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Establishment of *Sorangium cellulosum* So0157-2 Proteome Database Using Optimized Two-dimensional Electrophoresis Protocol*

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Abstract The rich classes of secondary metabolites from the genus *Sorangium* have been an important source of new drugs. The proteome analysis is an effective method to study the regulation of metabolism. However, the genus *Sorangium* contains a large amount of exopolysaccharides or slime that interferes with protein solubility, resolution, and repeatability in proteome analysis. To perform high-throughput screening of the specific proteins expressed by *Sorangium cellulosum* So0157-2, we optimized the two-dimensional electrophoresis (2-DE) protocol. Firstly, the proteins have better solubility in lysis buffer. The pH 3～10 NL strip is appropriate for the first-dimensional isoelectric focusing, improving the resolution of protein spots. 1 mg of protein was used in the isoelectric focusing, improving the expression of low-accumulation proteins. 15% SDS-PAGE improved the resolution and repeatability for separation of these proteins. Based on the optimal 2-DE protocol, the protein patterns of *S. cellulosum* So0157-2 cultured in M26 medium for three days were acquired, and 552 protein spots were detected. Further, the expressed proteins (85.9%) were identified by MALDI-TOF-MS. The identified proteins included components of cell structure and function, and cell metabolic enzymes. Worthy to be mentioned, 8 proteins were related to the transformation and metabolism of carbohydrate, which were contributed to the in-depth study of epothiloneoside A. This optimal protocol laid the foundation for the further construction of proteome expression database of *S. cellulosum* So0157-2 in various industrial culture conditions.

Key words two-dimensional electrophoresis, *Sorangium cellulosum* So0157-2, optimize, proteome
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Natural products which were produced by living organisms and their derivatives often have pharmacological or biological activities that provide the basis for medicines targeting a wide range of human diseases[1]. In particular, many of these products are important in the treatment of life-threatening conditions[2]. Microorganisms such as bacteria and fungi have been invaluable for discovering natural drugs and in leading compound synthesis. They produce a large variety of natural products, such as polysaccharides, enzymes, antibiotics, pigments, amino acids, organic acids, alcohols, ketones, vitamins, and nucleic acids. The Gram-negative myxobacteria, a remarkable group of δ-proteobacteria with a complex multicellular developmental program[3], is an important source of novel classes of secondary metabolites[4]. Of these, the genus *Sorangium* is particularly valuable, as 46% of metabolites derived from this group were isolated from it [4], including the potent anti-cancer metabolites, epothilones and derivatives [5]. These metabolites are well-known for their more effective anti-cancer function than taxol.

Sorangium cellulosum produces a large variety of natural products and has complex developmental cycles[6]. Carolacton, one of the secondary metabolites isolated from *S. cellulosum*, has been used in antibacterial therapy against biofilms of *Streptococcus mutans*. It interferes with the density-dependent signaling systems in *S. mutans* and provides a novel

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approach for the prevention of dental caries [7]. The *S. cellulosum* So ce56 was established previously as a model *Sorangium* strain [8] by virtue of its favorable growth characteristics and its ability to differentiate reproducibly under laboratory conditions [1]. To verify the importance of phosphorylation in gene regulation by *S. cellulosum* So ce56, phosphoproteome analysis was performed using two-dimensional electrophoresis (2-DE) coupled with matrix-assisted laser desorption ionization/tandem time-of-flight mass spectrometry (MALDI-TOF-MS)[1]. Next, shotgun proteome analysis for secondary metabolite biosynthesis was performed using 2D-HPLC-MS/MS with collision-induced dissociation (CID) and electron transfer dissociation (ETD)[9].

