Kor. J. Appl. Microbiol. Biotech.
Vol. 18, No. 3, 280-285 (1990)

Enhancement of Biotransformation Yield in 11 α-Hydroxylation of Progesterone by Continuous Addition of the Substrate

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Progesterone의 연속첨가에 의한 11α-hydroxyprogesterone으로의 생물전환수율의 증대

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Biotransformation of progesterone to 11 α-hydroxyprogesterone by growing cells of *Rhizopus nigricans* was investigated. As the concentration of progesterone increased, the specific growth rate of *R. nigricans* decreased linearly, and consequently the conversion yield lowered. The hyphae of the microorganism were observed to become thicker, shorter, and more densely branched at high concentrations of progesterone. In order to improve the process productivity, biotransformation was conducted with continuous addition of progesterone. When the substrate was added continuously at a rate of 0.86 g/hr for 30 hrs, overall conversion yield reached upto 56% while a single addition of the same amount of progesterone yielded about 40% conversion. When additional feeding of glucose was carried out upon its depletion, an improved bioconversion yield upto 68% was obtained.

Corticosteroids are, at present, produced from natural sterols via various chemical and biological conversion steps (1, 2). Biotransformation of progesterone to 11 α-hydroxyprogesterone has been known as a key step in the synthetic process of corticosteroids (3).

Low solubility of progesterone in water was considerd as one of the major drawbacks in the biotransformation at high concentration of progesterone is, therefore, considered essential for economy of the bioconversion process (4) However, biotransformation yield decreases as the concentration of progesterone increases, and much efforts have been made to increase the yield at elevated concentrations of the substrate (5-8). Various organic solvents and surfactants have been used to increase the concentration of progesterone in the broth (7-9). It has been known that the use of surfactant caused serious problems in separation processes (10). Organic solvents even have toxic effect on cell growth (9).

In our laboratory, we have been investigating the biotransformation of progesterone to 11 α-hydroxyprogesterone by using *Rhizopus nigricans*. It was observed that cell growth was inhibited by addition of progesterone which is practically insoluble in water, and consequently the conversion yield was significantly reduced. To improve the process yield, we have investigated the biotransformation process with a continuous addition of progesterone, and obtained quite promising results. The results are reported in this paper.

Key words: 11 α-Hydroxylation, progesterone, *Rhizopus nigricans*, continuous addition of progesterone, morphological change
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Experimental

Materials

Progesterone, 11 α-hydroxyprogesterone and Tween 80 were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Sabouraud dextrose agar and potato dextrose agar were obtained from Difco. The isopropanol and n-hexane used in mobile phase of HPLC were from Burdick and Jackson Labs (Muskegon, MI, U.S.A.). All other chemicals were of reagent grade.

Microorganism and culture media

Rhizopus nigricans ATCC 6227b was used to transform progesterone to 11 α-hydroxyprogesterone. Medium for slant culture was the same as that used by Hanish *et al.*, (10). Medium composition for cultivation of microorganism was as following; 2.5% glucose, 1.25% casamino acid, 0.1% KH_2PO_4, 0.05 KCl, 0.05% $MgSO \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 4.5 with 4 N-HCl.

Preculture in shake flasks

The spores from agar slant were inoculated to a 500 m*l* baffled flask containing 100 m*l* of culture medium, and progesterone was added for the induction of enzyme system to a final concentration of 0.5 g/*l*. The cultivation was carried out on a rotary shaker (200 rpm, Korea Vision Scientific, Seoul, Korea) at 28°C.

Biotransformation in a jar fermenter

Biotransformation was performed in a 2 liter jar fermenter (Bioengineering AG, Switzerland). Inoculum of 100 m*l* was transferred from the preculture flask into the fermenter with 1.3 liter working volume. Progesterone suspended in Tween 80 solution of 0.01% (v/v) was added to the fermenter at desired time of cultivation. The operating conditions were; temperature 28°C, agitation speed 450 rpm, aeration rate 0.5 vvm. When progesterone was added continuously, Masterflex pump (Cole Parmer, Chicago, IL, U.S.A.) was used at predetermined flow rates. 15 m*l* culture broth was taken for analysis at the desired intervals during biotransformation.

Determination of dry cell weight

Broth taken from the fermenter was filtered through a Whatman filter paper (No.1) and filtered mycelia were washed with four volumes of ethanol followed by one volume of distilled water. The mycelia were then dried at 105°C for 24 hr and dry cell weight was determined.

Microscopic observation of microorganism

Broth of about 2 m*l* was sampled, and morphology of microorganism was investigated by using a light microscope (CARL ZEISS Model JENAMED 2, East Germany).

Analysis of steroid compounds

Broth taken from the fermenter was extracted with two volumes of methylene chloride, and organic phase was filtered through a membrane filter (pore size 0.45 m, Millipore, MA, U.S.A) before injecting into HPLC (Hitachi Model 655A-12, Tokyo, Japan). The column (30 cm × 4.6 mm I.D.) used was packed with nominal 10 μm silica gel (μ-porasil, Waters Assoc., Milford, MA, U.S.A). The mobile phase was a mixture of n-hexane and isopropanol (80:20 (v/v)) and the flow rate was 1.5 m*l*/min. The column eluate was monitored at 254 nm. Peak areas were calculated with an integrator (Model D-2000, Hitachi Co., Tokyo, Japan).

