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Glycosylation and Production Characteristics of Epothilones in Alkali-Tolerant *Sorangium cellulosum* Strain So0157-2[§]

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3-O-α-D-ribofuranosyl epothilone A (epothiloneoside A) is a major component of glycosylated epothilones in *Sorangium cellulosum* strain So0157-2. The production and glycosylation ratios of epothiloneoside A in both solid and liquid culture conditions with various pH values and carbon sources were studied. The results showed that glycosylation occurs whenever epothilones are produced, regardless of changes in pH values, production time curves, and different carbon sources. We suggest that glycosylation is a stable process, paralleling the biosynthesis of epothilones in the So0157-2 strain.

Keywords*: *S. cellulosum, epothilones, glycosylation, epothiloneoside A

Antifungal and cytotoxic epothilones are a type of polyketide macrolide that are produced by a few strains of the myxobacterium, *Sorangium cellulosum* (Gerth *et al*., 1996; Höfle *et al*., 1996). Bollag *et al*. (1995) reported the strong stabilizing activity of epothilone compounds on polymerized microtubules, which mimicked the mechanism by which taxol affects tumor cells. This discovery led to the following extensive studies on epothilones regarding their biochemical mechanisms, chemical modifications, *in vivo* and *in vitro* activity tests, clinical trials, production, and biosynthesis, which showed that epothilones have enormous promise in cancer chemotherapy (Reichenbach and Höfle, 2008). There are several epothilones or their chemically-modified derivatives undergoing clinical trials for cancer treatment, one of which (ixabepilone) has been authorized for use in clinics by the U. S. Food and Drug Administration (Fornier, 2007).

Epothilones are biosynthesized in *Sorangium* cells in a stepwise polymerization sequence from short carboxylic acid precursors, catalyzed by a set of modular polyketide synthases (PKSs), which are combined with a non-ribosomal peptide synthase (NRPS) module (Julien *et al*., 2000; Molnár *et al*., 2000). In *S. cellulosum* So ce90, 8 analogs of 16-membered macrolide epothilones (A to H) containing 29 variants have been described (Hardt *et al*., 2001). One 18-membered (epothilone I), one 14-membered (epothilone K), and several linear epothilones have also been shown to be present in the broth of this strain. Epothilones A and B are the major epothilone products in the So ce90 strain, while others are produced in trace amounts. The biosynthetic gene cluster of epothilones has also been engineered into other hosts, such as *Streptomyces* (Tang *et al*., 2000) or *Myxococcus* (Julien and Shah, 2000), in search for efficient production. Other than the analogs that have been discovered in *Sorangium*, some new derivatives of epothilones have also been produced by genetic engineering of the biosynthetic genes in recombinant epothilone-producing *Myxococcus* strains (Starks *et al*., 2003).

In our screening programs, many epothilone-producing *Sorangium* strains have been obtained from different soil samples (Li *et al*., 2001; Dong *et al*., 2004; Hu *et al*., 2004; Li *et al*., 2007). The So0157-2 strain is an alkali-tolerant epothilone producer. The major components of epothilones in So0157-2 are also epothilones A and B (Gong *et al*., 2007). Interestingly, some glycosylated epothilones have been detected in the broth of this strain (PCT/CN2008/001946; Wang *et al.*, 2009). Because of their potential applications in therapy, not only providing great solubility, but also decreasing their toxicities, the glycosylation and production characteristics of epothilones have been analyzed under different culture conditions.

Materials and Methods

Microorganism and culture conditions

So0157-2 is a cellulolytic myxobacterial strain that was first isolated from an alkaline soil sample. The strain was morphologically and phylogenetically classified using previously reported methods (Yan *et al*., 2003; Jiang *et al*., 2008). The genomic DNA of *Sorangium cellulosum* strain So0157-2 was extracted by a Genome Extract kit (Tiangen, China). The 16S rRNA gene sequence was amplified using the primer pair of 27F/1492R (Yan *et al*., 2003). Several clones were selected for sequencing at the TaKaRa Sequencing Center (China). Phylogenetic reconstruction of the sequences was carried out using distance/neighbor joining programs with the Poisson correction model in the MEGA v.3.1 software package (http://www.megasoftware.net/). The interior branch length supports were from 1,000 replicates. The strain has been routinely cultivated on solid CNST agar plates (Yan *et al*., 2003) and in liquid M26 medium (Nguimbi *et al*., 2003) at 30°C. The pH value of the medium was adjusted using a KOH solution

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before autoclaving.