Previously, an epothilone-producing strain *S. cellulosum* So0157-2 was isolated from the soil of lakeside and screened in our laboratory. At present, research on *S. cellulosum* So0157-2 mainly focuses on two aspects: (i) "social behavior", including cell movement by gliding, biofilm formation, and morphological differentiation; (ii) identification of the novel anti-cancer metabolites epothilones and their derivatives [10–12]. The epothilones A and B are an important class of antitumor secondary metabolites produced by *S. cellulosum* So0157-2, which were found to kill dividing cells by stabilizing microtubules [13]. Two 18-membered epothilones M and N were identified from *S. cellulosum* So0157-2 using extensive NMR analysis [13]. Five novel natural epothilone derivatives identified as 3-alpha-D-arabinofuranosides of epothilones have better water solubility and absorbability, and weaker cytotoxic activity comparing with other antitumor agents [11]. The glycosylation of epothilones was demonstrated in paralleled with the biosynthesis processes of epothilones in the *S. cellulosum* So0157-2, and the production ratio of epothiloneoside A was improved in the specific medium components [14]. However, the proteins involved in the production of epothilones and derivatives were less well understood. The research of gene regulation and proteome information of epothiloneosides has become a hot spot.

S. cellulosum owns a huge genome. As the largest genome among sequenced prokaryotes, the circular genome of the *S. cellulosum* So0157-2 strain is beyond 14 M and has an extremely high GC-content of 69% ～72% . Considering the difficulty of genetic manipulation, 2-DE turns out to be the best method to illustrate the specific proteins that participate in biosynthesis of primary and secondary metabolite. 2-DE is one of the most common and effective techniques in proteome research. Acquiring high-quality protein patterns is essential for constructing the species proteome database. However, it requires great effort to perform a high resolution and coverage 2-DE. During uninterruptible operations, multi-conditions are all needed to be optimized effectively, such as method of protein purification, conditions of first-dimensional isoelectric focusing, choice of pH gradient ranges, amount of protein applied to the IPG strip, and concentration of SDS-PAGE. Furthermore, analyzing total cellular proteins of *S. cellulosum* So0157-2 encountered a new big problem: the cell produces a large amount of exopolysaccharides or slime, which severely interfered with protein solubility, resolution, and repeatability in proteome analysis [15]. Therefore, it became an exigence to establish an optimized 2-DE protocol for *S. cellulosum* So0157-2. In this study, we improved the methods for sample preparation and electrophoresis on the basis of the classical methods described by Gorg *et al*. and Otani *et al*. [15–17], and established the optimal protocol for analyzing the total cellular proteins of *S. cellulosum* So0157-2. The proteome database of *S. cellulosum* So0157-2 cultured in M26 fermentation medium was established using this protocol. And the special proteins related to the glycosylation of epothilones were acquired by high-throughput screening.

1 Materials and methods

1.1 Strains and culture conditions

S. cellulosum So0157-2 produced low levels of epothilone A and B(1.7 and 0.8 mg/L)[18] in M26 liquid medium (pH 7.0) containing (*w*/*v*) potato starch 0.8%, glucose 0.2%, yeast extract powder 0.2%, $CaCl_2 \cdot 2H_2O$ 0.1%, and trace elements solution 1 ml[19–20]. The strain was routinely cultured in M26 liquid medium at 30℃ with shaking at 200 r/min for three days [10]. The cells were harvested by centrifugation (5 000 *g*, 4℃ for 10 min).

1.2 Sample preparation

The *S. cellulosum* So0157-2 cells were washed twice using TM buffer containing 10 mmol/L Tris (pH 8.0) and 5 mmol/L magnesium sulfate, and

ultrasonically disrupted using two different extraction methods. (i) The samples were placed in 10 ml TM buffer, ultrasonic at 30% power (power on 5 s, power off 30 s) for 10 min; (ii) The samples were placed in 10 ml lysis buffer (8 mol/L urea, 2 mol/L thiourea, 4% (*w*/*v*) CHAPS, 40 mmol/L dithiothreitol, and 100 mmol/L PMSF), ultrasonic at 10% power (power on 2 s, power off 30 s) for 10 min. Then the samples were placed on ice overnight with the addition of 100 mg of DTT and 100 mmol/L PMSF. The lysate was centrifuged at 12 000 *g*, 4℃ for 30 min, and the supernatant was collected and stored at –80℃. The protein concentration was assayed using the BCA kit (Promega, Madison, WI, USA).