Glucose determination

Concentration of glucose was measured by DNS method (11).

Results and Discussion

General behavior of biotransformation

Wnen a single addition of progesterone was conducted, the total quantity of progesterone suspended in Tween 80 solution was added 9 hours of cultivation.

Time course of biotransformation of progesterone to 11 α-hydroxyprogesterone by *R. nigricans* was shown in Fig. 1. As increasing the concentration of progesterone, the conversion yield decreased; the yield lowered from 60% to 40% as progesterone concentration increased from 5 g/*l* to 20 g/*l*. It was also observed that the production rate of 11 α-hydroxyprogesterone decreased at the early phase of the biotransformation as the concentration of progesterone increased. Studies on the biotransformation of progesterone proposed (4) that when progesterone was added at a level far exceeding its solubility, solid progesterone would dissolve gradually into solution

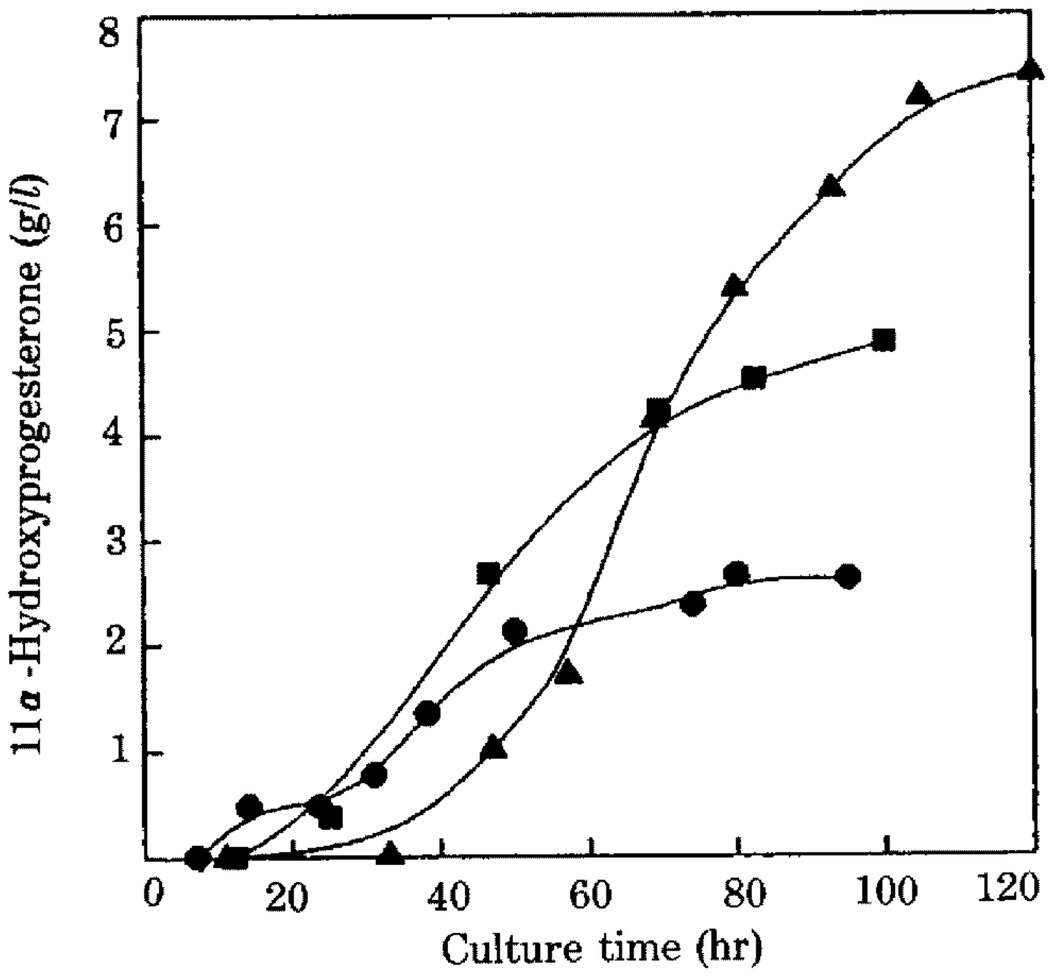


Fig. 1. Time course of biotransformation of progesterone to 11-α hydroxyprogesterone by *Rhizopus nigricans* at different concentration of progesterone.
Progesterone was added at 9 hr of cultivation.
Progesterone concentrations were; 5 g/*l* (●), 10 g/*l* (■), 20 g/*l* (▲).

