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Enhanced Production of Epothilones by Carbon Sources in *Sorangium cellulosum*

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Abstract To improve epothilones production and the ratio of epothilone B/A, carbon sources were investigated in flask culture of *Sorangium cellulosum*. Depending on the initial concentration, starch significantly enhanced cell growth, but the maximum epothilones productivity and the maximum epothilones production (0.3 mg/l · day and 2.6 mg/l at 15 g/l starch, respectively) were relatively low compared with cell growth. On the other hand, addition of glycerol did not stimulate cell growth, but epothilone production was increased from 2.81 mg/l to 7.59 mg/l. Addition of glycerol to culture medium resulted in more significant enhancement of the production of epothilone A, whereas epothilone B levels were relatively constant. Furthermore, when sodium propionate was added as a precursor of methylmalonyl-CoA, it resulted in increase of both total epothilones production and epothilone B/A resolution. Maximum epothilone A and B concentrations reached 10.9 mg/l and 8.58 mg/l, respectively, at 5 mM sodium propionate.

Key words: *Sorangium cellulosum*, epothilones, carbon sources

Epothilones are secondary metabolites that are naturally produced by Gram-negative *Sorangium cellulosum*, which was collected at the banks of the Zambesi river in the Republic of South Africa [5, 7]. They are potential anticancer drugs that bind to microtubules and stabilize them in a polymerized state, thereby blocking cell-cycle progression with an action manner similar to that of paclitaxel (Taxol) [1]. In addition, their efficiency against Taxol resistant tumor cell lines and their comparatively higher water solubility than that of Taxol make epothilones potential chemotherapeutic compounds with great commercial value [2, 14].

Polyketide synthase (PKS) contains seven gene clusters (EpoA, EpoB, EpoC, Epo D, EpoE, EpoF, and EpoK) and spans approximate 56 kb. These enzymes catalyze the successive condensation of carboxylic acid residues in a stepwise process. The thioesterase (TE) domain of EpoF then releases the epothilone chain by intramolecular cyclization, thereby creating the 16-membered ring of epothilone C or D. Finally, post-assembly-line epoxidation of the olefin is affected by the EpoK cytochrome P450 monooxygenase, converting epothilone C or D to epothilone A or B, respectively (Fig. 1) [10].

The ratio of epothilones A/B or C/D is a function of the epothilone PKS acyl transferase module 4(AT4) specificity for both malonyl-CoA and methylmalonyl-CoA, as well as the intracellular concentrations of these precursor pools [3, 6]. In brief, epothilones B and D are derived from methylmalonyl-CoA, and epothilones A and C are from malonyl-CoA. Accordingly, many research groups have studied epothilones that are derived from substrates of acetyl-CoA, propioyl-CoA, malonyl-CoA, and (2S)-methylmalonyl-CoA using several carbon sources [8, 11, 15]. Janice and coworkers showed that oils enhance epothilone D production (from 0.16 mg/l to 23 mg/l) in *Myxococcus xanthus* fed-batch fermentation [19]. Oils are carbon sources that could serve as a source of energy for cell, and formation of acetyl-CoA as a degradation product can also provide precursors for epothilones biosynthesis by the oxidation of fatty acid [9]. However, this approach is not applicable to *S. cellulosum*, since there have been no evidence to show that *S. cellulosum* has ability to

