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Research Article

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Preparation and regeneration of protoplast from antitumor agent epothilone-producing microbes myxobacteria

Guo-li Gong*, Xuan-ming Wei, Yu-ying Huang and Xue-feng Chen

College of Life Science and Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi, China

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ABSTRACT

Myxobacteria are amongst the top producers of natural products. The diversity and unique structural properties of their secondary metabolites is what make these microbes highly attractive for drug discovery, particularly epothilones, cytotoxic macrolides that mimic the effects of paclitaxel on cancer cells (i.e., microtubule stabilization) are highly promising prospective anticancer agents that naturally produced by myxobacteria. In this paper, some factors affecting the preparation and regeneration of protoplasts from myxobacteria were discussed, including enzymes, osmotic stabilizers, and regeneration medium, et al. This study laid the foundation to improve epothilone-producing strains by protoplast mutagensis, transformation, fusion, and even genome shuffling.

Keywords: Myxobacteria, Protoplast preparation, Protoplast regeneration, genome shuffling, Epothilone

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INTRODUCTION

In recent years, myxobacteria have matched fungi, actinomycetes as well as some species of the genus *Bacillus* as top producers of microbial secondary metabolites [1-6]. More importantly, screening campaigns have revealed a large proportion of the myxobacteria secondary metabolism to have activities against human diseases such as cancer, bacterial and viral infections [6-8].

Myxobacteria are a group of proteobacteria which reside mainly in soil [9,10]. These social microbes move by an axonal cellular motion called gliding [11,12], and although cells grow independently, they form collective swarms to prey and generate transient structures, called fruiting bodies, when resources are scarce [13]. During cooperative feeding, individual cells organize in waves which travel in a rippling-like motion [12,14]. As waves of cells collide, they aggregate in mounds that grow in size forming fruiting bodies that can harbor about 10^5 individuals. Cells within these structures become myxospores. Sporulation is triggered by signaling at the cell-cell contact surface when nutrients are available, and the myxospores germinate to eventually develop new swarms [11]. To control these processes, myxobacteria have evolved a unique mechanism of extracellular and intracellular signals, including diverse proteins and small metabolites [15].

Protoplasts are cells from which the cell wall has been removed by the digestion of enzyme. The cytoplasm can be considered equivalent to cytoplasm in higher cells. Protoplasts contain all the intracellular organelles of cells and form a vital link in transfer of micromolecules between cyto-organelles.

The myxobacteria produce rich extracellular polysaccharides, and tend to aggregate, which greatly hampered the enzymatic digestion of the peptidoglycan layer of the cell wall for producing protoplasts. Therefore, we attempted to develop methods for preparation and regeneration of myxobacterial protoplasts. In addition, strain improvement by genome shuffling whose main characteristics is recursive protoplast fusion has become a useful method for the improvement of microbiology. Thus, the process of preparation and regeneration of protoplast must be established before genome shuffling improves myxobacteria.

In this paper, we describe conditions for the preparation and regeneration of the protoplasts from the antitumor agent epothilone-producing microbes myxobacteria. The study laid the foundation to improve myxobacteria, especially epothilone-producing strains by protoplast mutagensis, transformation, fusion and genome shuffling.

EXPERIMENTAL SECTION

Microorganisms and culture conditions

Myxobacterium *Sorangium cellulosum* So07-9 [16] produces low-level of epothilone B (0.45mg l^{-1}). The strain was routinely inoculated on M26 agar [17] and cultured at 30°C. For convenience, the starting strain was frozen in aliquots. It was cultured in liquid M26 at 30°C with shaking at 200 rpm until the cells reach the exponential growth stage. The cells were centrifuged (3,000×g, 5min, 4°C), and resuspended in a 20% (v/v) sterile glycerol solution. One-ml aliquots containing 1×10^9 cells were frozen in cryo-vials and stored at -80°C.