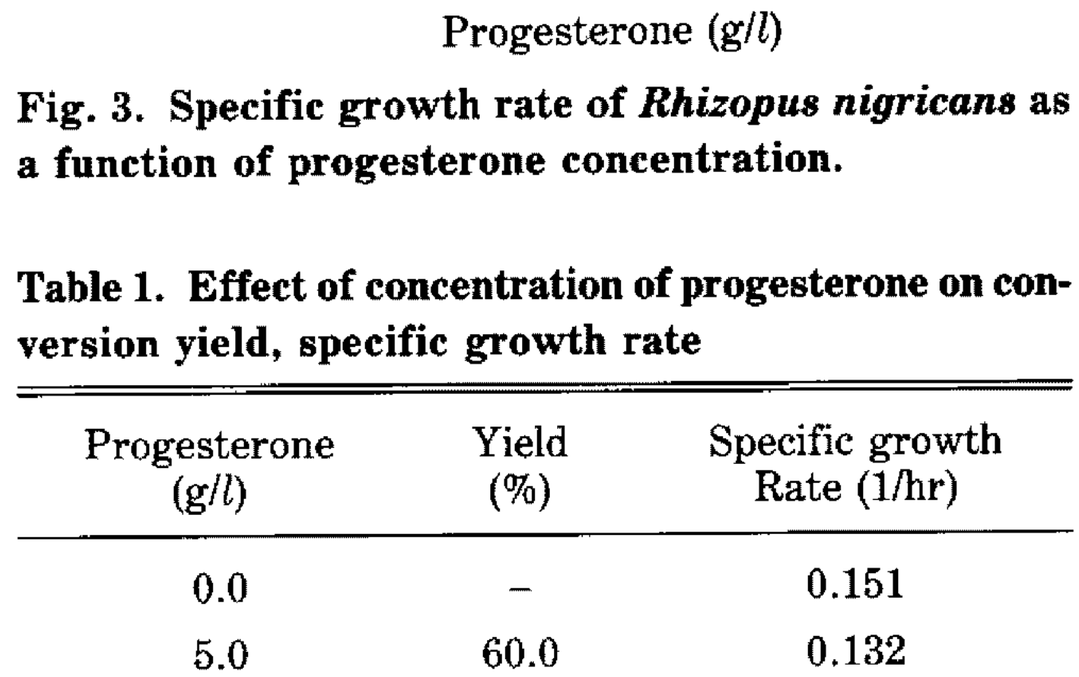


Fig. 3. Specific growth rate of *Rhizopus nigricans* as a function of progesterone concentration.

Table 1. Effect of concentration of progesterone on conversion yield, specific growth rate

Progesterone (g/*l*)	Yield (%)	Specific growth Rate (1/hr)
0.0	–	0.151
5.0	60.0	0.132
10.0	55.7	0.111
20.0	40.0	0.091