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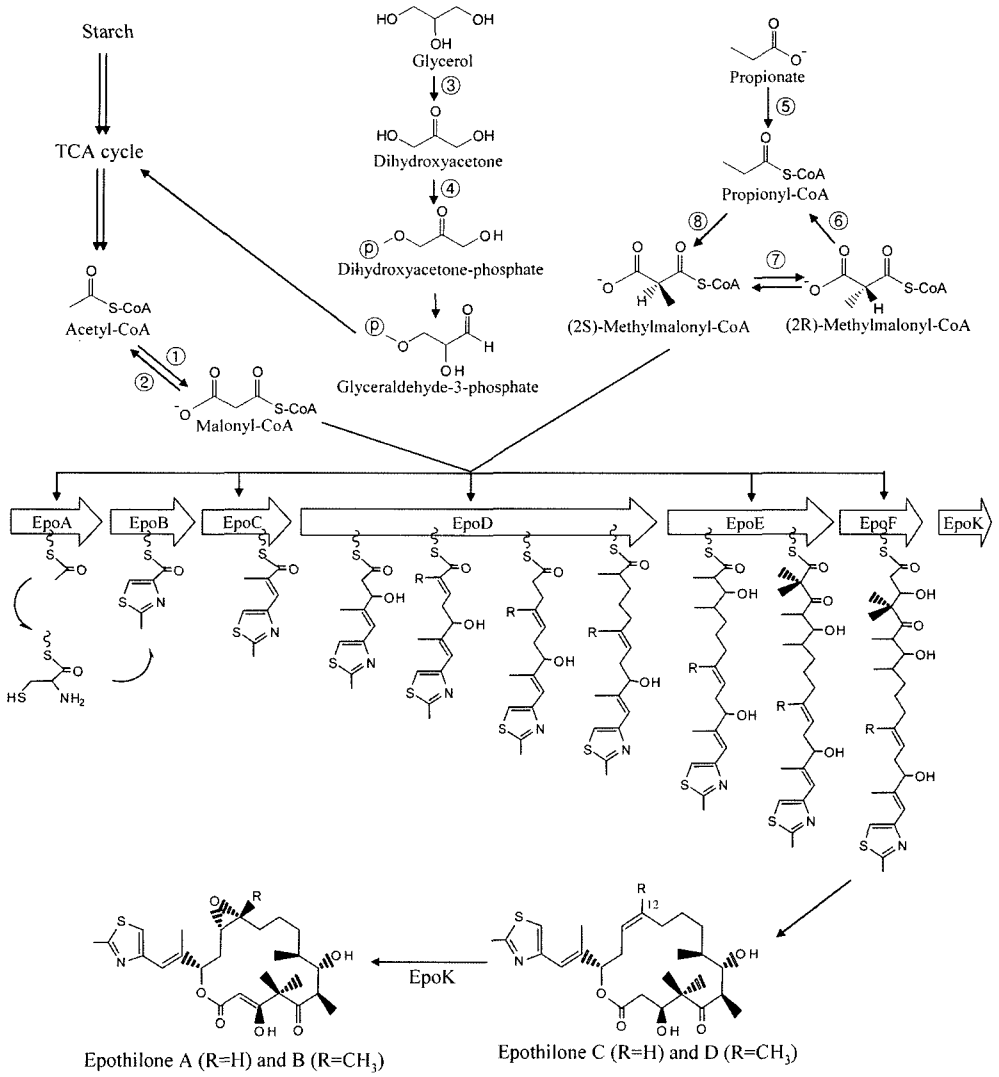

Fig. 1. Epothilone synthase substrate routes and structure of epothilones; ①, acetyl-CoA carboxylase; ②, malonyl-CoA decarboxylase; ③, glycerol dehydrogenase; ④, DHA kinase; ⑤, propionyl-CoA synthase; ⑥, methylmalonyl-CoA decarboxylase; ⑦, methylmalonyl-CoA epimerase; ⑧, propionyl-CoA carboxylase.

utilize oils as a carbon source. Epothilone D, the most commercial by available anticancer drug, is also produced in very low quantity by natural *S. cellulosum* [9]. However, it could be a major advancement to inactivate P450 monooxygenase using genetic manipulation or by an oxygen-controlling system [4].

In this study, three different carbon sources, including starch, glycerol, and propionate, were investigated to enhance epothilone B, the final material of epothilone D. First, the myxobacterium *S. cellulosum* was cultured in different initial concentrations of starch and glycerol to increase cell growth and epothilone production. Then, we studied the effect of initial sodium propionate concentration to increase the ratio of the methylmalonyl-CoA pool to that of malonyl-CoA and epothilones B production by adding starch, glycerol, and trace elements.

MATERIALS AND METHODS

Microorganism and Culture Conditions

S. cellulosum Soce90 was obtained from DSMZ (German collection of microorganism and cell culture, Germany) and plated on PM medium (pH 7.3). PM medium (per liter) proposed by DSMZ contained the following: peptone 0.4 g, $MgSO_4$ 1.5 g, Tris-HCl 2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, Na-Fe(III)-EDTA 80 mg, KNO_3 0.2 g, K_2HPO_4 6.25 mg, glucose 0.35 g, dithionite 0.06 mmol, and agar 15 g. To prepare batch flask cultures, agar plugs containing cells were transferred into 20 ml of E medium in a 250-ml Erlenmeyer flask and grown for 10 days in a shaking incubator at 32°C and 220 rpm. Seed and production media were based on E medium (pH 7.6) [6, 16]. The E medium (per liter) contained the following: skim milk 4 g, soy grits 4 g,

