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A chemically-defined medium supporting growth and providing cells converting compactin to pravastatin

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Actinomadura sp strain 2966, which converts compactin to pravastatin, requires vitamins to support its growth. Addition of folic acid, thiamine and cyanocobalamine allowed growth in chemically-defined medium. Cells grown in a chemically-defined medium were as capable of converting compactin to pravastatin as cells grown in a complex medium.

Keywords: *Actinomadura*; pravastatin; compactin; nutrition; vitamins

Introduction

Actinomadura sp strain 2966 hydroxylates compactin to pravastatin [1,3,8], a specific HMG-CoA reductase inhibitor which is used widely to reduce the cholesterol level in human blood [1,5,6]. The hydroxylase of *Actinomadura* [4] is different from the cytochrome P-450 hydroxylation system of *Streptomyces carbophilus* [2,7]. A chemically-defined medium for growth of *Actinomadura* sp strain 2966 would be useful to elucidate factors which affect the hydroxylase activity in cells. In this paper, we present nutritional requirements of this microorganism.

Materials and methods

Chemicals

Compactin was obtained from Fluka, Buchs, Switzerland, and pravastatin from Bristol-Myers Squibb, Princeton, USA.

Organism and media

Actinomadura sp strain 2966 (ATCC 55678) was isolated from soil by SH Bok in South Korea as a rare actinomycete [8]. NZ medium, used for preparing slants, contains the following ingredients (per liter): glucose 10 g, soluble starch 20 g, yeast extract (Difco Laboratories, Detroit, MI, USA) 5 g, N-Z amine A (ICN Biochemicals, Cleveland, OH, USA) 5 g and agar 18 g (pH 7.3). YM medium, used as a liquid seed medium, contains (per liter): glucose 10 g, yeast extract 3 g, malt extract (Difco) 3 g and peptone (Difco) 5 g (pH 6.5). Chemically-defined medium (medium A) contains (per liter): sucrose 30 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g and salt solution 1 ml. The salt solution contains (per liter): $FeSO_4 \cdot 7H_2O$ 1.0 g, $MnCl_2 \cdot 4H_2O$ 1.0 g, $ZnSO_4 \cdot 7H_2O$ 1.0 g and $CaCl_2$ 1.0 g. The organism was inoculated from a slant grown for 7 days at 30°C into 250-ml Erlenmeyer flasks containing 20 ml of YM medium as primary seed culture. After growth at 28°C with rotary shaking (220 rpm, 1 inch throw) for 2 days, 1.0 ml of the YM seed culture was inoculated into 250-ml Erlenmeyer flasks containing 20 ml of medium A. This culture was grown for 3 days as the secondary seed culture. One milliliter of the secondary seed culture was inoculated into 250-ml Erlenmeyer flasks containing 20 ml of medium A with different additives as growth media. All incubations were at 28°C with shaking at 220 rpm.

Growth determination

One milliliter of 2.5 N HCl was added to 1 ml of culture followed by 3 ml water. The suspension was sonicated for 30 s at output 5 (Model W185, Heat System-Ultrasonics, Plainview, NY, USA). The absorbance was measured as Klett units on a Klett Summerson Colorimeter (Klett Manufacturing Co, New York, NY, USA) with a red filter.

Determination of compactin and pravastatin

Compactin and pravastatin were determined by high-performance liquid chromatography (HPLC) [8].

Bioconversion

The bioconversion was carried out for 4 h as described previously [3].

Results and discussion

Effect of a 12-component vitamin mixture on growth

Earlier experiments showed that when strain 2966, previously grown in YM seed medium, was inoculated into chemically-defined medium A (using 10% inoculum), the organism grew well. However, subsequent inoculation from a medium A culture to another medium A flask failed to result in the growth of strain 2966. A vitamin mixture (V_m) containing folic acid, thiamine, cyanocobalamine (vitamin B_{12}), niacin, pantothenic acid, myo-inositol, ascorbic acid, choline, biotin, pyridoxine, riboflavin and para-aminobenzoic acid (PABA) was used to examine the growth factor requirements of strain 2966. Growth from the medium A secondary seed culture was inoculated into medium A (using 5% inoculum) containing V_m at two concentrations