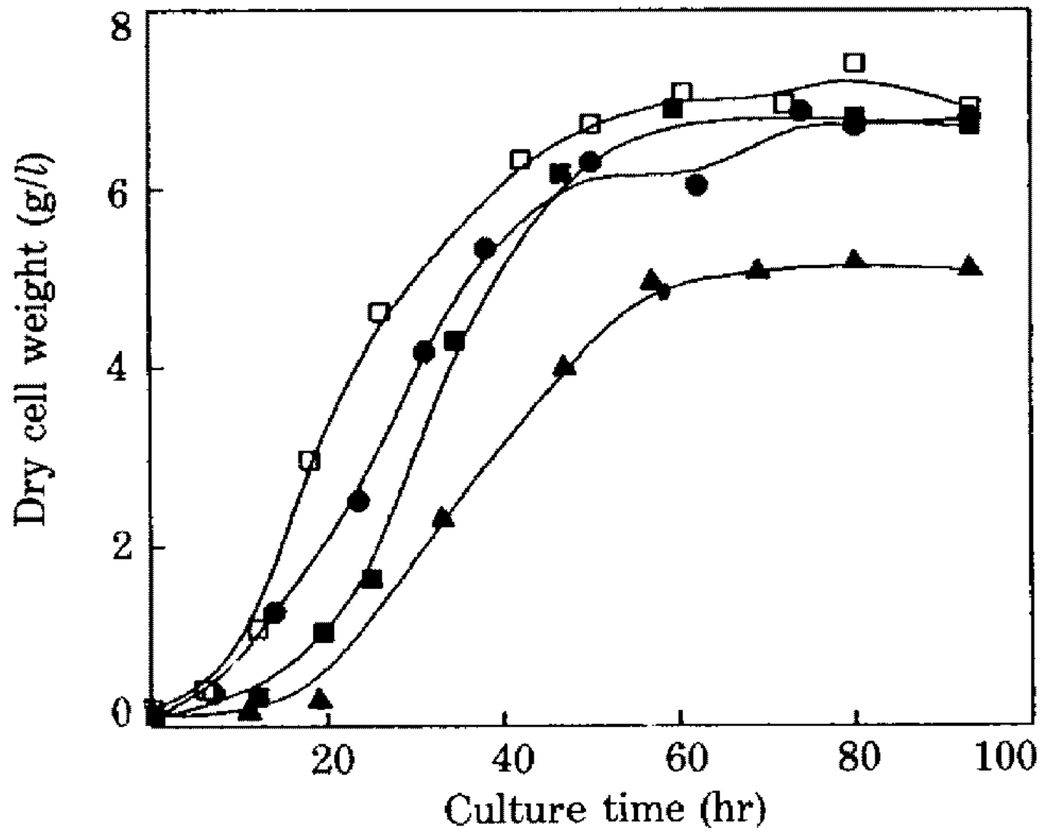


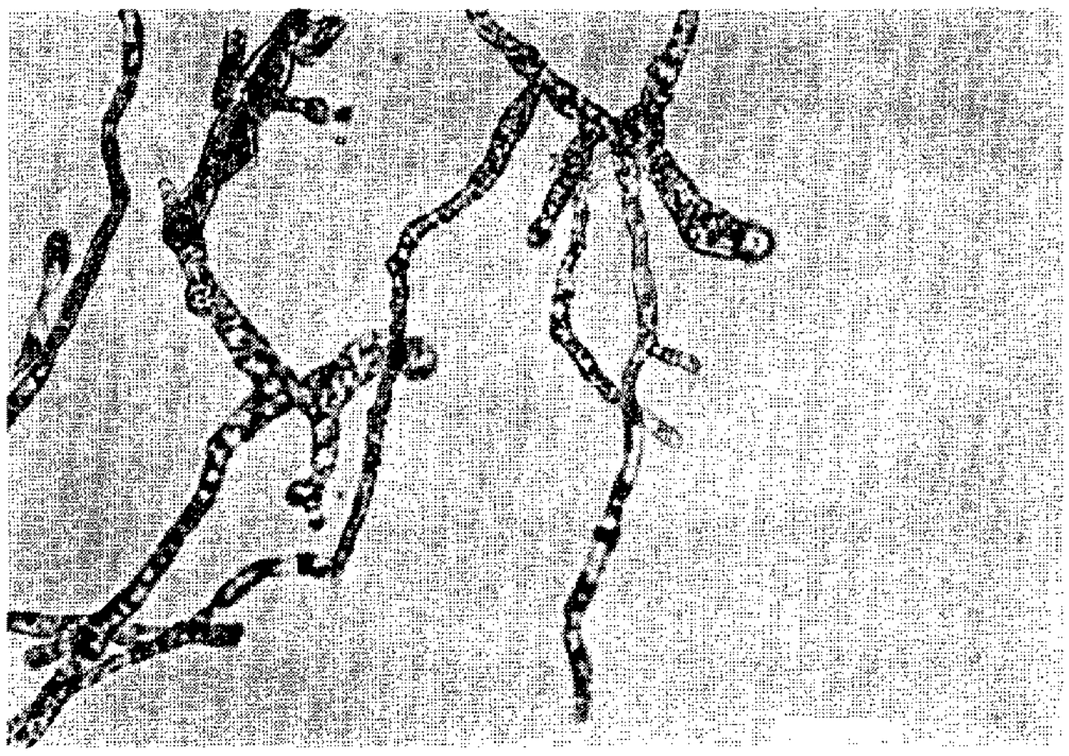
Fig. 2. Time course of cell growth at different concentration of progesterone.
Experimental conditions were the same as described in Fig. 1.
Progesterone concentrations were 0 (□), 5 g/*l* (●), 10 g/*l* (■), 20 g/*l* (▲).

and dissolved progesterone was transformed by the action of hydroxylase of the microorganism. It was also proposed (4) that the dissolution rate of progesterone is directly proportional to the concentration of solid progesterone dispersed in solution, and consequently, the conversion rate would increase as the concentration of progesterone increases.

