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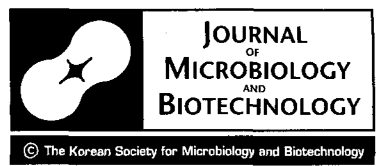

Repeated Batch Production of Epothilone B by Immobilized *Sorangium cellulosum*

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Abstract Production of extracellular epothilone B, one of the potent anticancer agents, by free and immobilized *Sorangium cellulosum* was studied using the repeated batch culture process. The concentration of alginate used in immobilization was directly related to the mass transfer rate of nutrients, mechanical stability, and the epothilone B production yield. With the optimized 3% (w/v) calcium alginate carrier, a prolonged repeated batch culture was investigated for the 5 repeated batches for 24 days. The maximum productivity of epothilone B obtained from the alginate-immobilized cells was 5.03 mg/l/day, which is 3 times higher than that of free cells (1.68 mg/l/day).

Keywords: Epothilone, immobilization, repeated batch culture, calcium alginate, *Sorangium cellulosum*

Epothilones are extracellular secondary metabolites naturally produced by the Gram-negative myxobacterium *Sorangium cellulosum,* which was originally identified in the Republic of South Africa [7, 8]. They are potential anticancer drugs that bind to microtubules, stabilize them in a polymerized state, and thereby block the cell multiplication, in a manner of action similar to that of paclitaxel (Taxol) [1]. In addition, the effect of epothilones on Taxol-resisting tumor cell lines and its comparatively higher water-solubility in comparison with Taxol make epothilones a potential chemotherapeutic compound with a great commercial value [3, 12].

However, the natural *S. cellulosum* has a relatively long doubling time. It is approximately 16 h, and is the longest time among all myxobacteria [11]. This biological characteristic may be a problem when the strain is used in the industrial applications. Tang and coworkers [20] introduced all the genes of the polyketide synthase (PKS) into *Streptomyces coelicolor* CH999, which is a common host used for production of a variety of polyketides from actinomycetes. It has a growth rate that is about 10 times faster than that of the natural *S. cellulosum* strains, which can reduce the long doubling time and enhance the epothilone production yield [20]. Furthermore, Julien and Shah [11] inserted a 65.4 kb DNA fragment of *S. cellulosum* that encompassed the entire epothilone gene cluster into the chromosome of *Myxococcus xanthus*. The result showed that the growth rate increased about 3.2 times faster than the native *S. cellulosum.* Although the recombinant strains usually grow more rapidly than the natural *S. cellulosum,* they produced a small quantity of epothilones. Therefore, it was necessary to improve the process by enhancing the epothilone production. Lau and coworkers [13] studied on optimizing the heterologous *Myxococcus xanthus* using a continuous fed batch process. They could improve the epothilone production by 140-folds from an initial titer of 0.16 mg/l of epothilone D.

In this study, we investigated the development of a novel process for epothilone B using the whole-cell immobilization and repeated batch culture technique to overcome the long doubling time of the natural *S. cellulosum* and to increase the production yield. The cells were immobilized on calcium alginate and the immobilized cells were cultured in a batch reactor. The batch culture was repeated for an extended time by replacing the exhausted culture medium with a fresh medium several times.

Sorangium cellulosum Soce90 was obtained from the DSMZ (German Collection of microorganism and Cell Culture, Braunchweig, Germany). Cells were cultured at 32°C with shaking at 200 rpm. The medium for cell inoculation was E medium, which contained, per liter of

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deionized water, pH 7.6, 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 buffer 50 mmol, and $FeCl_3$ 21 µmol [17, 18]. The culture medium for epothilone production (the production medium, hereafter) was a modified E medium, which contained the same components as the inoculation medium E, except for potato starch 15 g, glycerol 8.3 ml, sodium propionate 5 mmol, and 10 ml of trace elements solution [13]. The production medium contained 20 g/l of hydrophobic XAD-16 resin (Rohm and Haas Electronic Materials Korea, Ltd., Korea) to bind and stabilize the product [8]. Separated XAD-16 resins from the culture broth were washed 3 times with 50 ml of water and filtered with a DB Falcon strainer (Becton Dickinson & Co., NJ, U.S.A.) and then epothilone B was extracted using 25 ml of methanol for 30 min. Assay of epothilone B was carried out by a Shimadzu HPLC system (Shimadzu, Japan) [18]. The concentration of soluble starch was measured using the iodo-starch reaction method [10], and Megazyme's lactose/galactose assay kit was used (Bison Corp., Korea) for determination of the lactose concentration. One ml of sample was taken aseptically from each flask and the cells were removed by centrifugation at 12,000 ×*g*, 10 min, at room temperature. The supernatant was diluted with distilled water if necessary, and starch and lactose were analyzed at 550 nm and 340 nm, respectively.