Preparation of the strain for protoplast

One cryo-vial *S. cellulosum* So07-9 was used to inoculate 50 ml M26 medium, and the culture was shaken at 200 rpm at 30°C for 3-4 days. The cells were gently homogenized with glass beads (3 mm in diameter), collected by centrifugation (3,000×g, 5min, 4°C), and resuspended with sterilized water at approximately 1×10^7 cells ml^{-1}. Then they were vortexed for 2 min to form a homogeneous suspension. The cell suspension was used to prepare protoplast.

Protoplast preparation

The cells suspension of strain were harvested by centrifugation (3,000×g, 5 min, 4°C), gently homogenized with glass beads, and washed three times with 0.01M Tris-HCl (pH8.0). The cells (1×10^8 ml^{-1}) were then mixed and centrifuged at 3,000×g for 5 min at 4°C. The cell mass was resuspended in 30 ml MMM buffer containing 0.3 M mannitol, 0.02 M $MgCl_2\cdot6H_2O$ and 0.02 M maleic acid buffer (pH 6.5). Potassium ethylene diamine tetraacetate (EDTA, 0.1M, pH 8.0) was added slowly to a final concentration of 0.01 M. The mixture was shaken at 100 rpm for an additional 10 min at 30°C. Then the cells were collected by centrifugation (3,000×g, 5 min, 4°C), washed twice with MMM buffer, and resuspended in 30 ml MMM buffer containing 2mg ml^{-1} lysozymes (Sigma Co.) for enzymatic digestion of the cell wall. The cells were shaken at 100 rpm for 30 min at 30°C. The efficiency of protoplast formation was determined by microscopy.

Protoplast regeneration

The prepared protoplasts were washed twice with MMM buffer and centrifuged at 2,000×g for 20 min at 4°C. The protoplasts were resuspended in liquid regeneration medium (liquid VY/2 containing 0.3 M mannitol), and immediately spread on regeneration plates (liquid regeneration medium plus 1.5% agar). The regeneration plates were incubated at 30°C for 7~10 days. Some of the individual colonies that appeared were transferred to fresh agar. Next, the regeneration ratio was determined.

RESULTS

The formation and release of protoplasts

The formation conditions of protoplasts for the myxobacteria were optimized, including concentrations of lysozyme and mannitol (osmotic stabilizer), enzymatic digestion time and temperature. The myxobacteria are Gram-negative, unicellular bacteria with rod-shaped cells that are stout, cylindrical rods, sometimes almost cube-shaped, with broadly rounded ends, about 2.5–8 μm long and 0.6–1.0 μm wide. The shape of rod began to change after enzymolysis for 10 minutes or so and spherical protoplasts began to form. With the increasing of enzymolysis time, more protoplast sphere emerged gradually, the contents presented to be beaded and protoplasts began to be prepared in large amounts. The diameter of protoplast ranges between 0.5 μm and 1.5 μm. (Figure 1)

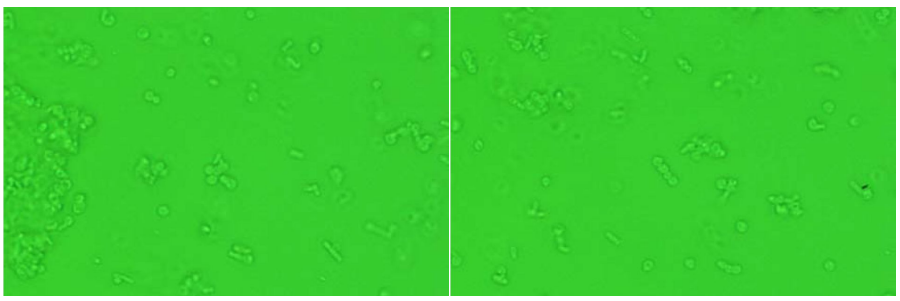

Fig. 1 The sphere-shape protoplasts and rod-shape cells of myxobacteria

Effects of various enzymes on preparation of protoplast

The effect of snailase, lysozyme and cellulase on the preparation of the protoplast was researched. The result indicated that the optimal condition of the preparation of protoplast is 2 mg ml^{-1} lysozymes (Table 1).