1.3 Optimization of 2-DE

Based on the methods described by Gorg *et al*. and Otani *et al*.[15–17], we optimized the 2-DE protocols with different IPG Strips, concentrations of sample solution and SDS-PAGE, respectively.

The sample solution (500 μg or 1 mg of protein) and rehydration buffer(8 mol/L urea, 2 mol/L thiourea, 4% (*w*/*v*) CHAPS, 20 mmol/L dithiothreitol, 0.5% IPG buffer) were mixed for a total volume of 350 μl and poured into the isoelectric focusing tray (Bio-Rad, Hercules, CA, USA). Three different pH-Range ReadyStrip™ IPG Strips (pH 3～10, pH 4～7, or pH 3～10 NL, 17 cm) were used separately for the first-dimension isoelectric focusing gel. The strips were covered with mineral oil to prevent the samples from evaporation and were rehydrated at 17℃, 50 V for 16 h. First-dimension isoelectric focusing gel electrophoresis was conducted using an electrophoresis apparatus at 500 V for 2 h, at 700 V for 1 h, at 1 000 V for 1 h, at 2 000 V for 1 h, at 4 000 V for 1 h, and at 8 000 V for 50 000 Vh. After isoelectric focusing, the strips were placed on Kimwipes to remove mineral oil from the surface and soaked in equilibration buffer (0.375 mol/L Tris-Cl, pH 8.8, 6 mol/L urea, 20% glycerol, 2% SDS, supplemented with 2% dithiothreitol or 2.5% iodoacetamide) at room temperature with gentle shaking separately. SDS-PAGE was performed with different concentrations of acrylamide gel (12%, 15%, or 17.5%) at 10 mA/gel for the first 1 h and then at 30 mA/gel for 5 h. After electrophoresis, the gels were fixed in 20% methanol and 10% acetic acid solution for 1 h and then stained using Coomassie Brilliant Blue G-250, destained with a solution containing 10% methanol and 10% acetic acid[15,21].

1.4 Proteome database construction of *S. cellulosum* So0157-2

S. cellulosum So0157-2 was grown in M26 medium at 30℃ with shaking at 200 r/min for three days until the cells reached the production stage of epothilones. The cells were harvested by centrifugation at 5 000 *g*, 4℃ for 10 min. The proteomic patterns of *S. cellulosum* So0157-2 were acquired using the optimized 2-DE protocol. Electrophoresis was carried out three times to obtain repeatability. Patterns were analyzed by the software PDQuest 8.0 (Bio-Rad, Hercules, CA, USA). All protein spots were excised from the stained gels and digested in gels with trypsin. Excised gel pieces were washed with ammonium hydrogen carbonate (50 mmol/L, Sigma Aldrich, USA) for 10 min, next with ammonium hydrogen carbonate/acetonitrile (1 ∶ 1) for 10 min, and finally with acetonitrile (Acros, Belgium). The supernatants were removed, and then the spot gels were air dried before digestion in ammonium hydrogen carbonate (50 mmol/L, Sigma Aldrich, USA) containing trypsin (50 ng, Promega) at 37℃ overnight. The peptides were extracted twice by addition of 0.1% trifluoroacetic acid. Peptide extracts (0.5 μl) were spotted onto the 384 Opti-TOF plate, and then mixed with matrix (0.5 μl). The protein identification was performed using matrix-assisted laser desorption ionization/tandem time-of-flight mass spectrometry (MALDI-TOF-MS) (ABI, Foster, CA, USA) at Nankai University. Peptide mass fingerprinting (PMF) searching was performed at http://www.matrixscience.com to establish the protein database.