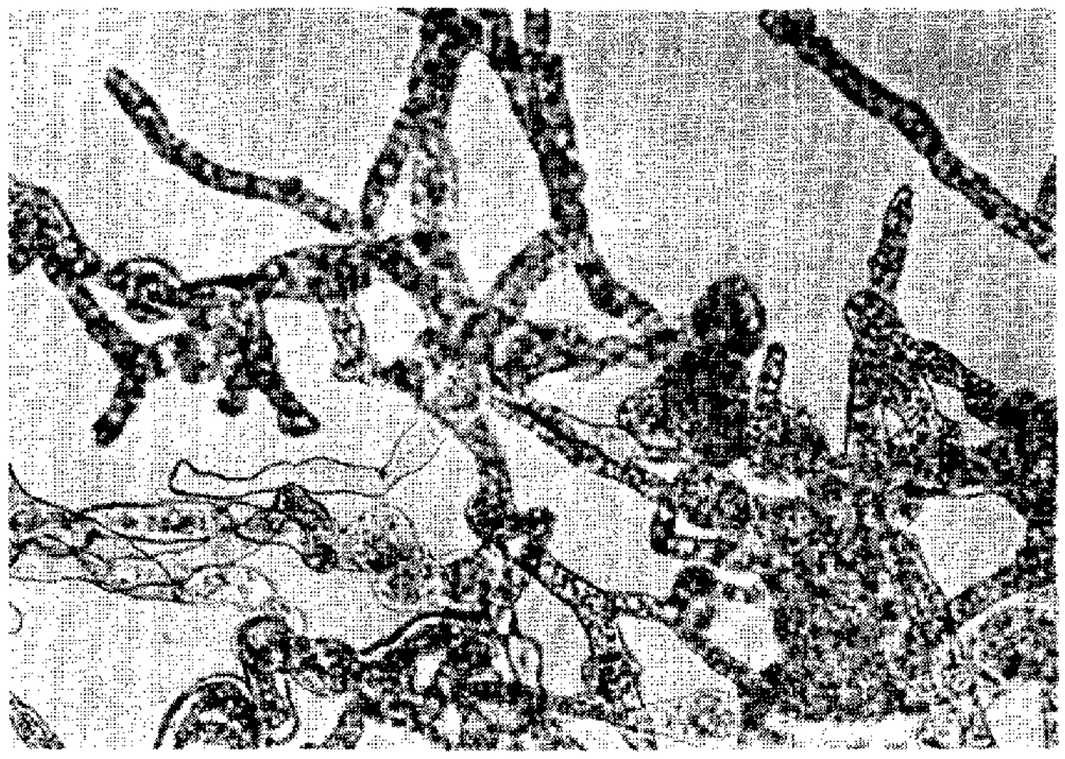
As an attempt to explain the result obtained in Fig. 1, the time course of cell growth was determined at different concentration of progesterone. As shown in Fig. 2, an increase in the concentration of progesterone resulted in reductions of specific growth rate and final biomass concentration in the broth. The specific growth rate of *R. nigricans* was calculated from Fig. 2 as a function of progesterone concentration (Fig. 3). The specific growth rate decreased linearly as the concentration of progesterone increased. When considering the above results, it is likely that the reduction of conversion rate at the early phase of biotransformation was due to the decrease cell growth in the presence of progesterone. Results obtained are summarized in Table 1. It seems most rational to say that biotransformation is related to the cell mass and the growth of *R. nigricans* is also inhibited by excess progesterone dispersed in the broth.

Microscopic observations

In order to investigate the morphology of *R.*



(a)



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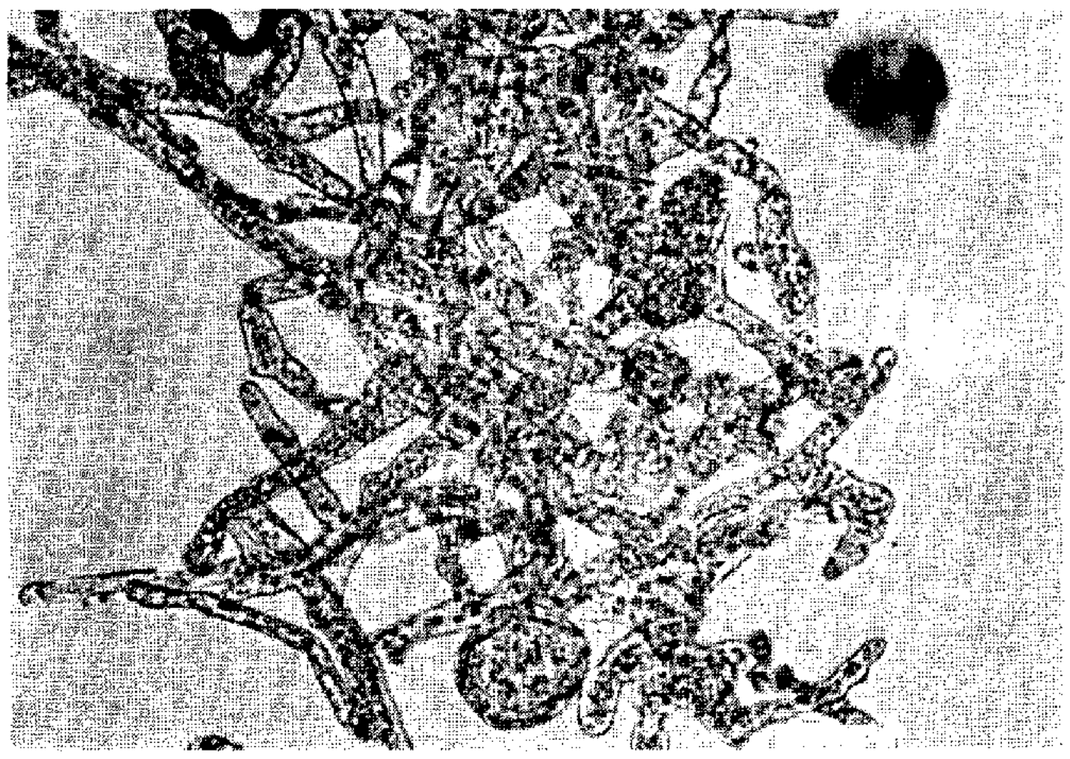