potato starch 10 g, yeast extract 2 g, glycerol 4.3 ml, $CaCl_2 \cdot 2H_2O$ 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, HEPES 50 mmol, and $FeCl_3$ 21 μmol. The production medium contained 20 g/l XAD-16 that binds and stabilizes epothilones (Rohm and Haas electronic materials, Korea, Ltd.) [9]. *S. cellulosum* was grown for 4–5 days at 32°C and 220 rpm until OD_{600} reached to 2–6 and used to inoculate 50 ml of production medium in a 250-ml Erlenmeyer flask at 10% (v/v). Each experiment was carried out in duplicate flasks and data are reported as an average of the two flasks. Trace element solution was composed of H_2SO_4 10 ml, $FeCl_3 \cdot 6H_2O$ 14.6 g, $ZnCl_3$ 2.0 g, $MnCl_2 \cdot 4H_2O$ 1.0 g, $CuCl_2 \cdot 2H_2O$ 0.43 g, H_3BO_3 0.31 g, $CaCl_2 \cdot 6H_2O$ 0.24 g, and $NaMO_4 \cdot 2H_2O$ 0.24 g, per liter of distilled water, and was sterilized by filtration [9].

Analytical Methods

XAD-16 resins were separated from the culture broth by gravity, washed three times with 50 ml of deionized water, and then filtered with a BD Falcon strainer. Epothilones were extracted from the resin with methanol for 30 min, and epothilones A and B were assayed by a Shimadzu HPLC system (Shimadzu, Japan). The HPLC system was equipped with two LC-10AD pumps and a SPD-10A UV-Vis detector. Fifty μl of the methanol extract was injected across a 4.6×10 mm guard column (Inertsil, ODS-3, 5 μm) and a 4.6×150 mm separation column (Inertsil, ODS-3, 5 μm). The column was eluted with mobile phase, which consisted of 60% acetonitrile and 40% water for 40 min at a flow rate of 1.0 ml/min, and eluates were monitored at 250 nm [16].

Results and Discussion

Effect of Initial Starch Concentration on Epothilones Production

To develop a new medium that improves cell growth and epothilones production, the effect of initial concentration of starch from 10 g/l to 20 g/l was investigated in the batch fermentation. The cell growth increased, depending on the initial starch concentration in the medium (data not shown). In this case, maximum epothilones production and total epothilones production were 0.3 mg/l · day and 2.6 mg/l at 15 g/l starch, respectively (Fig. 2). However, in the case of 20 g/l starch, there was little increase of total epothilones production in spite of the higher cell concentration than at 15 g/l starch. The result, therefore, shows that the addition of starch was not correlated with increasing epothilones production, even though cell growth was dose-dependent. Therefore, there is a need to study other carbon sources that serve as better precursors for epothilones biosynthesis rather than as a source of energy for the cell, such as starch.

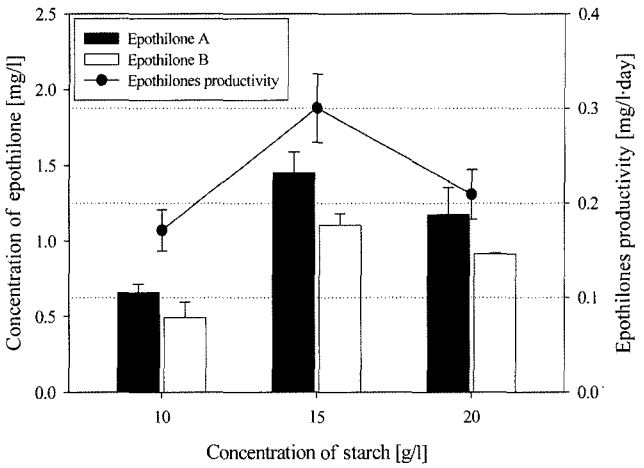

Fig. 2. Effect of potato starch on epothilones production and epothilones productivity.

Effect of Glycerol on the Epothilones Production

Alternative carbon sources were evaluated as supplements to the production medium with 15 g/l starch. In epothilones biosynthesis in *S. cellulosum*, alternative selection of malonyl-CoA and methylmalonyl-CoA specify the epothilone derivations A and B by the acyltransferase domain [3, 6]. We, therefore, added glycerol to the cell culture media to enhance epothilone production. Generally, glycerol kinase converts glycerol to glycerol-6-phosphate by transferring a phosphate group of ATP to glycerol: Glycerol-6-phosphate dehydrogenase sequentially catalyzes the synthesis of dihydroacetone phosphate, and then to glyceraldehydes-3-phosphate, ultimately resulting in the formation of acetyl-CoA by the glycolysis pathway (Fig. 1) [18]. Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase or to succinyl-CoA that is converted to methylmalonyl-CoA by methylmalonyl-CoA mutase by the TCA cycle [15]. In the present study, glycerol did not significantly stimulate cell growth in all ranges of initial glycerol concentration (data not shown). As shown in Fig. 3, total epothilones production was enhanced by increasing glycerol concentration, with total epothilone production reaching about 7.59 mg/l at 4 ml/l glycerol concentration. More significant enhancement of epothilone A production was observed. Lactose also showed a result similar to glycerol (data not shown). These results might be due to the fact that the pathway of malonyl-CoA to epothilones A is more highly activated by the addition of glycerol than that of methylmalonyl-CoA to epothilones B. The supplementation of increasing concentration of acetate results in decrease of the epothilones D/C ratio in *M. xanthus* culture in a manner similar to that of glycerol [3]. Glycerol and lactose also increase intracellular pools of acetyl-CoA and malonyl-CoA in *Escherichia coli* K-12 [17]. However, since epothilones D, an intermediate metabolite of the epothilones B pathway, is the most