Fermentation

Myxobacteria are characterized by their social lifestyle, which leads myxobacterial cells to have different characteristics under liquid and solid conditions (Reichenbach and Dworkin, 1992). Thus, both solid state and submerged (liquid state) fermentations have been carried out for the production of epothilones. To prepare the inoculum, *Sorangium* strain So0157-2 was inoculated on CNST agar plates (pH 8.0) and incubated for 4 days at 30ºC, followed by shaking in liquid M26 (pH 8.0) for 4-5 days. The cells were collected from the M26 broths and inoculated in liquid M26 or on solid CNST plates for the production of epothilones. Sterilized XAD-16 resin (Gerth *et al*., 1996) was added (2%, v/v) into liquid cultures from the beginning of inoculation and or spread onto solid cultures (covering the cultures) after 4 days of incubation for the absorption of products. Following the addition of resin, cultures were incubated for an additional 10 days in the solid state fermentation or 6 days in the submerged fermentation. To assay the production curves of epothilones, resin was added at the beginning of inoculation, and the cultures were harvested every day from the second day of inoculation. After fermentation, resin was collected, and the metabolites absorbed in the resin were extracted using methanol. Components in the extracts were isolated, and epothilones A and B, and epothiloneoside A was identified and quantified using purified compounds.

Single-factor test

For the single-factor test (SFT) of carbon sources, the So0157-2 cells were cultivated in a base medium containing 0.1% glucose, 0.05% KNO_3, 0.015% Na_2HPO_4, 0.015% K_2HPO_4, 0.15% $MgSO_4$, 0.15% $CaCl_2$, 0.001% $FeCl_3$-EDTA, 1 ml/L of trace element solution (Reichenbach and Dworkin, 1992), and 0.5 mg/L of V_{B12}. The pH value of the medium was adjusted to pH 7.5 using KOH solution before autoclaving. The base medium was shown to be unable to produce epothilones. Single carbon sources were added into the base medium to test the effects on glycosylation and the production of epothilones. Greater than 40 carbon sources and two concentrations of each were selected for the test. Cultivation performances were the same as the above description.

Detection of epothilone production

Yields of epothilones were detected using HPLC chromatography with a 5 μm RP-C18 column (4.1×250 mm; Agilent, USA). The methanol extract from resin was eluted with methanol:water (60:40) at 1 ml/min. Epothilones were detected in HPLC with the Photo-Diode Array (PDA) detector using an absorption range from 200-800 nm. The production of epothilones was calculated on the basis of the absorption of purified epothilone standards at 249 nm. The MS analysis was carried out under the following conditions: ESI positive; probe temperature, 450°C; cone 75 V; and full scan mass range from 100-1,000 for epothiloneoside A.

Cytotoxic activity assays

A colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cytotoxic activities of the compound. The assays were performed on human breast cancer cells (MDA-MB-435), human lung adenocarcinoma cells (A-549), and mouse lympholeukemia cells (P-388). The IC_{50} values were determined.

Results

Culture and morphologic characteristics of the So0157-2 strain

The So0157-2 strain is able to grow in a wide pH range (5.0-14.0), and the optimal pH value for growth is between 7.0 and 10.0. On the nutrient-limited medium, CNST (mineral salts plus filter paper as the only organic resources), the strain aggregated, but formed no fruiting body structures. However, if the pH value of the medium was adjusted to ≥9.0, sparse and unpacked sporangiole-like structures were formed in colonies (Fig. 1A). The 10-day CNST cultures of different pH values were assayed for heat tolerance at 55ºC for 10 min. Following heat treatment, only the cultures with a pH≥9.0 were able to survive. Thus, the sporangioles formed in the conditions with high pH values were real anti-adversity structures. The vegetative cells of So0157-2 were 3-5 μm long with a diameter of 1-2 μm, while the myxospores, embedded in sporangioles, were in short bacilli form, 2-3 μm in length and 0.8-1 μm in width. Together with the morphologic characteristics, the phylogenetic analysis of 16S rDNA gene sequences (Fig. 1B) indicated that So0157-2 is a typical strain of *Sorangium cellulosum* (the accession number of the 16S rRNA gene sequence of So0157-2 in GenBank is DQ256394). Furthermore, it is interesting to note that the So0157-2 strain is rather easy to grow in liquid, where cells formed loose clumps (Fig. 1A).

