Optimization of Culture Conditions for the Bioconversion of Vitamin D_3 to 1α,25-Dihydroxyvitamin D_3 Using *Pseudonocardia autotrophica* ID9302

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Abstract We assessed the ability of a *Pseudonocardia* sp. from soil samples to bioconvert vitamin D_3. The optimal culture conditions for the bioconversion of vitamin D_3 to active 1α,25-dihydroxyvitamin D_3 were investigated by varying the carbon and nitrogen sources, the metal salt concentrations, the initial pH, and the temperature. Microbial transformations were carried out with the addition of vitamin D_3 dissolved in ethanol. They were sampled by extraction with methanol-dichloromethane and the samples were examined by HPLC. Optimum culture conditions were found to be 0.4% yeast extract, 1% glucose, 3% starch, 1% fish meal, 0.2% NaCl, 0.01% K_2HPO_4, 0.2% $CaCO_3$, 0.01% NaF, and pH 7.0 at 28°C. The optimal timing of the addition of vitamin D_3 for the production of calcitriol by *Pseudonocardia autotrophica* ID9302 was concurrent with the inoculation of seed culture broth. Maximum calcitriol productivity and the yield of bioconversion reached a value of 10.4 mg/L and 10.4% respectively on the 7th day in a 75 L fermenter jar under the above conditions.

Keywords: bioconversion, calcitriol, calcifediol, *Pseudonocardia autotrophica* ID9302, fermentation

INTRODUCTION

Vitamin D_3 is converted in the liver to 25-hydroxycholecalciferol (calcifediol) by 25-hydroxylase [1,2]. There is limited negative feedback by calcifediol on the activity of hepatic 25-hydroxylase, and its activity is not influenced by plasma calcium and phosphorus concentrations. Subsequently calcifediol becomes metabolically activated to 1α,25-dihydroxyvitamin D_3 (calcitriol) in the kidneys by 1-α-hydroxylase [3,4]. The rate of renal 1-α-hydroxylase activation is limited by plasma concentrations of parathyroid hormone, calcium, phosphorus, and calcitriol [5,6]. Calcitriol has potent biological effects in the treatment of calcium metabolism dysfunction and calcium deficiency. Synthetic calcitriol production involves regio- and stereo-specific hydroxylation at C-1, which necessitates a multi-step reaction and is therefore very expensive [7-9]. The microbial hydroxylation of drugs has a large cost advantage over chemical synthesis and so has become commercially viable and competitive in the drug industry [10,11]. Transformation with enzymes or intact cells of microorganisms takes place under moderate reaction conditions. The bioconversion rate is affected by the medium composition, the culture process and the bioconversion conditions such as pH, temperature, and precursor [12-14]. Moreover, the optimization of the culture conditions for calcitriol production is very important in order to achieve the maximal bioconversion rate. This study aimed to optimize the culture conditions of vitamin D_3 bioconversion in order to enhance the production of calcitriol for industrial-scale calcitriol production. The bioconversion parameters such as the supply rate of precursor, pH, temperature, aeration concentration, and agitation speed were optimized first for culture in Erlenmeyer flasks and 5 L fermenter jars, then scaled-up to culture in 75 L fermenter jars [15].

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MATERIALS AND METHODS

Bacterial Strain

Pseudonocardia autotrophica ID9302 was isolated from soil samples collected in Korea and was improved by a series of UV mutations.

Media and Bioconversion Methods

Vitamin D_3-hydroxylating strains separated from soil samples were cultured in basal medium (0.4% soluble