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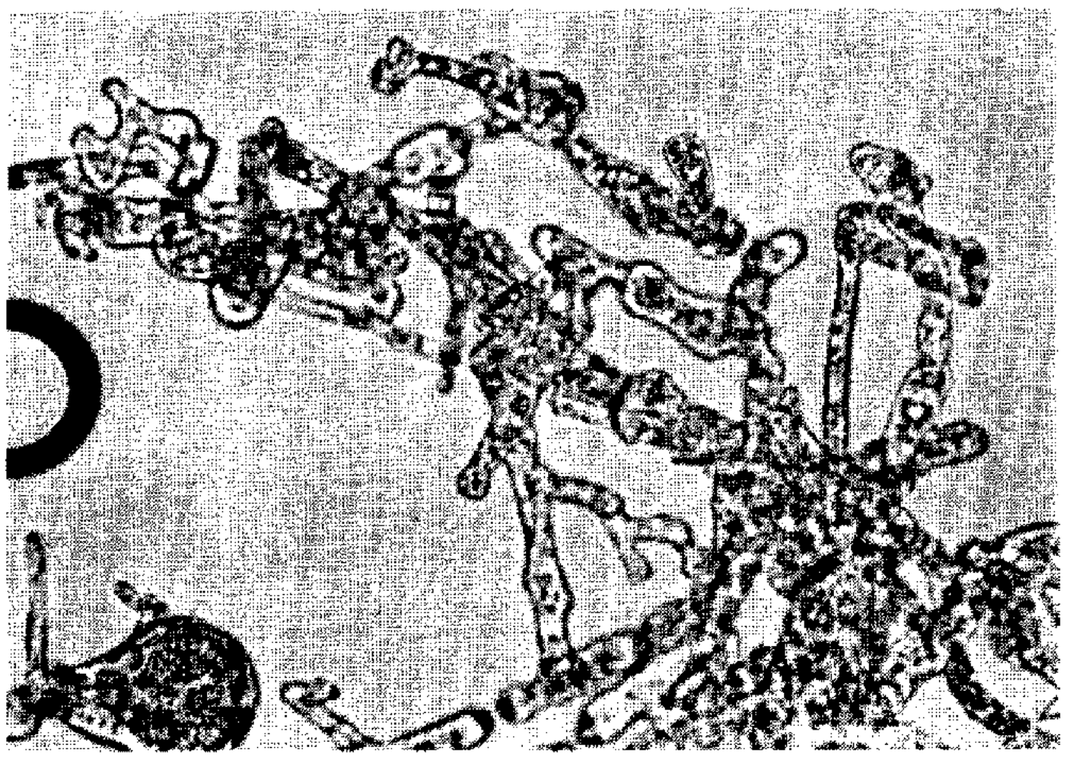

(d)

Fig. 4. Microphotographs of *Rhizopus nigricans* before and after addition of progesterone.
Progesterone of 20 g/*l* was added at 9 hr of cultivation.
Magnification was 80 fold.
(a) before addition of progesterone
(b), (c) and (d) were at 12 hr, 24 hr and 36 hr after addition of progesterone, respectively.

nigricans in the presence of progesterone, microscopic observations were carried out. Fig. 4 shows the change in mycelial morphology before and after the addition of 20 g/*l* progesterone. The hyphae were long, thin, and sporadically branched before addition of progesterone (Fig. 4 (a)) and they became thicker, shorter, and more densely branched after its addition (Fig. 4 (b), (c), (d)). It was reported that when *Aspergillus niger* was grown at higher agitation speed, the hyphae of microorganism were shown to be thicker, highly septated, more densely branched, and twisted (12, 13). Generally, a mycelial hypha only extends in length at the extension zone although apical growth is supported by protoplasm in the non-extending part of the hypha.

Morphological change of *R. nigricans* in the presence of progesterone seems due to many factors such as shear stress exerted to mycelia, certain physiological impact under high substrate concentration and some physical damage on the extension zones of hyphae. These factors are considered to result in, in a complex way, abnormal morphologies and some retardation of cell growth. Further detailed researches are necessary on the mechanism of morphological change and its possible utilization to improve the process productivity.

Effect of continuous addition of progesterone on biotransformation

In order to reduce the inhibition of cell growth by progesterone, and there by to increase the bioconversion yield, continuous addition of progesterone was carried out. The addition rate was determined by div-