Glycosylated epothilones in the So0157-2 strain

Several analogs of glycosylated epothilones were found in the culture of the So0157-2 strain (PCT/CN2008/001946; Wang *et al.*, 2009). All of these compounds were glycosylated at the hydroxyl group of the 3-C site. The major glycosylated epothilone had a molecular mass of 625.29 and a chemical formula of $C_{31}H_{47}NO_{10}S$. This compound was determined to be 3-O-α-D-ribofuranosyl epothilone A (epothiloneoside A; the molecular structure is shown in Fig. 2A and the MS analytic results are provided in the Supplementary datas). The other glycosylated epothilones were all in trace amounts. In a methanol solution, epothiloneoside A possessed a typical absorption spectrum of epothilones (Fig. 2B). Under the HPLC conditions provided in the 'Materials and Methods', this glycosylated epothilone compound was eluted at 14.52 min, while the retention time for epothilones A and B was 20.73 and 25.94 min, respectively (Fig. 2C). The glycosylated epothilone A was cytotoxic to human breast cancer cells (MDA-MB-435) and human lung adenocarcinoma cells (A-549; Table 1). The IC_{50} value of the compound was very low (0.07 μg/ml) on mouse lympholeuke mia cells (P-388). The cytotoxic activity of epothiloneoside A was similar to that of epothilone A (Altmann *et al.*, 2000). Until now, glycosylation on epothilones has only been found in *Sorangium cellulosum* strain So0157-2. It is thus interesting to investigate the conditions for glycosylation.

Table 1. IC_{50} Values of epothiloneoside A to tumor cells

Strains	Human breast cancer cell MDA-MB-435	Human lung adenocarcinoma cell A-549	Mouse lympholeukemia cells P-388
IC_{50} (μg/ml)	2.02	6.47	0.07

