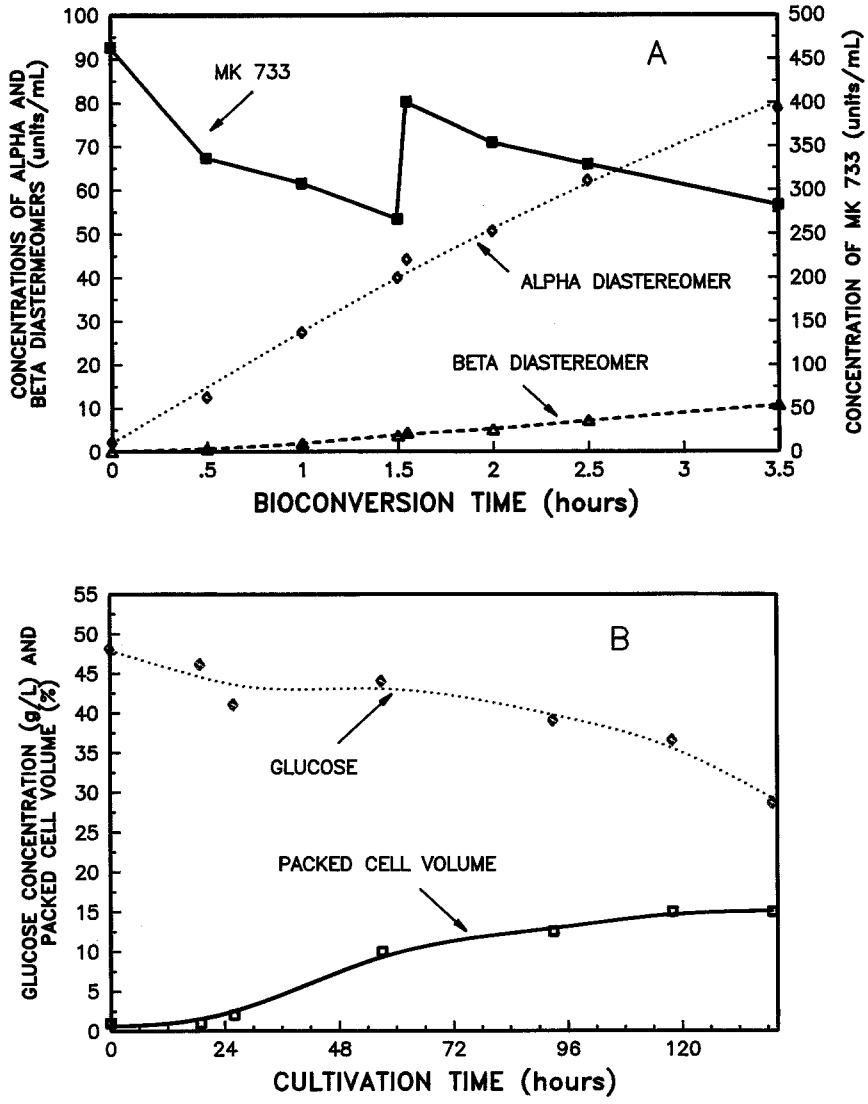

Fig. 5. A 23-l bioconversion profile using demonstrated process improvements (pH control, magnesium sulfate addition, increased medium strength). **A.** MK-733 bioconversion kinetics by the actinomycete MA 6474. **B.** MA 6474 growth kinetics and glucose consumption kinetics.

development studies showed that both the rate of bioconversion and the α/β diastereomeric ratio were controlled by magnesium ion and pH, respectively. Process improvements achieved a two-fold increase in α-diastereomer production and a 23-fold increase in the bioconversion rate over shake flask performance. The ability to enhance α diastereomer isolation through pH control greatly facilitated down stream isolation and purification (data not shown).

Magnesium appears to stimulate the rate of the bioconversion, but does not appear to increase the rate by increasing the amount of cells present and it does not effect the yield of bioconversion products. The reason why magnesium stimulates the enzymatic activity has yet to be determined. Similar stimulation of other microbial biotransformations by inorganic ions ($MgSO_4$, K_2HPO_4, $FeSO_4$) have been reported in the literature [3,7]. One hypothesis is that magnesium may be required by the enzyme(s) of the hydroxylation reaction as a cofactor that couples with the enzyme or with the substrate [16]. It is also possible that magnesium may serve as a membrane stabilizer and/or aid in transport. The faster rates of biotransformation have resulted in a shorter cycle time for maximum bioconversion.

Controlling pH of the MK-733 bioconversion provides a way to enhance production of desired bioconversion

products (α-diastereomer) while minimizing the production of undesirable products (β-diastereomer). It appears that the diastereomeric position at which the enzyme(s) hydroxylates the 6 prime methyl of MK-733 is influenced by the pH of the bioconversion phase. All three bioconversion products (α- and β-diastereomer, and 6-ring-hydroxy) were produced at the various pH values tested. However, enhanced production of the α-diastereomer was achieved when the bioconversion was controlled at a pH of 8.2. Similar reports of pH influencing bioconversions exist in the literature [2,12,14]. The ability of pH to influence the position of the hydroxylation may imply that three distinct enzymes exist, each having their own pH optimum. Such was the case in the transformation of syringic and vanillic acids by the laccases of *Rhizotonia praticola* and *Trametes versicolor* [12]. Studies focusing on identifying the enzyme(s) responsible for the MK-733 bioconversion reaction have not been completed and warrant further investigation.

The scale-up of the bioconversion from shake flasks to laboratory fermentors (23-l) was successfully performed. As was previously stated, all process improvements ($MgSO_4$ additions, pH control, increased medium strength) were implemented. The 23-fold increase in the rate of α-diastereomer synthesis was the combined result of all process improvements. The 1.8-fold increase in α-diastereomer production over initial shake flask studies was most likely attributed to the increase in cell yields from the greater medium strength. Substrate did not appear to be a limiting factor in the bioconversion nor was β-diastereomer production suppressed by high concentrations of MK-733. Even though the undesirable β-diastereomer was not suppressed, its concentration was maintained at an acceptable level (less than 12%) through pH control at 8.2.

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