2 Results

2.1 Sample preparation

Utilizing the extraction method (ii), most of the *S. cellulosum* So0157-2 proteins were dissolved out from cell debris in the lysis buffer, whereas they could not be separated in the TM buffer. The lysate produced much higher solubility and expressed more protein spots on the pattern (Figure 1a). According to the method (i), a large quantity of ions contained in the TM buffer led to the appearance of horizontal streaks. The solubility of *S. cellulosum* So0157-2 proteins was lower in the TM buffer and shown fewer proteins in the pattern (Figure 1b). This protein extraction method (ii) greatly reduced the influence of exopolysaccharides and slime.

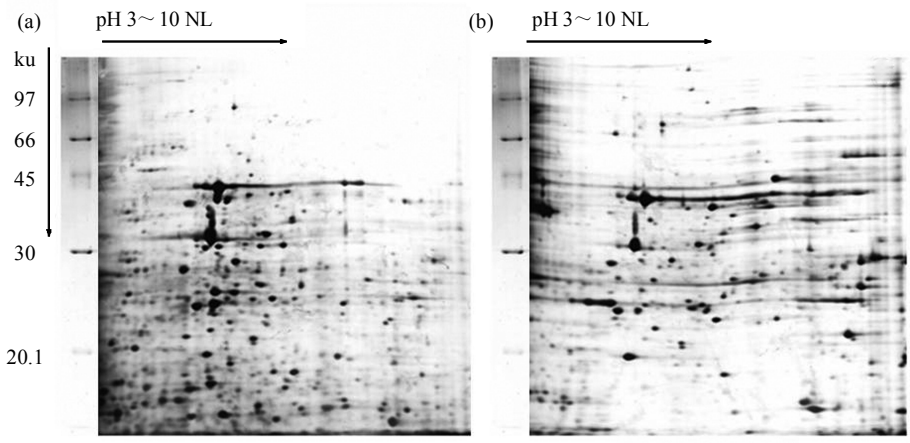

Fig. 1 2-DE of *S. cellulosum* So0157-2 total proteins with two different extraction methods

(a) Method (ii): 10 ml lysis buffer, ultrasonic at 10% power, power on 2 s, power off 30 s. (b) Method (i): 10 ml TM buffer, ultrasonic at 30% power, power on 5 s, power off 30 s.

2.2 Optimization of 2-DE

Three different pH-Range IPG strips were used separately for the first-dimension isoelectric focusing gel. With the pH 3～10 strip, almost all of the protein spots expressed by *S. cellulosum* So0157-2 were represented in the pattern, but many protein spots aggregated and crowded at about p*I* 5 of the pattern (Figure 2a). The pH 4～7 strip was used to enlarge the middle area and increase the resolution of the protein spots. The protein spots in the pH 4～7 range were improved, but the spots expressed in the alkaline range were lost(Figure 2b). Therefore, the pH 3～10 NL strip used in the first-dimensional isoelectric focusing not only improved the resolution of protein spots, but also covered as many protein spots as possible (Figure 2c).

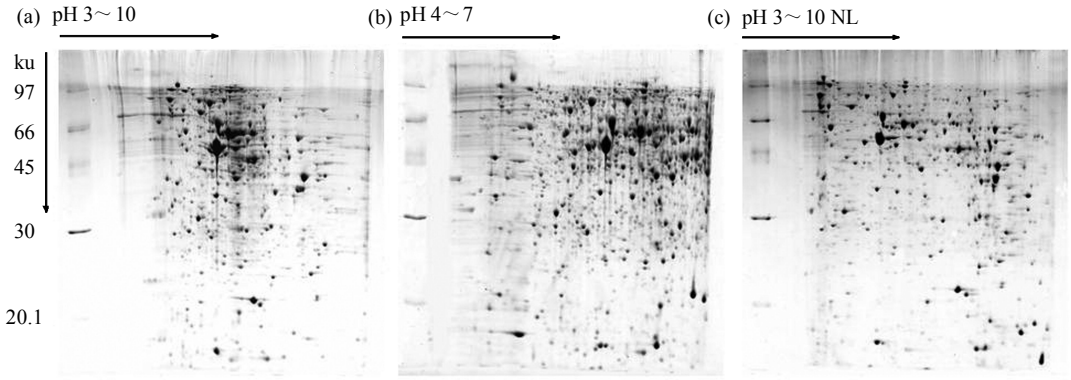

Fig. 2 2-DE of *S. cellulosum* So0157-2 total proteins with three different IPG Strips

(a) pH 3～10 strip. (b) pH 4～7 strip. (c) pH 3～10 NL strip.