starch, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$, 0.2% NaCl, pH 7.2) at 28°C for 4~7 days. Selected strains were stored in 4 mL vials containing 20% (w/v) glycerol in medium at −72°C. When needed for experiments, the stock cultures were rapidly thawed and utilized after two subculture passages. GS agar medium (1.5% glucose, 1.5% soytone, 0.5% NaCl, 0.2% $CaCO_3$, 2% agar, pH 7.0) was used for agar culture. The plate was cultivated in an incubator maintained at 28°C for 4 days. During the cultivation, the cells produced large amounts of white spores. About 1 cm^2 of agar plate was used to inoculate 50 mL GS medium without agar in a 250 mL Erlenmeyer flask with 4 baffles. The cells were cultivated at 28°C for 3 days on a rotary shaker (180 rpm). Two mL of the first seed culture were inoculated into 50 mL fresh GS medium in a 250 mL flask with baffles, and the culture was incubated at 28°C for 3 days on a rotary shaker. Two mL of the culture broth from the second seed culture were inoculated into 50 mL GSYF medium (1% glucose, 1% corn starch, 1% fish meal, 0.01% K_2HPO_4, 0.01% NaF, 0.2% $CaCO_3$, 0.2% NaCl and pH7.2) in a 250 mL flask with baffles, and simultaneously fed 100 μL vitamin D_3 solution (5% vitamin D_3 dissolved in ethanol), which initiated the bioconversion reaction. The bioconversion was carried out on a rotary shaker (180 rpm) at 28°C for 7 days. Three mL samples of culture broth were taken at appropriate times.

Bioconversions in 5 and 75 L Fermenter Jars

The calcitriol production experiments were done mainly in either 5 or 75 L fermenter jars. A 5 L fermenter jar containing 3.5 L GSYF medium was inoculated with 150 mL second seed culture and 7 mL vitamin D_3 solution were added to the vessel at the same time. The bioconversion reaction culture conditions were an agitation speed of 600 rpm, an aeration concentration of 1 vvm and the temperature was maintained at 28°C. The fermenter impeller was a six-blade 77 mm diameter turbine, and the diameter of the vessel was 170 mm.

A 75 L fermenter jar was used to further scale-up the reaction. The culture conditions of the 75 L fermenter jar were similar to those of the 5 L fermenter jar. The bioconversion reaction was initiated by the inoculation of second seed culture (2 L) with the simultaneous addition of vitamin D_3 solution (100 mL). The culture conditions included an agitation speed of 550 rpm and an aeration concentration of 1 vvm at 28°C. The impeller blade diameter was 120 mm and the vessel diameter was 350 mm.

Analytical Methods

After the fermentation, cells were harvested by centrifugation (11,000 × g, 10 min), and cell growth was estimated from cell dry weight after drying at 100°C for 1 h. Three mL culture broth samples were extracted with methanol-dichloromethane using a modified Bligh - Dyer method [16]. The extraction samples were concentrated under reduced pressure and extracted with 200 μL methanol. The extracts were separated by centrifugation at 14,000 rpm. The supernatants were analyzed by HPLC using a modified European Pharmacopoeia. Analytical HPLC was quantified by a TSP HPLC system which comprised an ODS column (4.6 × 250 mm) and a photodiode array, with a flow rate of 1 mL/min, and a mobile phase consisting of a mixture of acetonitrile/0.1% phosphoric acid (55/45, v/v). Calcitriol, calcifediol, calcidiol, vitamin D_3, and 1β,25-dihydroxyvitamin D_3 could be distinguished by this HPLC system according to the differences in their retention times.

Table 1. Effect of various nitrogen sources on hydroxylating activity

Nitrogen source	Calcitriol (mg/L)	Calcifediol (mg/L)	Yield[a] (%)
Peptone	0.07	0.52	0.59
Skim milk	0.84	2.46	3.30
Cottonseed meal	0.24	1.83	2.07
Tryptose	0.03	0.21	0.24
Tryptone	0.03	0.25	0.28
Casitone	0.02	0.21	0.23
Soybean meal	0.48	2.28	2.76
Fish meal	1.18	5.44	6.62
Yeast extract	0.22	1.26	1.48

Yeast extract in the basal medium was replaced with the nitrogen sources (0.5%, w/v) in a 250 mL Erlenmeyer flask with baffles. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2.
[a]Implies total yields of active vitamin D_3 such as calcitriol and calcifediol.