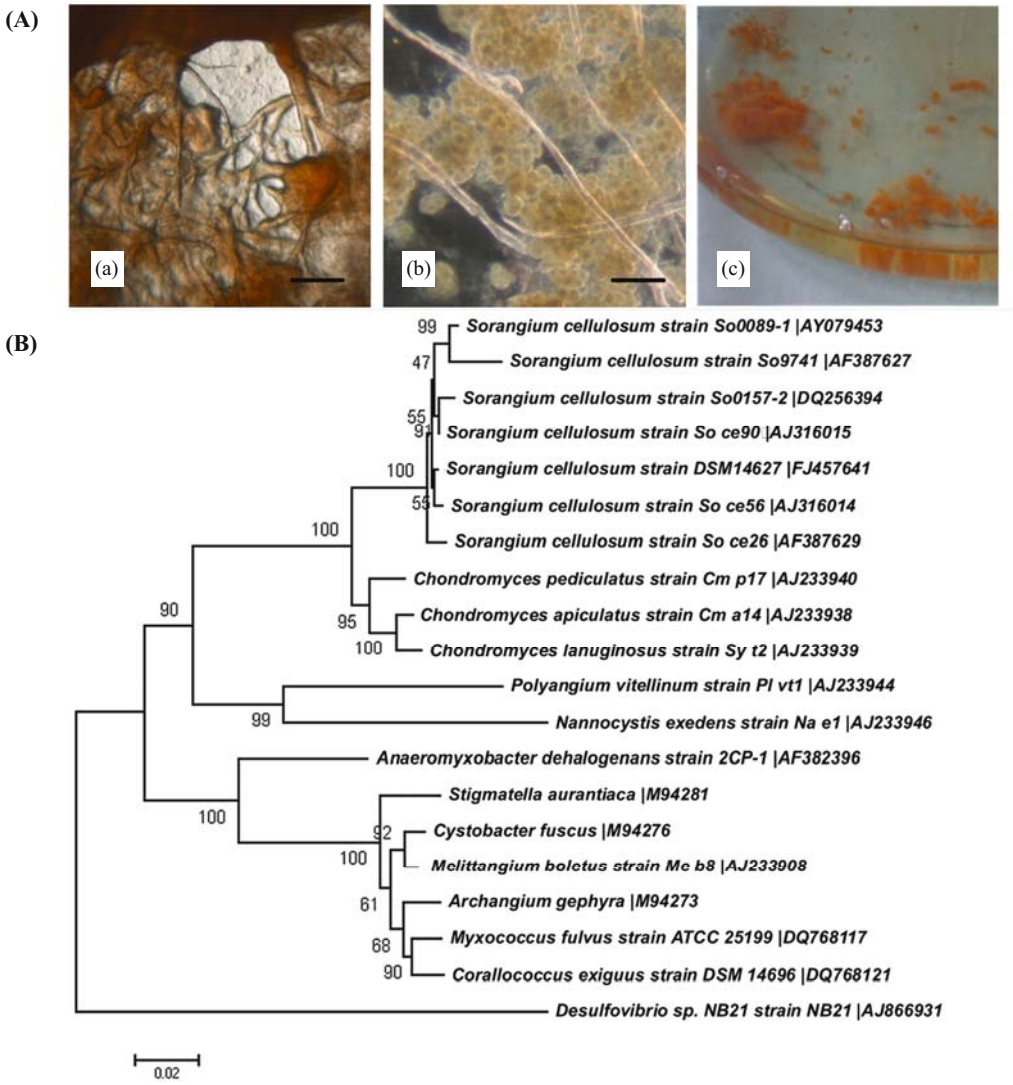

Fig. 1. (A) The swarms (a) and sporangioles (b) of *S. cellulosum* So0157-2 grown on CNST medium at pH 10.0. The pictures were obtained after 10 days of incubation. The bar is 0.5 mm for the swarms and 50 μm for the sporangioles. (c) Loose clumps of So0157-2 cells grown in M26 medium at pH 7.0. (B) Phylogenetic analysis of strain So0157-2 using the 16S rDNA gene sequences.

Effects of pH values on glycosylation

So0157-2 is an alkali-tolerant epothilone producer. In order to explore the potential relationships between glycosylation efficiency and the culture pH values, the strain was fermented in liquid and solid conditions with different pH values (6.5 to 11.5) at intervals of 0.5. The medium for the liquid culture was nutrient-rich medium (M26), and solid cultures were performed on nutrient-limited medium (CNST agar plates). In M26, epothilones were only produced within a pH range from 8.5-11.0. At lower pH values, no epothilones were detectable. The highest yield of total epothilones appeared at pH 8.5, and the production gradually decreased with an increase in the pH value (Fig. 3A). The glycosylation ratio of epothilone A changed from 3.3% at pH 9.5 to 12.6% at pH 10.5. However, on the solid nutrient-limited medium (CNST), epothilones were produced in wider pH values (6.5 to 11.5). The highest yield of total epothilones on CNST plates appeared at pH 9.5 (Fig. 3B). Similar to that in liquid, the glycosylation ratio from epothilone A to epothiloneoside A changed little with the changes in pH values, from 7.5% at pH 8.5 to 13.9% at pH 10 (some high glycosylation ratios at a low rate of epothilone production were not taken into account).

Glycosylation curves during the production of epothilones

To determine glycosylation changes during the production process of epothilones, solid state fermentation was performed on CNST medium at pH 7.5, 8.5, 9.5, and 10.5. Unlike the curves in liquid medium (Gong *et al*., 2007), the production process of epothilones on CNST agar plates with different pH values lasted a long time. After 15 days of incubation, the production of epothilones continued to increase (Figs. 4A-D). In fact, on CNST medium, epothilone production increased, even after 30 days of incubation (data not shown). The productions of epothilone A, epothilone B, or epothiloneoside