For the isoelectric focusing, 500 μg of protein and 1 mg of protein were used respectively. The results indicated that the sample solution containing 1 mg of protein was appropriate to acquire more protein spots (Figure 3).

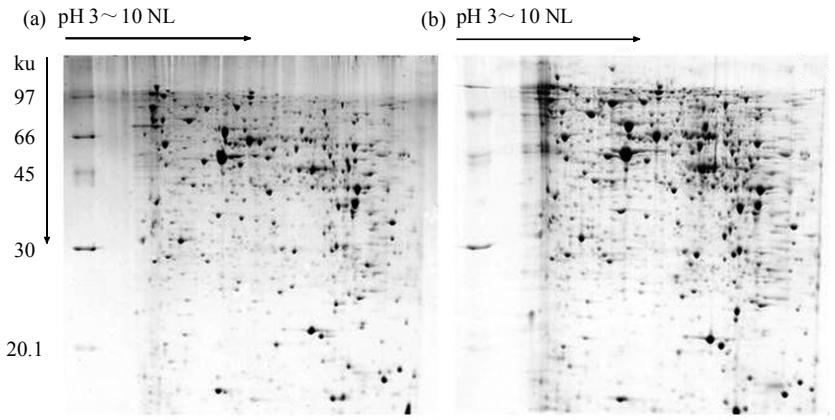

Fig. 3 2-DE of *S. cellulosum* So0157-2 total proteins in two different concentrations of sample solution in isoelectric focusing

(a) 500 μg of protein. (b) 1 mg of protein.

SDS-PAGE was performed using a 12%, 15%, or 17.5% acrylamide gel. In the 12% SDS-PAGE, many small proteins expressed by *S. cellulosum* So0157-2 were still not separated from the bromophenol blue until the termination of electrophoresis, and they were clearly stacked at the end of the SDS-PAGE(Figure 4a). However, in the 15% SDS-PAGE, many more low molecular mass protein spots were acquired. The integral protein spots moved up in the pattern (Figure 4b). In the 17.5% SDS-PAGE, the protein spots were compressed substantially, and no more protein spots were expressed in the pattern (Figure 4c). Above all, the 15% SDS-PAGE was most appropriate for the proteins expressed by *S. cellulosum* So0157-2 and improved the resolution of the patterns.

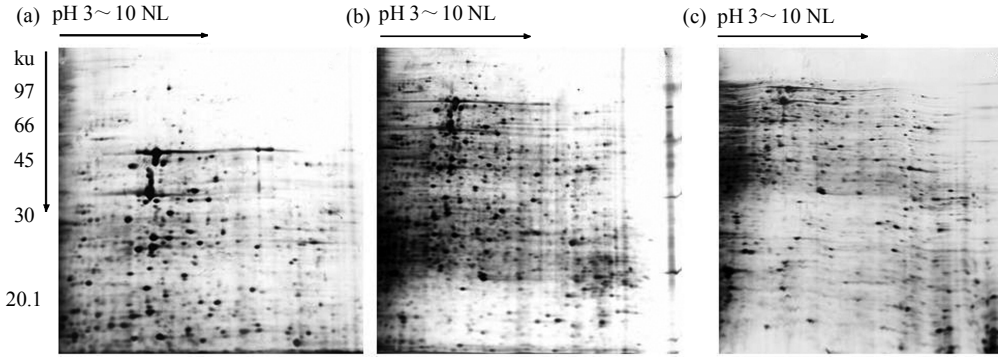

Fig. 4 2-DE of *S. cellulosum* So0157-2 total proteins in three different concentrations of SDS-PAGE

(a) 12% SDS-PAGE. (b) 15% SDS-PAGE. (c) 17.5% SDS-PAGE.