RESULTS AND DISCUSSION

Effects of Nitrogen Sources on Hydroxylation of Vitamin D_3

In order to determine the effects of the nitrogen source on cell growth and calcitriol production, the yeast extract of the basal medium was replaced with 0.5% (w/v) of various nitrogen sources. This reaction was maintained in a shaking incubator at 180 rpm at 28°C for 7 days. Out of the nitrogen sources investigated, fish meal was found to be the most effective at increasing calcitriol and calcifediol (Table 1). To further confirm the effects of fish meal on the bioconversion of vitamin D_3 to calcitriol, fish meal was added to the medium at a concentration range of 0~4% instead of the yeast extract. It can be seen in Fig. 1 that the optimum initial concentration of fish meal was 1% (w/v) and further addition of fish meal reduced the yield of bioconversion. The bioconversion of vitamin D_3 to calcitriol was enhanced about 5.4-fold by the addition of fish meal. Active vitamin D_3 yield showed an increase of 5.14% over the control. These results indicate that fish meal is likely to be the most effective nitrogen source for the bioconversion of vitamin D_3 to calcitriol.

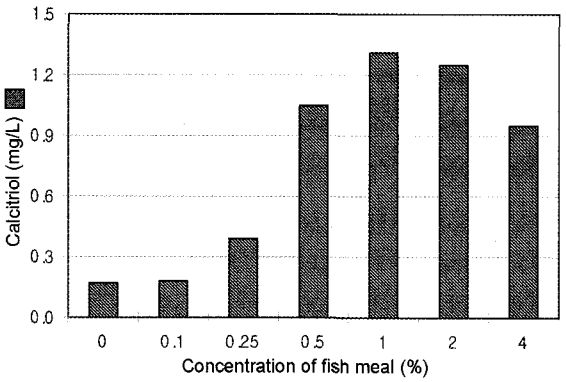

Fig. 1. Effect of fish meal concentration on calcitriol production. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2.

Table 2. Effect of carbon sources on hydroxylating activity

Carbon sources	Growth (g/L)	Calcitriol (mg/L)	Calcifediol (mg/L)	Yield[a] (%)
Soluble starch	9.1	0.21	2.01	2.32
Fructose	8.9	0.21	2.01	2.32
Sucrose	18.2	1.73	7.61	9.34
Trehalose	9.8	1.41	6.95	8.36
Lactose	9.2	0.70	3.58	4.28
Maltose	17.9	1.91	7.68	9.59
Malt extract	16.8	1.02	5.23	6.25
Glycerol	14.2	1.20	6.58	7.78
Galactose	10.3	1.03	4.33	5.36
Xylitol	9.8	1.22	5.20	6.42
Glucose	21.6	2.03	7.81	9.84

Soluble starch and malt extract in the basal medium were replaced with various carbon sources in a 250 mL Erlenmeyer flask with baffles. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2.

[a]Implies total yields of active vitamin D_3 such as calcitriol and calcifediol.

Effects of Carbon Sources on the Hydroxylation of Vitamin D_3

Soluble starch and malt extract in the basal medium were replaced with of the various carbon sources and the effects on growth and hydroxylation activity were assessed. As mentioned above, culture broth of a second seed culture was inoculated into 50 mL GSYF, with the simultaneous addition of 100 μL vitamin D_3 solution (5% vitamin D_3 dissolved in ethanol). This reaction was maintained in a shaking incubator at 180 rpm and 28°C for 7 days. The cells did not grow well on fructose, lactose, and soluble starch, but glucose, maltose and sucrose resulted in abundant growth and high yields. Of these carbon sources, glucose resulted in the highest yield (Table 2). Therefore, glucose at a concentration of 1% (w/v) was determined to be the optimal carbon source.

Table 3. Effect of NaF concentration on hydroxylating activity

NaF (%)	Calcitriol (mg/L)	Calcifediol (mg/L)	Yield[a] (%)
0	2.05	7.80	9.85
0.001	2.59	11.97	14.56
0.005	2.74	12.98	15.72
0.01	2.94	13.41	16.35
0.05	2.52	12.31	14.83
0.1	2.49	11.15	13.64

Various concentrations of NaF were added to the 250 mL Erlenmeyer flask with baffles. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2.