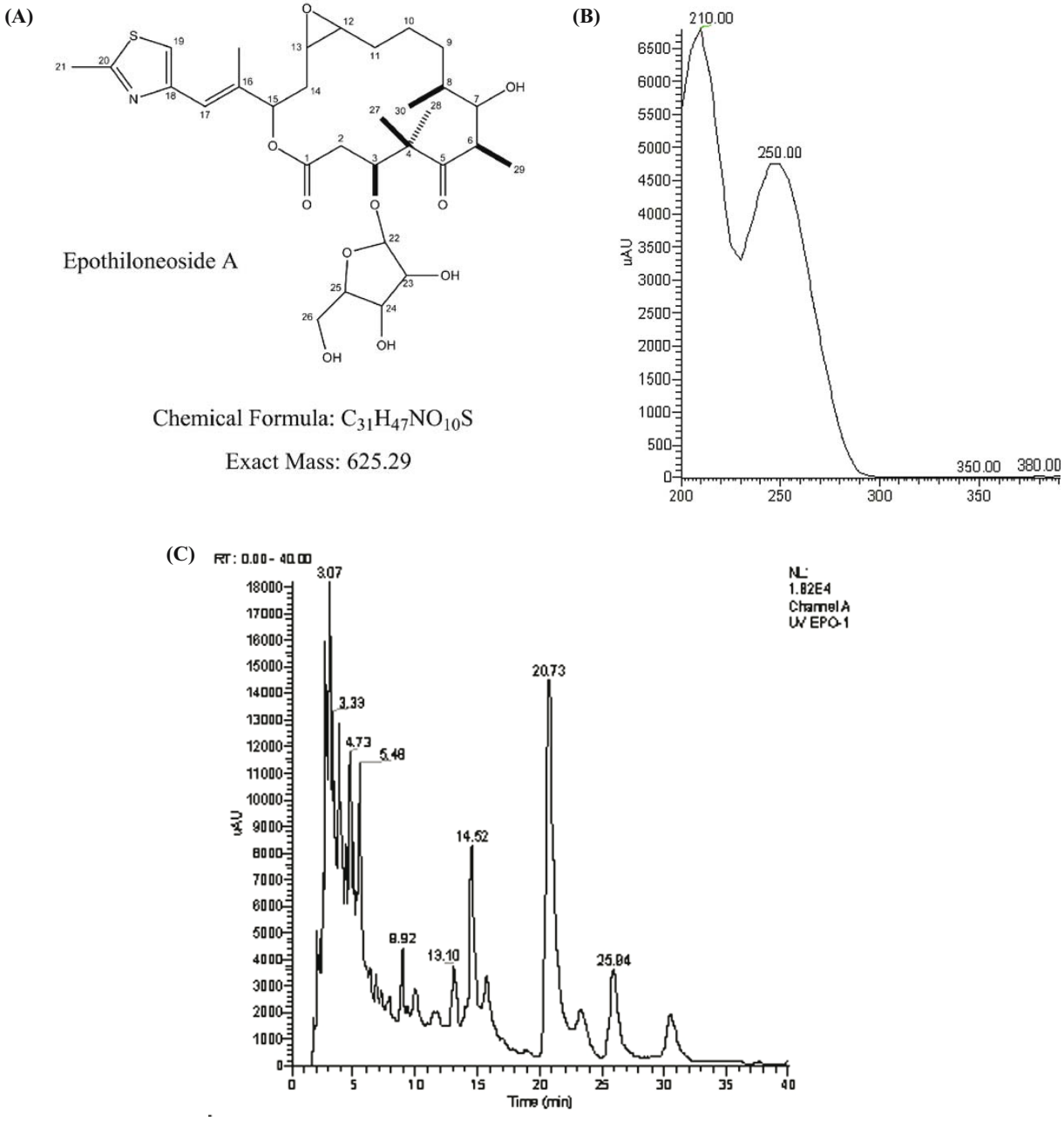

Fig. 2. (A) The molecular structure of epothiloneoside A. (B) Electronic absorption spectrum of epothiloneoside A in methanol which was detected by the PDA detector of HPLC. (C) The retention time of epothilones A/B and epothiloneoside A.

A had similar curves in the cultures with different pH values. Interestingly, during the production process of epothilones, glycosylation of epothilone A always occurred.