2.3 Protein patterns of *S. cellulosum* So0157-2

We acquired the patterns of *S. cellulosum* So0157-2 grown in M26 fermentation medium for three days *via* the optimal 2-DE protocol. The patterns maintained good resolution and repeatability(Figure 5). The results indicated that the method established was effective for the 2-DE of *S. cellulosum* So0157-2 proteins.

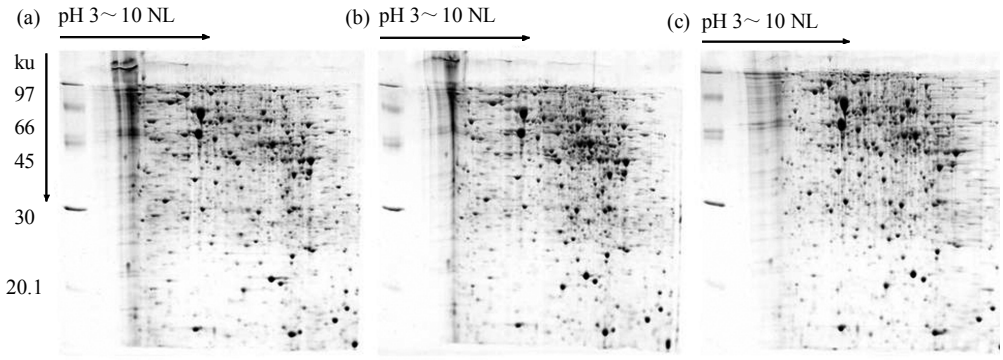

Fig. 5 2-DE of *S. cellulosum* So0157-2 total proteins in M26 medium
Electrophoresis was carried out three times to obtain reproducibility (a, b, c).

2.4 Protein identification and analysis

All protein spots expressed in the 2-DE patterns were analyzed. We finally acquired all information of protein spots expressed in the *S. cellulosum* So0157-2 2-DE patterns. The results indicated that the *S. cellulosum* So0157-2 cultured in M26 medium for three days expressed 552 proteins. Searching in the protein database, 474 proteins were identified, and the ratio of identification was up to 85.9%.

Most of the proteins were mainly identified as the components of cell structure and function, and cell metabolic enzymes. The components of cell structure and function included 16 S rRNA protein, a family of outer membrane protein, ribosomal protein, molecular chaperone protein, signal peptide protein, and heat-shock protein (HSP20) family. The cell metabolic enzymes mainly included ATPase, ATP synthase, and a variety of isomerases and dehydrogenases. Particularly, we acquired six classes of proteins participated in the carbohydrate metabolism (Table 1, marked in Figure 6), which may be related to the production of epothiloneosides. It is contributed to the research of epothiloneoside A.

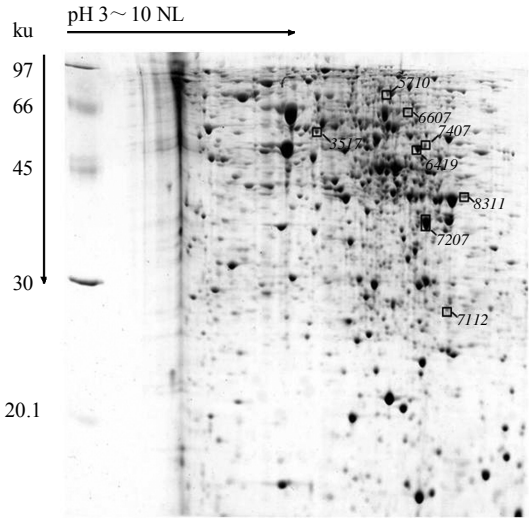

Fig. 6 The protein pattern of carbohydrate metabolism in *S. cellulosum* So0157-2
The related spots were marked with boxes.