[a]Implies total yields of active vitamin D_3 such as calcitriol and calcifediol.

Table 4. Effect of K_2HPO_4 concentration on hydroxylating activity

K_2HPO_4 (%)	Calcitriol (mg/mL)	Calcifediol (mg/mL)	Final pH	Yield[a] (%)
0	3.01	13.78	6.61	16.79
0.001	3.12	13.98	6.59	17.10
0.01	3.64	16.36	7.08	20.00
0.1	3.35	13.23	7.18	16.58
1	2.56	7.89	7.44	10.45
2	1.02	2.22	7.62	3.24

Various concentrations of K_2HPO_4 were added to the 250 mL Erlenmeyer flask with baffles. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2.

[a]Implies total yields of active vitamin D_3 such as calcitriol and calcifediol.

Effect of Sodium Fluoride on Hydroxylation of Vitamin D_3

In order to improve the bioconversion of vitamin D_3 to calcitriol, the effect of several salts were investigated. Sodium fluoride was chosen as the optimal salt (data not shown). As can be seen in Table 3, 0.01% sodium fluoride resulted in the highest productivity using an improved medium consisting of glucose, corn starch, fish meal and yeast extract. Calcitriol productivity reached 2.94 mg/L this concentration of sodium fluoride.

Effect of Phosphate on Hydroxylation of Vitamin D_3

In order to determine the effects of dipotassium phosphate on vitamin D_3 bioconversion, the production medium was supplemented with varying concentrations of dipotassium phosphate. The maximum productivity was achieved with 0.01% dipotassium phosphate (Table 4). The bioconversion yields for calcitriol increased in pro-

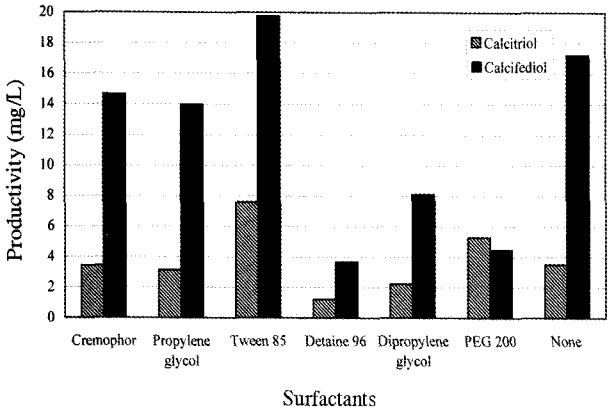

Fig. 2. Effects of various surfactants on hydroxylating activity. Various kinds of surfactants were added to 250 mL GYSF in an Erlenmeyer flask with baffles. One hundred μL vitamin D_3 solution (5% vitamin D_3 dissolved in ethanol) and 100 μL surfactant were added. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2.

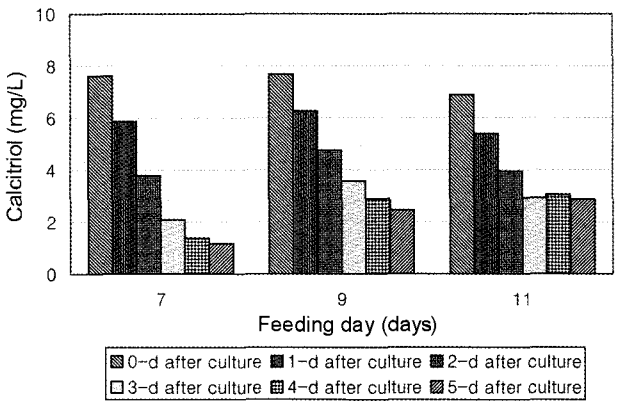

Fig. 3. Effect of time of addition of vitamin D_3. Vitamin D_3 was added various times after bacterial inoculation in 250 mL Erlenmeyer flask with baffles. The culture conditions were as follows: 28°C, 7 days, 180 rpm, pH 7.2. Sampling was at 7, 9, and 11 days.

portion to the increase in dipotassium phosphate concentration. Optimal phosphate concentration provides the right environment for the production of the best quality cells for the bioconversion, which provided the optimal level of p450 hydroxylase. It is clear that the proper regulation of phosphate concentration is important during the bioconversion of vitamin D_3 to calcitriol [17,18].