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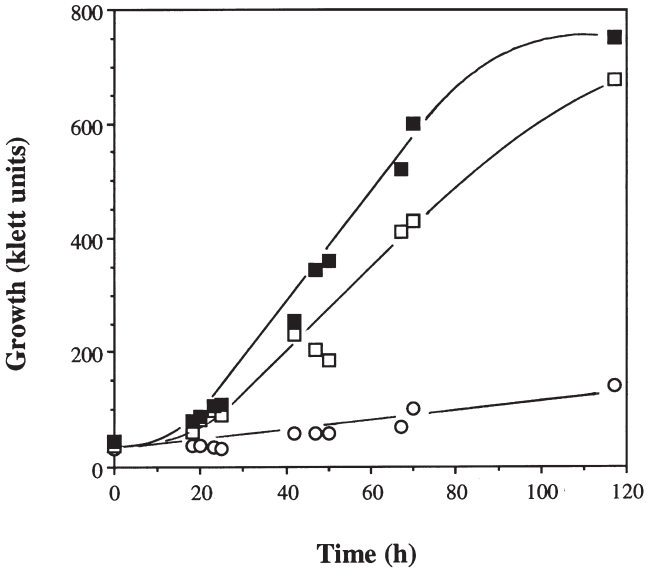

Figure 1 Effect of concentration of vitamin mixture V_m on growth of *Actinomadura* strain 2966. Final concentration of each vitamin in the medium: ○, zero; □, 1 mg L^{-1}; ■, 10 mg L^{-1}.

(final concentration in the medium = 1 mg L^{-1} and 10 mg L^{-1} of each vitamin). The results (Figure 1) show that without the addition of vitamins, strain 2966 grew only slightly. The slight growth was probably due to a small amount of vitamin(s) carried over from the YM primary seed medium. Growth was better at the higher level of vitamin mix than at the lower level.

Bioconversion by cells grown in chemically-defined medium

Cells grown in complex YM medium and chemically-defined medium containing vitamin mixture V_m at 0.25 mg L^{-1} of each vitamin were compared for bioconversion of compactin to pravastatin. As shown in Table 1, the cells grown in defined medium were fully capable of carrying out the bioconversion.

Effect of vitamin concentration on growth

A number of experiments were carried out by elimination of individual vitamins. Elimination of pyridoxine, riboflavin and biotin did not slow the growth of *Actinomadura* sp strain 2966. At this point, a new mixture containing the remaining nine vitamins (V_{m2}) was prepared for further studies. An experiment was conducted to determine the lowest vitamin mixture (V_{m2}) concentration needed to support good growth of strain 2966. It was found that a V_{m2} concentration of 0.05 mg L^{-1} of each vitamin was sufficient. Lower concentrations (0.001–0.01 mg L^{-1} of each vitamin) increased growth to a lesser extent and the cells experienced lysis.

Table 1 Bioconversion of compactin to pravastatin by cells grown in YM medium and in chemically-defined medium

Medium	Growth (Klett units [ku])	Pravastatin (μg ml^{-1})	Productivity (μg ml^{-1} h^{-1} ku^{-1})
YM	600	18.2	7.6×10^{-3}
Chemically-defined	440	12.6	7.2×10^{-3}

Seed growth in YM was for 3 days. Seed was inoculated (5% v/v) into YM and medium A containing V_m at 0.05 mg L^{-1} of each vitamin. After 4 days, the cells were used for a 4-h bioconversion of compactin to pravastatin.

Effect of individual vitamins on growth

In an attempt to determine which vitamins are essential for growth, we eliminated individual vitamins from V_{m2}. Elimination of individual vitamins from the mix of nine vitamins did not affect growth. This means that either a particular vitamin was not required or some vitamin(s) in the mixture could replace the eliminated one in support of growth.

Starting with the positive control (medium A supplemented with V_{m2}), one vitamin at a time was sequentially eliminated from V_{m2}. Sequential removal of pantothenic acid, myo-inositol, ascorbic acid, choline, folic acid and niacin had no effect. In the medium lacking these six vitamins, removal of thiamine and PABA virtually eliminated growth. Since PABA is a precursor of folic acid, the effect of folic acid, PABA, thiamine and B_{12} and combinations of these were tested in four sequential growth cultures. The concentration of each vitamin was 10 mg L^{-1}. A mixture of thiamine, folic acid and B_{12} supported growth for four sequential cultures to the same degree as V_{m2}.

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