Table 1 The proteins of carbohydrate metabolism expressed by *S. cellulosum* So0157-2 cultured in M26 medium for three days

Spot No.	Protein name	Peptide count	Protein p*I*	Molecular mass	Protein score
7207	UDP-glucose 4-epimerase	18	6.68	34 424	229
8311	UDP-glucose 4-epimerase	4	8.31	37 296	95.6
7112	Polyphosphate-glucose phosphotransferase	6	5.52	27 265	87.5
7407	Polyphosphate-glucose phosphotransferase	15	6.58	47 431	215
6607	Glucose-6-phosphate 1-dehydrogenase	17	6.45	59 312	258
6419	GDP-mannose dehydrogenase	24	6.48	48 840	325
5710	Glucosamine--fructose-6-phosphate aminotransferase	16	6.31	66 667	159
3517	Beta-glucosidase	29	5.72	52 437	361

Proteins from 2D analysis were identified by MALDI-TOF-MS peptide mass fingerprinting. The spot number, protein name, peptide count, protein p*I*, molecular mass and protein score are shown for each protein.

As shown in Figure 6, through the global analysis of protein expression, there were 8 protein spots related to carbohydrate metabolism. Most of these spots were at about p*I* 5～8, with molecular mass of 27 ku～66 ku. The proteome analysis indicated that the UDP-glucose 4-epimerase (spot *7207*), which participated in carbohydrate metabolism and galactose metabolism, expressed the most quantity among the carbohydrate metabolic enzymes. Identification of these proteins provided important insights into their functions of carbohydrate metabolism, which may be related to the production of epothiloneosides during *S. cellulosum* So0157-2 fermentation in M26 medium.

3 Discussion

A large number of metabolites produced by microorganisms and their derivatives have been discovered and widely applied to the medical treatment of human diseases. It has become an effective protocol to screen for therapeutic drugs from among natural metabolites. Myxobacteria produce a wealth of primary and secondary metabolites, of which 46% come from the genus *Sorangium*. Many of them show positive effects on the inhibition of infections and the treatment of diseases. It was reported that 952 proteins of *S. cellulosum* So56 were confidently identified by 2-DE and mass spectrometry that were related to the function of regulatory proteins in the social myxobacteria and their role in secondary metabolism [9]. Therefore, a 2-DE protocol for *S. cellulosum* So0157-2 was established by optimizing the conditions of sample preparation and electrophoresis for subsequent sequencing. This work acquired the protein patterns of *S. cellulosum* So0157-2 cultured in M26 medium for three days and high-throughput screened the proteins.

The method of sample preparation is critical for isoelectric focusing, which in turn affects the 2-DE result in terms of quality and protein species distribution [22]. The *S. cellulosum* So0157-2 cells were harvested and ultrasonically disrupted in the lysis buffer or TM buffer. The components of the lysis buffer were selected depending on the nature of the sample. The neutral chaotral, urea denatures proteins by disrupting noncovalent and ionic bonds between amino acid residues. Thiourea, in particular, has been shown to improve the solubilization of hydrophobic membrane proteins [23-24]. CHAPS, as a zwitterionic detergent, has since been demonstrated to be even more effective at solubilization [25]. And the free thiol-containing reducing agent dithiothreitol (DTT) reduces disulphide bonds to aid solubilization of complex mixtures of proteins [26]. PMSF is commonly used in lysis buffer as a serine protease inhibitor [27]. However, the sample solubilization under TM buffer conditions is incomplete, and the ions from the TM buffer affect the first-dimensional isoelectric focusing.