When a whole-cell system is immobilized by calcium alginate, optimization of the sodium alginate concentration is often necessary [14]. Sodium alginate concentration is known to determine mass transfer phenomena of nutrients and biomass, which, in turn, are directly related to the cell growth, mechanical stability of immobilized biomass for extended repeated batch culture, and maintaining a high production yield over the repeated batches [2, 6, 9, 15, 21]. Therefore, different sodium alginate concentrations from 2 to 4% (w/v) were tested, and their effects on the uptake rate of a primary carbon source, starch, biomass stability, and production of epothilone B were studied. In order to immobilize *S. cellulosum*, sodium alginate solutions (2%, 3%, 4% [w/v]) were first prepared by dissolving an appropriate amount of sodium alginate in a 0.9% (w/v) NaCl solution. The solution was autoclaved at 121°C for 20 min, and then cooled down at room temperature. Five ml of 3-day precultured cells in E medium were then mixed with 20 ml of a sodium alginate solution. The mixture was then added dropwise to a gently stirred 3% (w/v) $CaCl_2$ solution through a peristaltic pump (Kd Scientific, PA, U.S.A.). The final calcium alginate beads with an average diameter of 3 mm were incubated in a $CaCl_2$ solution for 1 h. These beads were washed four times with sterilized water.

Beads with the low concentration of alginate (2%) were observed to be too soft and fragile, resulting in problems such as overgrowth of cells, expansion of bead diameter, and deformation of bead shape (data not shown). The high

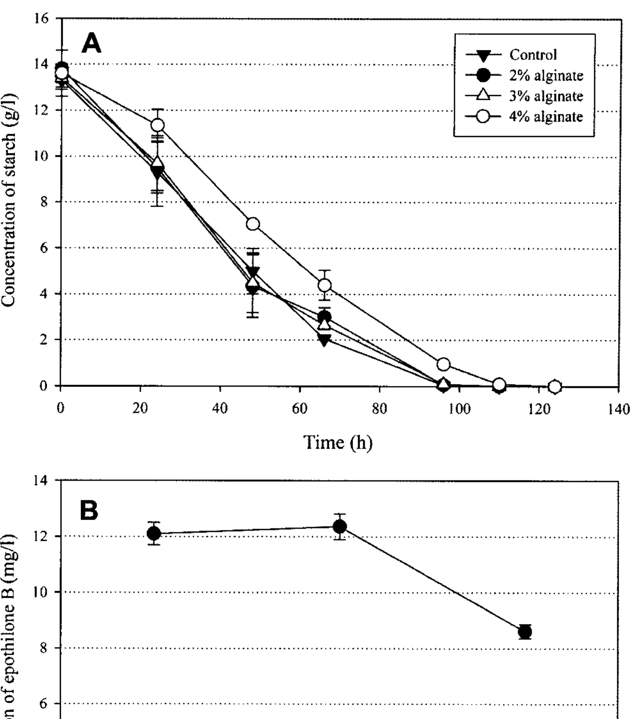


Fig. 1. Effect of sodium alginate concentration on the uptake rate of starch (**A**) and the production of epothilone B (**B**).
Cells were immobilized in three different sodium alginate beads having different concentrations of sodium alginate; 2, 3, and 4% (w/v).

concentration-composed alginate beads (4%) were very rigid, and therefore, a low diffusion rate of the nutrients seemed to be the most probable cause of the decline of epothilone B production, which totaled 12.36±0.46 and 8.61±0.25 mg/l for 3% and 4% (w/v) sodium alginate, respectively (Fig. 1). Finally, the suitable alginate concentration based on the uptake rate of starch and the epothilone B production yields was determined as 3% (w/v). In addition, we analyzed the inside of the calcium alginate bead in order to visualize the morphology and density of the *S. cellulosum* biomass using an FE-SEM (Field Emission Scanning Electron Microscope, JEOL, U.S.A.). The yellowish orange vegetative *S. cellulosum* is known to form fairly compact, dark cylindrical rods with broad, rounded ends, with the average dimensions of 3–6 µm and 1 µm in length and thickness, respectively [8, 16]. As shown in Fig. 2, *S. cellulosum* was rod shaped with rounded ends, and the length of the vegetative phase could be appropriate to be cultured in alginate beads.