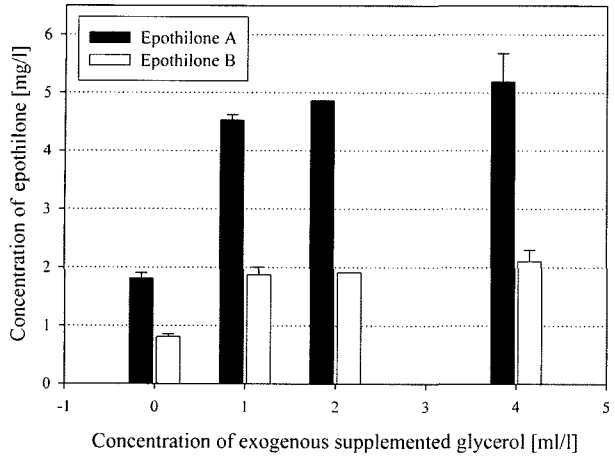

Fig. 3. Effect of glycerol on epothilones production; Control cell was cultured for 8.5 day in E medium with 15 g/l starch.

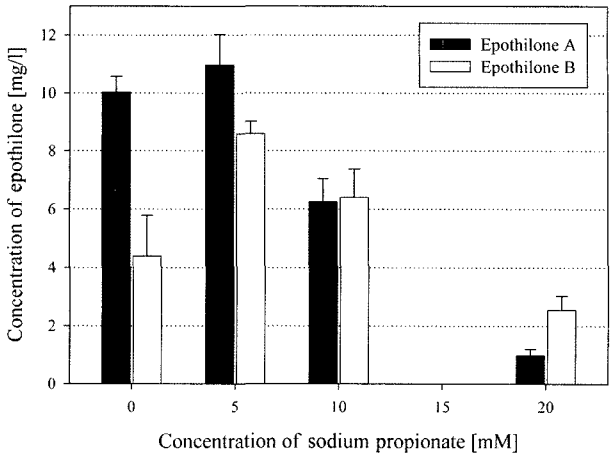

Fig. 4. Enhancement of epothilone B/A ratio by sodium propionate; Control cell was cultured for 8.5 day in E medium with 15 g/l starch, 8.3 ml/l glycerol, and 10 ml/l trace elements.

commercially promising of the four compounds as an antitumor drug [2], glycerol addition results in setback of reduced epothilones B resolution.

Enhancement of Epothilones B/A Ratio by Sodium Propionate

Frykman and coworkers studied the effect of initial concentration of sodium propionate to improve epothilone resolution in *M. xanthus* [3]. Sodium propionate is converted to propionyl-CoA by propionyl-CoA synthase, and propionyl-CoA carboxylase catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA in *M. xanthus* [12, 13, 15]. In this study, we also investigated the effect of initial concentration of sodium propionate on the epothilone biosynthesis to increase the pool of methylmalonyl-CoA by feeding starch, glycerol, and trace elements. The addition of trace element solution resulted in enhancement of total epothilones production while maintaining epothilones ratio (data not shown). This is similar to the previous result obtained with *M. xanthus* [9]: In the case of *M. xanthus*, increasing the sodium propionate concentration upto 20 mM resulted in an increase of the resolution of epothilone D/C. In contrast, the ratio at higher concentration of sodium propionate gradually decreased. Therefore, the result indicates that the rate of cell growth and cell densities are dependent on the sodium propionate level.

On the other hand, sodium propionate in our experiment was found to inhibit *S. cellulosum* growth at over 20 mM (data not shown), and the epothilone B/A ratio also increased to about 50:50 with increasing sodium propionate concentration at 10 mM sodium propionate. The maximum total epothilone concentration and epothilones B concentration were 19.4 mg/l and 8.58 mg/l, respectively, at 5 mM sodium propionate. However, total epothilone production declined at concentrations greater than 5 mM sodium propionate (Fig. 4).

In conclusion, the feeding condition of glycerol and sodium propionate was very important to improve the specific epothilones B production and resolution in natural *S. cellulosum* culture. The results presented from this study would be expected to be useful in a system design for epothilone production.

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