Three different pH-range strips were tested for isoelectric focusing. The results indicate that the proteins of *S. cellulosum* So0157-2 expressed much higher resolution in the pH 3～10 NL strip than in the others. Since a large number of proteins were expressed in the range of pH 4～8, the linear pH 3～10 strip with a uniform distributed pH gradient could not make these proteins migrate and separate well with isoelectric focusing. However, the pH 3～10 NL strip not only enlarged the range of the middle pH gradient, but also maintained proteins on both sides of the pH range, which were very different from the pH 4～7 strip resulting in duplication and loss of proteins. Meanwhile, using 1 mg of proteins has improved the resolution of low-accumulation proteins and facilitated protein identification in mass spectrometry. 15% SDS-PAGE was determined to separate the proteins of *S. cellulosum* So0157-2 based on the size range of the proteins. This concentration of SDS-PAGE had the appropriate size of pores produced by raising or lowering the concentrations of acrylamide and bisacrylamide in the gel.

The high-quality protein patterns of *S. cellulosum* So0157-2 were acquired in fermentation conditions, using the optimal 2-DE protocol. The high-resolution protein patterns of the strain producing the anti-cancer drug epothilone were acquired for the first time. Comparing with former work [1], the patterns are significantly improved with more protein spots, higher resolution and better effects. Our optimized 2-DE protocol had the universality to apply for the valuable samples which have a large amount of extracellular secretion and cytochrome, and difficult genetic manipulation. This work provides a practical method for the proteome research on the same or similar materials.

We acquired the whole protein expression pattern and established the proteome database. The results indicated that the strain *S. cellulosum* So0157-2 cultured in M26 fermentation medium for three days expressed 552 proteins. Most of the proteins were

identified by MALDI-TOF-MS. The identification rate of the expressed proteins was up to 85.9%. The identified proteins included components of cell structure and function, and cell metabolic enzymes. Worthy to be mentioned, 8 proteins were related to the transformation and metabolism of carbohydrate. These proteins were contributed to the combination of carbohydrate and epothilone, through which epothiloneoside A was produced. Meanwhile, the optimal 2-DE protocol achieved high-throughput screening of the proteins of *S. cellulosum* So0157-2 that participated in the production of secondary metabolite-cured diseases, as well as enzymes involved in the regulatory network with potential utility in industry. Based on the study, the proteome expression databases of *S. cellulosum* So0157-2 in various industrial culture conditions are under construction in our laboratory.

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应用优化的双向电泳技术建立纤维堆囊菌 So0157-2 蛋白质组数据库 *

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摘要　堆囊菌丰富的次级代谢产物是新药的重要来源，而蛋白质组学分析是研究代谢调控的有效方法．然而堆囊菌含有大量的胞外多糖以及黏液，干扰了蛋白质组学分析中蛋白质的溶解度、分辨率及重现性．为了高通量地筛选 *Sorangium cellulosum* So0157-2 表达的特异性蛋白，实验优化了 *S. cellulosum* So0157-2 双向电泳方法．首先，*S. cellulosum* So0157-2 蛋白在裂解液中有更好的溶解度．pH 3～10 非线性胶条和 1 mg 的蛋白上样量适用于第一向等电聚焦，分别提高了蛋白质点的分辨率和低丰度蛋白质的表达．15% SDS-PAGE 改善了 *S. cellulosum* So0157-2 蛋白分离的分辨率和重现性．最终，通过优化的双向电泳方法获得了 *S. cellulosum* So0157-2 在 M26 培养基中培养 3 天的全蛋白质表达谱，并检测到 552 个蛋白质点．进而对表达蛋白通过 MALDI-TOF-MS 进行质谱鉴定，其中 474 个蛋白质得到鉴定，鉴定率 85.9%．得到鉴定的蛋白质包括细胞结构和功能组分，以及细胞代谢合成酶类，其中 8 个蛋白质与糖类的转化和代谢相关，这有助于糖基化埃博霉素 A 的深入研究．该优化方法为进一步建立纤维堆囊菌 So0157-2 在各种培养条件下的蛋白质组表达数据库打下基础．

关键词　双向电泳，纤维堆囊菌 So0157-2，优化，蛋白质组

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