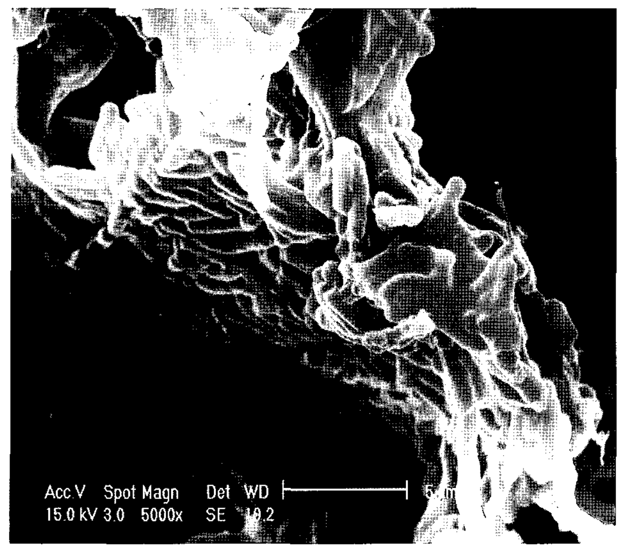


Fig. 2. Scanning electron micrograph of the immobilized *S. cellulosum* in calcium alginate bead (3% [w/v] sodium alginate).

Repeated batch processing is a well-known method for enhancing the productivity of microbial culture through extending the production phase of the culture. A portion of the original culture medium is replaced with fresh substrates and the culture continues in a subsequent batch [4, 5, 19]. In this study, free or immobilized cells were cultivated in 50 ml of production medium with 1.0 g of XAD-16 resin wrapped in a cotton cloth membrane. The substitution of exhausted medium with a fresh production medium was carried out at the time of maximum epothilone B production. Free or immobilized cells were separated from the culture broth and cultivated in 50 ml of the fresh production medium with XAD-16 resin after washing with sterile deionized water. Since the epothilone B production was saturated when lactose in skim milk was completely depleted in the *S. cellulosum* culture (data not shown), each of the batch culture time was determined by the lactose concentration.

As shown in Fig. 3A, the consumption rate of starch and lactose increased during the three time-repeated batches in the free *S. cellulosum* (8.5, 7.0, and 6.1 days for the first, the second, and the third batches, respectively). However, the maximum epothilone B concentration and its maximum productivity were 11.79±0.658 mg/l and 1.68± 0.092 mg/l/day, respectively, at the second batch. Although the third batch culture time was shorter than the other batch times, the epothilone B concentration and its productivity declined (Fig. 4), which was a major limitation for an extended repeated batch process using free cells. The total production of epothilone B reached 1.36 mg and productivity was 0.063 mg/day.

On the other hand, for the 3% calcium alginate beads, each culture time decreased even further than that of the free cell culture. As shown in Fig. 3B, the uptake of starch and lactose and each of the batch culture times declined sequentially during the 3 repeated batch cultures; 8.5, 5.3, and 2.8 days for the first, the second, and the third batch cultures, respectively. Batch culture times reduced after the initial batch, and then increased again in the final two batches (3.7 and 4.0 days) (Fig. 3B). It is thought that the immobilized biomass became unstable as the number of dead cells increased and/or the toxic byproducts accumulated when the repeated batch culture continued. As a result, the batch culture times continued to increase after the third batch. Moreover, because of the increase of biomass, the mass transfer rate of nutrients might have possibly decreased, which affected the batch culture time.

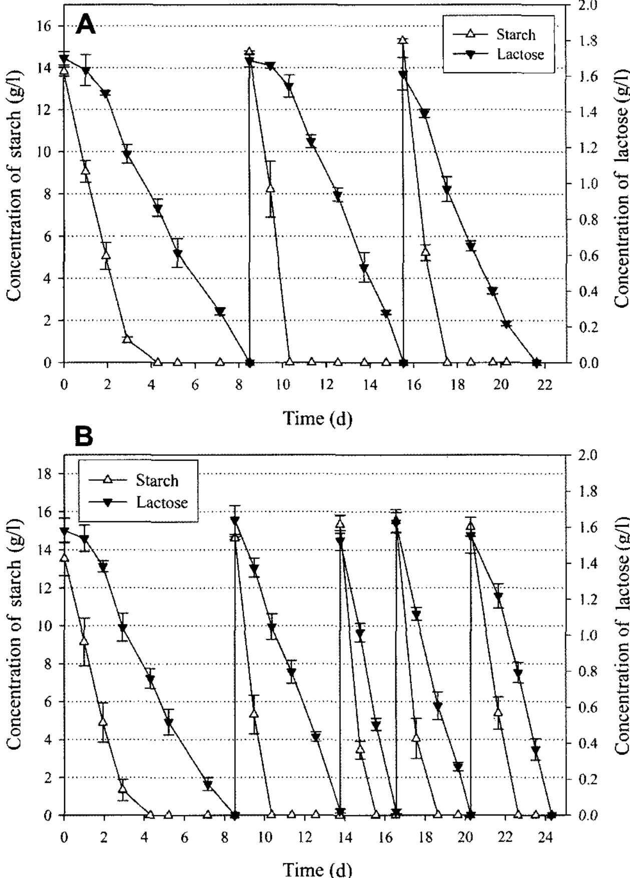
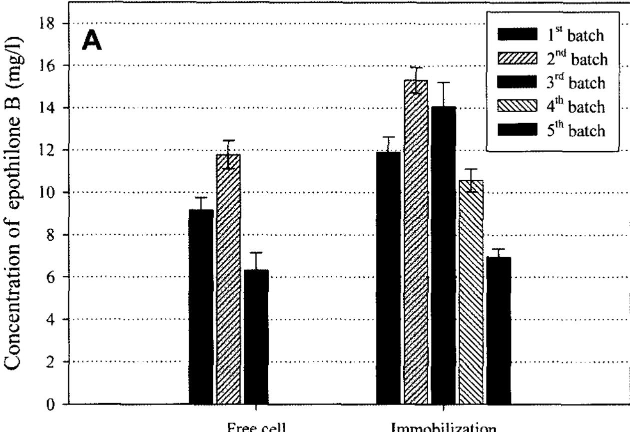