Optimal Conditions of Temperature and pH for the Bioconversion Reaction

Calcitriol productivity was stable at temperatures ranging between 22 to 32°C, but calcitriol productivity of the strain decreased drastically above 37°C. The highest productivity was observed at 28°C. The effects of pH on the bioconversion reaction were also examined. The pH of the culture media was adjusted with 10% NaOH. The bioconversion activity was stable from pH 5 to 9 but decreased at pHs above 9 or below 5. Calcitriol production and yield were maximum at pH 6.5~7.5.

The Effects of Surfactants on Solubility of Vitamin D_3

Various surfactants are well known to improve the solubility of insoluble materials. We added various surfactants to the vitamin D_3 solution in ethanol. The surfactants had varying effects on the productivity as can be seen in Fig. 2. Among them, Tween 85 significantly increased calcitriol productivity in the GSYF medium to the maximum value (Fig. 2), whereas calcifediol productivity was relatively low. This indicates that calcifediol was actively metabolized to calcitriol during the bioconversion. The increase of the calcitriol productivity by the addition of PEG 200 or Tween 85 implies higher solubility of vitamin D_3, insoluble in water, which was effective in contact with cells. The bioconversion of vitamin D_3 to calcitriol was enhanced about 2-fold above the control in the GSYF medium when Tween 85 was added.

The Determination of the Timing of Precursor Addition

Fig. 3 shows the importance of the timing of the addition of vitamin D_3. Unexpectedly, addition of vitamin D_3 simultaneously with bacterial inoculation resulted in the highest production rate. The lowest calcitriol production was seen when vitamin D_3 was added 5 days after inoculation. These findings differ from those seen in Taisho's method for the bioconversion of vitamin D_3 to calcitriol [19]. After the cells had been cultured for a while, many functions of the cells including p450 monooxygenase functioned normally [20,21]. Therefore it is good to determine the precursor addition time after fermentation. However, production of calcitriol by *P. autotrophica* ID9302 in this study was optimal when the inoculation of seed culture broth and the addition of vitamin D_3 were at the same time. In further experiments, the effect of raising the vitamin D_3 concentration and that of an extra addition of vitamin D_3 were investigated. These changes increased the volumetric productivity of active vitamin D_3 significantly under optimal conditions (data not shown).

The Effect of Agitation Speed on Bioconversion in a 5 L Fermenter Jar

In the bioconversion process, the cells can be cultured in different morphological ways, such as filamentous culture and pellet-forming culture. Divergent morphology results in differences in calcitriol productivity. Agitation speed has been shown to affect pellet formation. The effect of agitation speed was investigated in a 5 L fermenter jar for 9 days. As shown in Fig. 4, agitation speed significantly affected calcitrol production [22]. Below 400 rpm, pellet formation was observed and calcitriol productivity was low. Above 400 rpm, the number of filamentous type cells and calcitriol production increased in proportion to the agitation speed up to 550 rpm. This suggests that the supply of nutrients and oxygen to the cells is the limiting

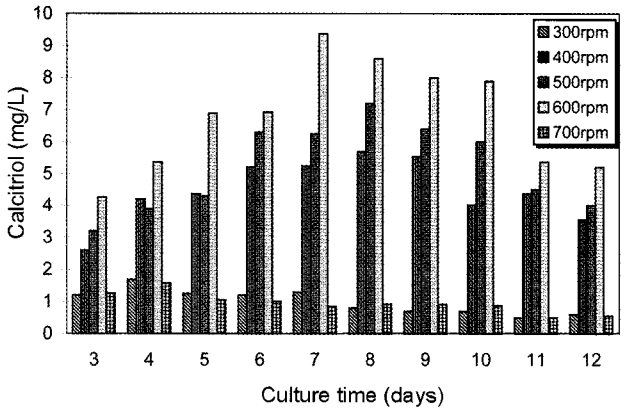

Fig. 4. Effect of agitation speed of impeller in a 5 L fermenter jar. Various agitation speeds were tested in a 5 L fermenter jar. The fermentation was continued in the GSYF medium for 12 days. The samples were analyzed by HPLC. Aeration concentration was fixed at 1 vvm. Tip speed was increased 0.4 m/s per 100 rpm and was 2.42 m/s at 600 rpm.