Effects of carbon sources on glycosylation

To screen whether or not there was a special type of saccharide able to trigger the glycosylation of epothiloneoside A, different carbon sources and two concentrations of each were added into a base medium (see 'Materials and Methods'). So0157-2 cells grew well in this base medium. In either liquid or solid state culture conditions, the So0157-2 cells reached the growth summit (assayed by dry cell mass) after 4 or 5 days of incubation, followed by a gradual decrease of cell mass. However, this base medium did not support the production of epothilones (no epothilones were detectable by the HPLC method in either liquid or solid state cultures).

After the addition of different carbon sources, a few were able to support the production of epothilones, but in varied efficiencies (Table 2). Interestingly, similar to the above experiments, whenever epothilones were produced, glycosylation of epothilones occurred. In liquid state fermentation, celluflor (0.3%), maltose (0.5%), potato starch (0.5%), and dextrin (1.2%) were suitable for the production of total epothilones, among which dextrine gave the highest yield. However, the

Table 2. The production of epothilones triggered by the addition of carbon sources in liquid and on solid cultures in the base medium

Carbon source	Epothilone A (mg/L)	Epothilone B (mg/L)	Total yield (mg/L)	Glycosylation efficiency (%)
In liquid culture				
L-arabinose 0.3%	3.23	1.8	5.79	19
Celluflor 0.3%	5.7	2.19	8.95	15.7
Maltose 0.5%	6.07	2.81	9.66	11.4
Potato starch 0.5%	4.64	1.95	7.61	18
Citric acid 0.3%	2.36	0.96	3.69	13.6
Fructose 0.3%	1.55	0.64	2.8	28.2
Dextrin 1.2%	8.1	2.91	12.58	16.2
Sodium acetate 0.3%	2.38	0.82	4.12	27.9
Glucose 0.1%	2.29	1.43	4.24	18.5
Xylitol 0.9%	0.59	0.16	1.03	32.2
On solid culture				
Mannose 0.2%	0.92	0.09	1.25	20.7
L-Arabinose 0.3%	0.62	0.22	1.12	31.1
D-Arabinose 0.1%	0.5	0.28	0.92	21.9
Filter-paper	28.03	7.67	43.04	20.8
Maltose 0.5%	28.42	14.96	47	11.3
Potato starch 0.5%	1.53	0.48	2.61	28.2
Potato starch 0.2%	5.67	1.42	10.14	35
Fructose 0.1%	0.85	0.22	1.92	50
Dextrin 1.2%	2.16	0.33	2.8	12.6
Sodium propionate 0.1%	0.59	0.14	1.75	63.4

glycosylation ratio of epothilone A to epothiloneoside A changed in a different manner. A high glycosylation ratio appeared when 0.9% xylitol, 0.3% fructose, or 0.3% sodium acetate were mixed in the base medium, and reached 32.2%, 28.2%, and 27.9%, respectively. The lowest glycosylation ratio in liquid was 11.4% with the addition of 0.5% concentrations of maltose. In solid state fermentation, the triggering carbon sources for the production of epothilones were also limited to a few. However, the compounds that were suitable for the production of epothilones in solid state fermentation were different from those in the submerged fermentation. Some compounds, such as cellulose (filter paper and celluflor were used in solid and liquid state fermentations, respectively) produced a high yield of the total epothilones in both liquid and solid state fermentation. Similar to liquid, the glycosylation ratio of epothilone A to epothiloneoside A in solid state fermentation also differed. For example, 0.2% potato starch and 0.3% L-arabinose had a 35.0% and 31.1% glycosylation ratio, but both yielded a low production of total epothilones. Interestingly, in solid state fermentation, 0.1% fructose produced a 50.0% glycosylation ratio, whereas in liquid the compound at this concentration did not produce any HPLC-