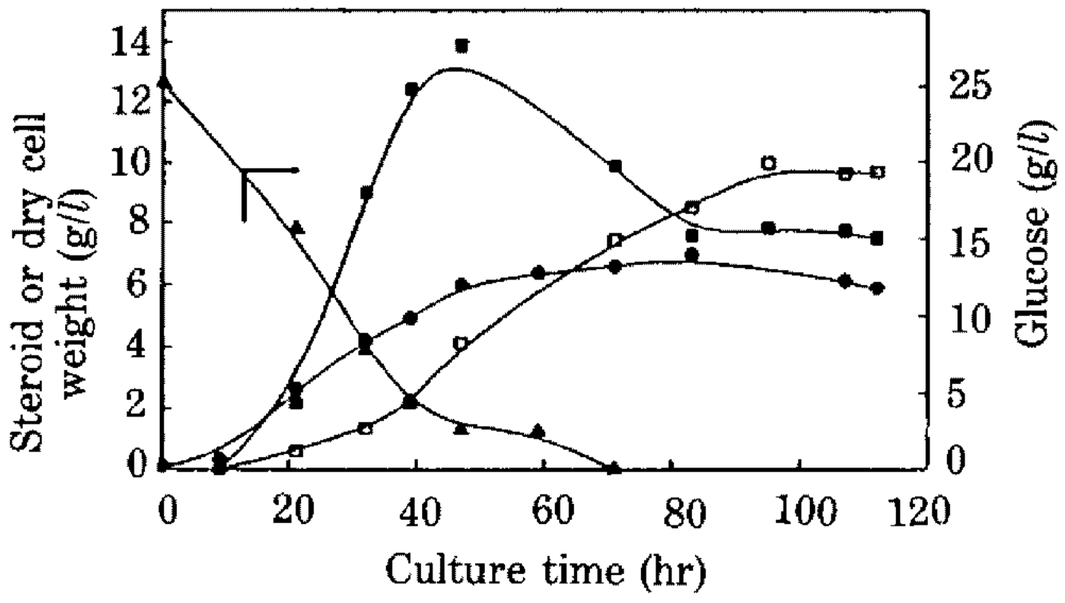

Fig. 5. Effect of continuous addition of progesterone on biotransformation.
Progesterone was added continuously at the rate of 0.86 g/hr for 30 hours.
Symbols are; dry cell weight (●), progesterone (■), 11α-hydroxyprogesterone (□), glucose (▲).

ing the total amount of progesterone to be added by the approximated time of exponential growth phase. When progesterone was continuously added at a rate of 0.86 g/hr for 30 hours after 9 hour cultivation, both growth rate and conversion yield were improved (Fig. 5). The conversion yield obtained was about 56% while a single addition of the same amount of progesterone yielded about 40% conversion. Thus, continuous addition of progesterone was considered to reduce the inhibition on cell growth, and therewith the bioconversion yield improved.

Since single addition of progesterone during the early logarithmic growth phase caused a serious growth inhibition, its continuous addition during the exponential growth phase was found most effective to increase