Fig. 3. Time course of starch and lactose concentrations in free and immobilized *S. cellulosum* on calcium alginate (3% [w/v] sodium alginate).

A. Free *S. cellulosum* was cultured for 22 days over three batch cultures; **B.** Immobilized *S. cellulosum* was cultured for 24 days over five batch cultures.

The maximum epothilone B concentration was observed to be 15.31±0.608 mg/l in the second batch, and its maximum productivity was 5.03±0.58 mg/l/day in the third culture (Fig. 4). The total production of epothilone B reached 2.94 mg and productivity was 0.121 mg/day.



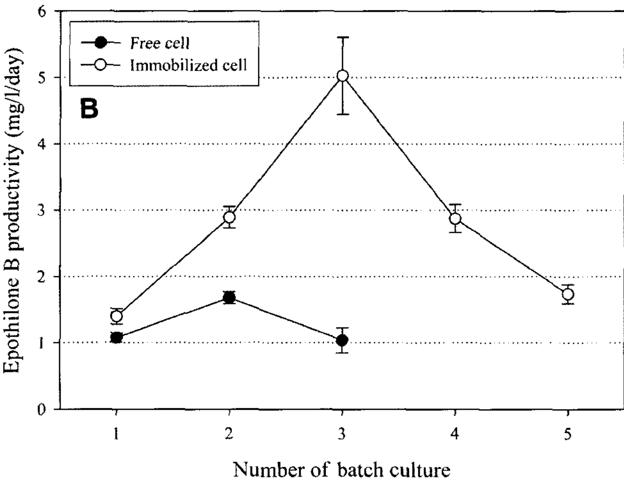


Fig. 4. Repeated batch production of epothilone B by free and immobilized *S. cellulosum*.

Epothilone B production (**A**) and epothilone B productivity (**B**) were measured at the end of each batch. Free and immobilized cells were cultivated in 50 ml of production medium and then the medium was replaced with a fresh one. Cultures continued for 3 and 5 batches for free and immobilized *S. cellulosum*, respectively.

Although both the immobilized *S. cellulosum* using calcium alginate and free cells were cultured during similar periods (22–24 days), the number of batch culture repetitions and the epothilone production yields for the immobilized system were greater than those of the free cells. This result indicates that calcium alginate helped maintain either the biological stability of *S. cellulosum* or protected the cells from toxic byproducts that were formed during the cellular metabolism. Moreover, by using the immobilized cells, it was possible to achieve a 1.7-times increase in the number of batch culture repetitions, which resulted in a two-times higher epothilone B productivity.

In the previous study of Gerth *et al.* [8], natural epothilone B production and its productivity were 11 mg/l and 1.571 mg/l/day, respectively, in batch fermentation. In this study, we obtained a maximum productivity of 5.03 mg/l/day using the immobilized *S. cellulosum* system in calcium alginate beads, which is 3 times greater than that of the free cells (1.68 mg/l/day). This result demonstrated that one of the problems, the long doubling time, was resolved by the repeated batch culture method.

To the best of our knowledge, this study was the first attempt in which an immobilized *S. cellulosum* system was used for epothilone B production in the prolonged (*i.e.*, extended) repeated batch process; 5-time repetitions for 24 days. Repetition of the batch culture made it possible not only to overcome the relatively long doubling time of *S. cellulosum*, but it also enhanced the epothilone B production. In addition, the sodium alginate concentration and the period of repeated batch culture were found to be very important factors in enhancing both the total epothilone B production and epothilone B productivity. Further scale-up and optimization studies are still necessary in order to apply the proposed process to commercial processes and to evaluate its techno-economical feasibility.

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