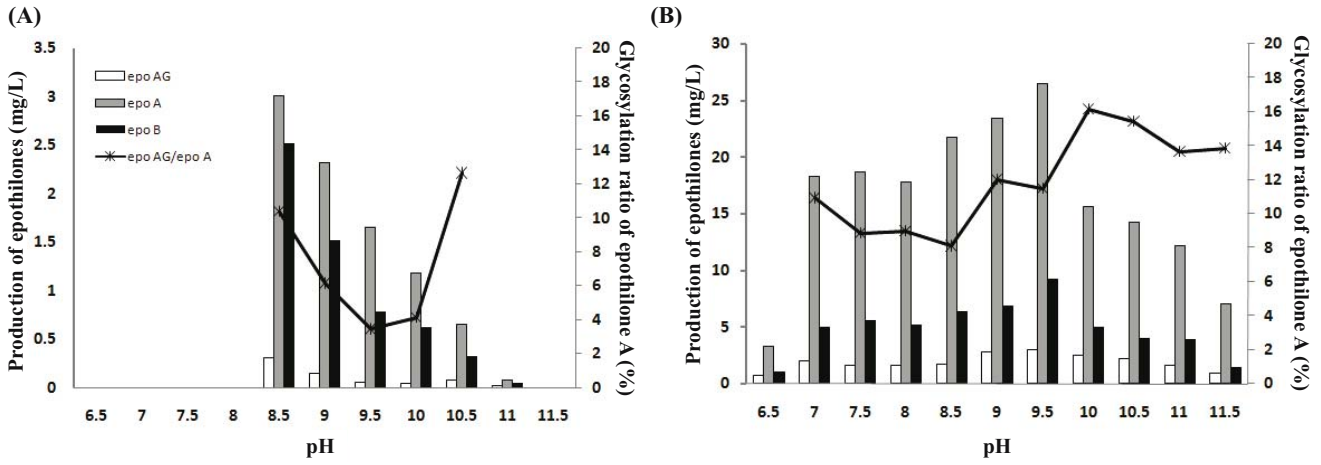

Fig. 3. The production of epothilones A/B and glycosylated epothilone A in liquid M26 (A) and on solid CNST (B) with different pH values. The glycosylation ratio of epothilone A was calculated by dividing the yield of epothiloneoside A with the yield of epothilone A.