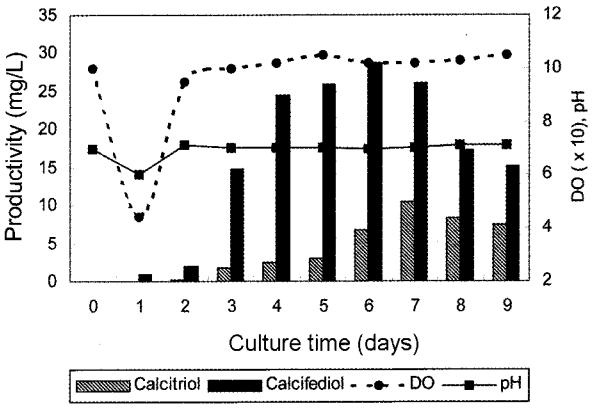

Fig. 5. Production of calcitriol in batch culture in a 75 L fermenter jar. The fermentation was continued for 9 days in the GSYF medium. Agitation speed and aeration concentration were fixed at 500 rpm and 1 vvm, respectively.

factor during the bioconversion process. Our findings imply that tip speed and aeration concentration are also important in creating the optimal conditions for vitamin D_3 bioconversion as they affect the formation of filamentous cells which are essential for the maximum production of calcitriol.

Scale-up of Production of Calcitriol in a 75 L Fermenter Jar

The optimal fermentation conditions such as aeration concentration, agitation speed, dissolved oxygen concentration, and tip velocity were considered for calcitriol production in a 75 L fermenter jar. Based on the results from the 5 L fermenter jar, aeration concentration was fixed at 1 vvm. The agitation speed was fixed at 550 rpm according to the tip velocity. As shown in Fig. 5, calcitriol production in a 75 L fermenter jar reached the maximum on the 7th day. The determination of agitation speed based on tip speed must take into consideration the ratio of vessel diameter to the impeller diameter. If this variable is calculated, 3.46 m/s tip speed is the appropiate value for a 75 L fermenter jar. The production profile of a 75 L fermenter jar was the same as that at the 5 L scale; therefore, impeller tip velocity can be a useful criterion from the viewpoint of scale-up. As calcitriol is very expensive and only small doses are used in treatment of calcium disorders, mass production at the ton-scale is not necessary. This study demonstrated a practicable and effective fermentation strategy to bring about an outstanding increase in the production of calcitriol by *P. autotrophica* ID9302 for small-scale operation.

CONCLUSIONS

We studied the effects of many media and culture conditions on the production of calcitriol from vitamin D_3 using *P. autotrophica* ID 9302. Many kinds of culture media are available for the production of calcitriol, and it is well known that the addition of fish meal is effective for the bioconversion of vitamin D_3 to calcitriol. The addition of Tween 85 improved the solubility of vitamin D_3 and so increased calcitriol productivity. The bioconversion of vitamin D_3 to calcitriol was enhanced about 5.4- and 2-fold above control by the addition of fish meal and Tween 85 respectively. We have demonstrated here that optimizing the culture conditions and the timing of precursor addition is an excellent strategy for the improvement of bioconversion of vitamin D_3 to calcitriol. The culture conditions for scale-up of calcitriol production in a 75 L fermenter jar were determined. A combination of optimization of the cultivation media, the culture processes and the addition of vitamin D_3 may facilitate higher scale-up in a fermenter jar.

Acknowledgments The Project of Ministry of Commerce, Industry and Energy, Korea Supported this study.

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[Received April 18, 2006; accepted September 15, 2006]