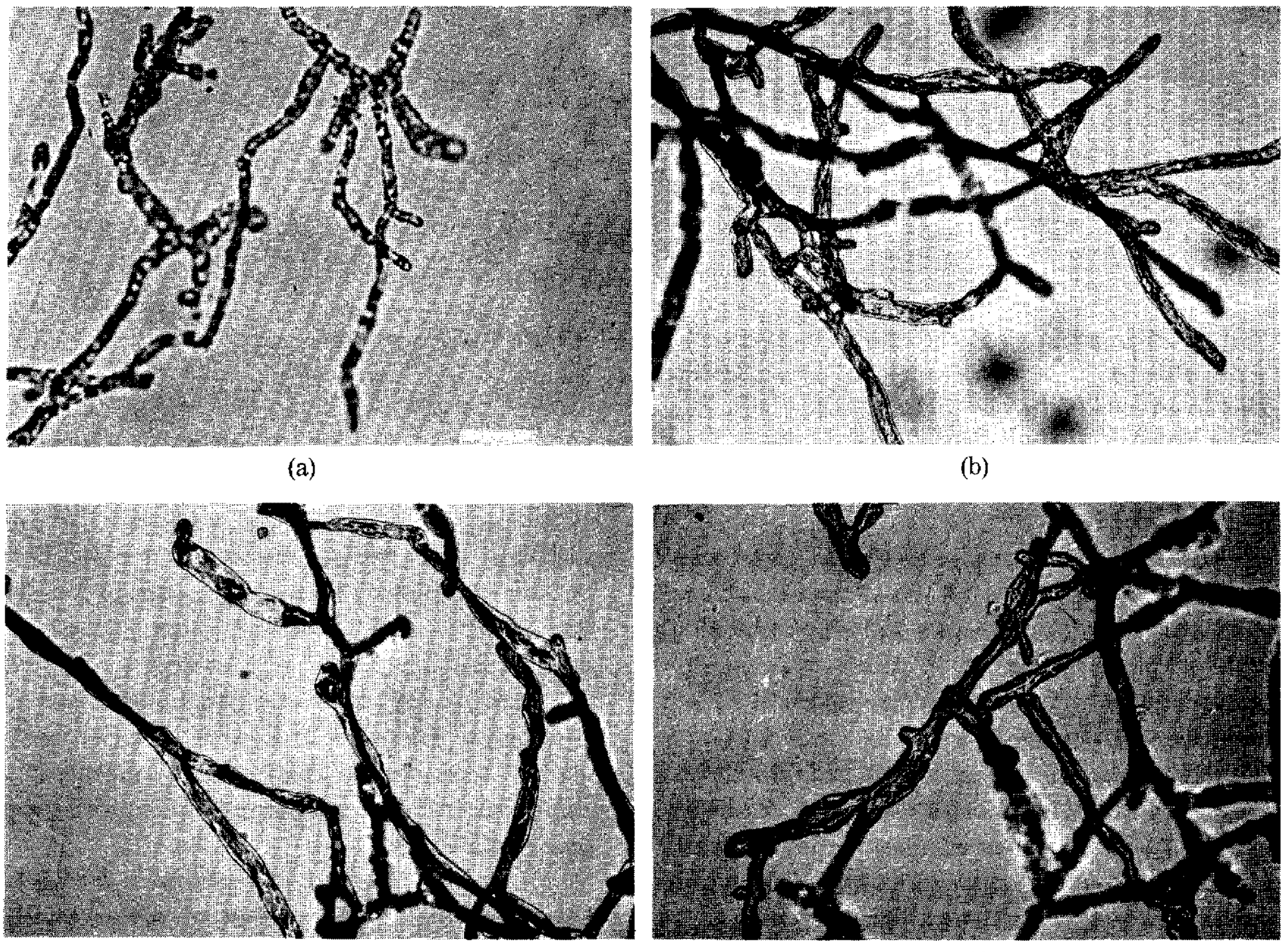

Fig. 6. Microphotographs of *Rhizopus nigricans* at continuous addition of progesterone.
(a) before addition of progesterone
(b), (c) and (d) were at 10 hr, 20 hr, and 30 hr after initiation of continuous addition, respectively. Addition rate of progesterone was the same as described in Fig. 5.

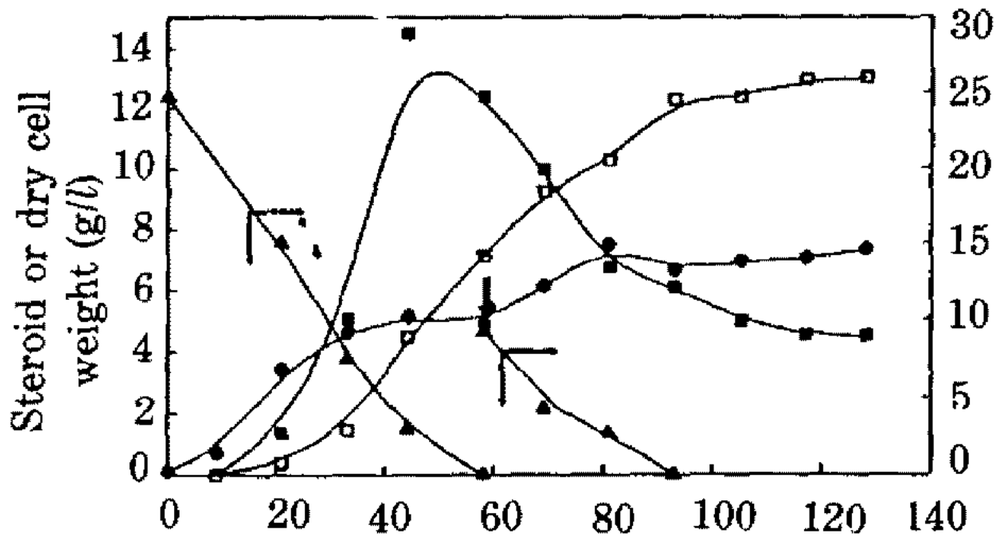

Fig. 7. Effect of additional glucose feeding on biotransformation.
Addition rate of progesterone was the same as described in Fig. 5 and glucose (10 g/*l*) was added at arrow indicated. Symbols are; dry cell weight (●), progesterone (■), 11α-hydroxyprogesterone (□), glucose (▲).

Table 2. Effect of continuous addition of progesterone on conversion yield, specific growth rate

Case	Yield (%)	Specific growth rate (1/hr)
Control	40.0	0.091
A	56.0	0.120
B	67.7	0.119 (0.018)*

Control: Single addition of 20 g/*l*progesterone.
A: Continuous addition of 20 g/*l* progesterone.
B: Continuous addition of 20 g/*l* progesterone and additional feeding of 10 g/*l* glucose.
*: Specific growth rate after additional feeding of glucose.

the cell growth and the bioconversion yield.

Morphology of *R. nigricans* was examined at continuous addition of progesterone. As shown in Fig. 6, its hyphal morphology hardly changed when compared with that before the addition of progesterone.

Effect of glucose feeding on biotransformation

Since the biotransformation of progesterone to 11 α-hydroxyprogesterone was shown to be related with the growth of *R. nigricans*, glucose was fed upto a final concentration of 10 g/*l* upon its depletion, while the feeding rate of progesterone was kept the same as described in Fig. 5.

As shown in Fig. 7, when an additional feeding of glucose was conducted, the bioconversion yield was enhanced upto 68%. This implies that the additional energy is required for bioconversion and the biotransformation of progesterone to 11 α-hydroxyprogesterone is associated with the growth of *R. nigricans*. Further research shall be necessary about the mechanism of biotransformation. Effects of both continuous addition of progesterone and glucose feeding on biotransformation are summarized in Table 2. Improved productivity by continuous addition of progesterone could be attributed to the increase of bioconversion rate.

The approach described in this article shall well be applied to other biotransformation systems of water insoluble steroids. It is also believed that such an innovative effort on process engineering can significantly improve the overall process economics of steroid biotransformation.

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(Received April 14, 1990)