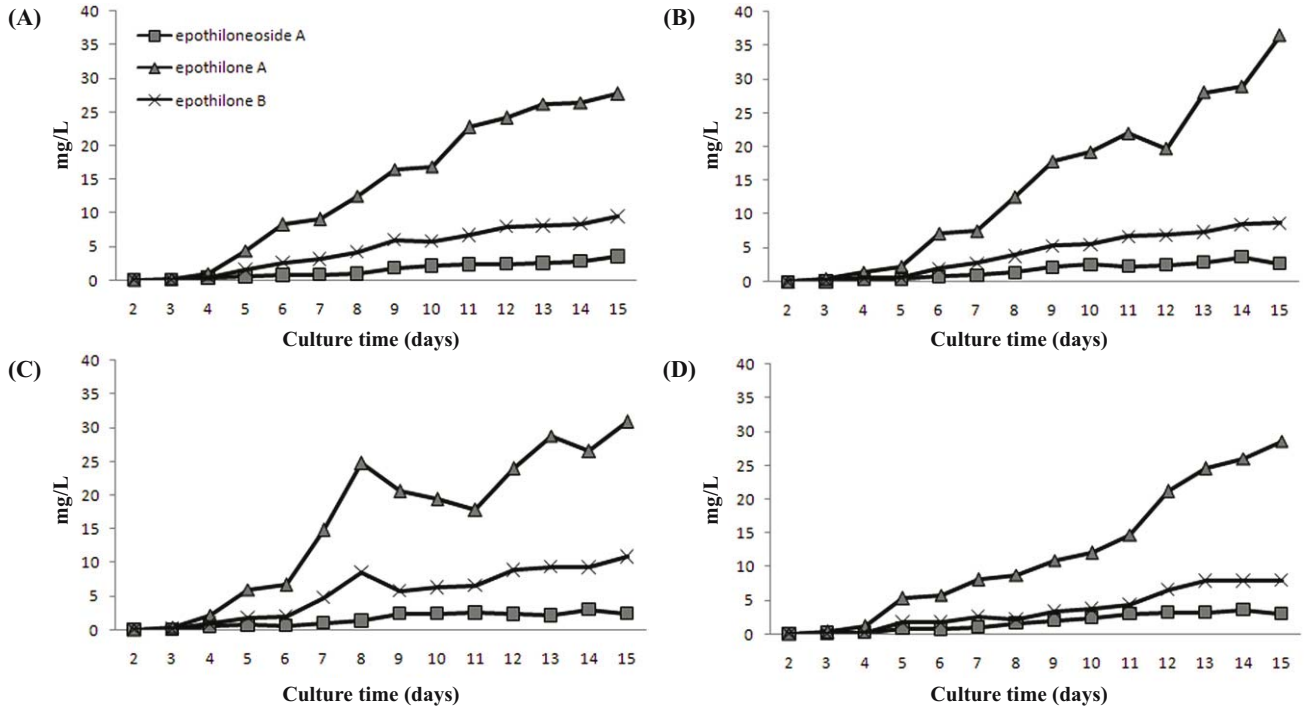

Fig. 4. The production curves of epothilones on CNST with pH values of 7.5 (A), 8.5 (B), 9.5 (C), and 10.5 (D).

detectable epothilones. Furthermore, 0.1% sodium propionate produced 63.4% of glycosylated epothiloneoside A from epothilone A; this is the only example that glycosylated epothilone A was higher in concentration than epothilone A, but the production of the total epothilones was low. Although there were several high glycosylated ratios, the most practical media were filter-paper and 0.5% maltose in low ratios in the solid fermentation.

Discussion

Sorangium strains have an excellent ability for producing diverse secondary metabolites. The secondary metabolites discovered from this genus account for approximately 48% of the total discoveries arising from myxobacteria (Gerth *et al.*, 2003). Epothilones, as a type of polyketide, are the major secondary metabolites produced by some *Sorangium* strains. Although greater than 40 analogs of epothilones have been identified from the broth of a 10-ton fermentation of the So ce90 strain, no glycosylated epothilone it was found (Hardt *et al*., 2001). Genome sequencing has revealed many genes encoding enzymes potentially for glycosylation in *Sorangium* (Schneiker *et al*., 2007). Glycosylated epothilones were, however, only reported in So0157-2 (PCT/CN2008/001946). Until now, the glycosides that were identified in the glycosylated epothilones were all pentosides (PCT/CN2008/001946). Furthermore, after the discovery of glycosylated epothilones in So0157-2, we screened greater than 50 epothilone-producing *Sorangium* strains that were isolated from different soil samples, but no glycosylated epothilone was found in these strains (data not shown). Thus, it is suggested that glycosylation of epothilones is probably catalyzed by some specialized enzymes encoded by the So0157-2 strain. Glycosylated epothilones, with similar cytotoxicities against different tumor cells, have potential clinic interests, not only providing greater solubility, but also decreasing their toxicities. It is interesting to elucidate the mechanisms responsible for the glycosylation of epothilones in So0157-2.

So0157-2 is an alkali-tolerant *Sorangium* strain. We have demonstrated that the glycosylation process of epothilones occurs in So0157-2 under many culture conditions. In the nutrient-limited conditions (the CNST medium), epothilones were able to be biosynthesized in a wide range of pH values; whereas, in the nutrient-rich medium (M26), the compounds only appeared in the alkaline conditions. The production of epothilones appeared to respond to the alkali-tolerant characteristics of the strain. However, whenever epothilones are produced, glycosylation of epothilones occurs, regardless of changes in pH values, production time curves, and different carbon sources. The glycosylation activity changed slightly in response to culture conditions. In those conditions that gave a high yield of epothilones, the glycosylation ratios are similar (about 20%). It should be noted that under various culture conditions the production of other glycosylated epothilone analogs is always in trace amounts. The glycosylation of epothilones is a rather stable physiologic process during the biosynthesis of epothilones in So0157-2 cells. The enzymes that are responsible for the glycosylation of epothilones are probably co-expressed with the biosynthetic enzymes of epothilones. Bioinformatic analysis of the incomplete genome sequence reveals at least 53 possible glycosylase genes in So0157-2, 7 of which are nearby or in the biosynthesis gene cluster of epothilones. Further heterogonous expression and characteristic analysis are being performed to determine the

corresponding enzyme and mechanisms for the glycosylation.

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