Table 1 Effects of enzyme on preparation of protoplast

pH	preparation ratio
snailase	10%
lysozyme	80%
cellulase	0

Effects of pH value on preparation of protoplast

The relationship between pH 4.5~7.0 and the forming ratio of protoplast was studied (Table 2), the results showed that the achievable maximum forming ratio of protoplast was 98% per milliliter when the pH value was 6.5.

Table 2 Effects of pH on preparation of protoplast

pH	preparation ratio
4.5	10%
5.0	40%
5.5	80%
6.0	90%
6.5	98%
7.0	89%

Effects of enzymolysis temperature on the preparation of protoplast

The optimal enzymolysis temperature was studied when the pH value was 6.5. The forming ratio of protoplast at different enzymolysis temperature were presented in Table 3. The results showed that 30 ℃ was the optimal enzymolysis temperature.

Table 3 Effects of enzymolysis temperature on preparation of protoplast

temperature	preparation ratio
25	30%
27	87%
30	98%
32	90%
37	20%

Effects of enzymolysis time on the preparation of protoplast

Effects of enzymolysis time on the preparation ratio of protoplasts were studied under the condition of 2 mg ml^{-1} lysozyme, 30 ℃ and pH 6.5. The result showed that with prolonging of enzymolysis time, the forming ratio of protoplasts increased, the ratio of protoplasts came to almost 100% when enzymolysis time was 35 minutes (Figure 2). If the enzymolysis time is longer than 35 minutes, the forming ratio of protoplast decreased.

Effects of pretreatment on the preparation of protoplast

The collected cells were treated for 30 min using 0.01 M potassium ethylene diamine tetraacetate (EDTA) before they would be enzymolysized. At the same time, untreated cells as control. The results showed that pretreatment could dramaticlly raise the preparation ratio of protoplasts.

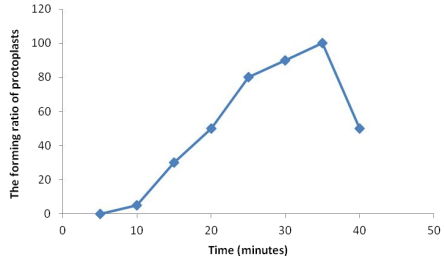

Fig.2 Effect of enzymolysis time on the forming ratio

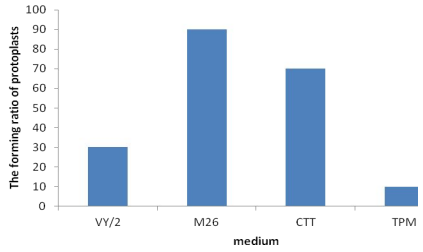

Fig.3 Effects of medium on forming ratio

Effects of medium on the preparation of protoplast

On the condition of 2 mg ml^{-1} lysozyme, 30℃ and pH 6.5, the experiment studied the effect of VY/2 medium, M26 medium, CTT medium, TPM medium on the preparation of the myxobacterial protoplast (Figure 3). The results

showed that the forming ratio of protoplasts varied greatly with medium, among which M26 medium showed the best result and TPM medium showed the most dissatisfactory result.

Effects of regenerative medium on the regeneration of protoplast

Three kinds of high osmotic agar media including VY/2 medium, M26 medium and CTT medium were applied to study on the effects of medium on regeneration of myxobacterial protoplasts. We could saw from Table 4, the regeneration ratio of protoplasts was highest using VY/2, moderate using M26, and lowest using CTT.

Table 4 The effect of regeneration medium on the regeneration ratio of protoplast

Regeneration medium	Regenerative colonies	Regeneration ratio (%)
VY/2	200	60
M26	80	24
CTT	10	3

Effects of enzymolysis time on the regeneration of protoplast

After the concentrations of prepared protoplasts from different enzymolysis time were modulated, using dilayer plate method of the prepared protoplasts was regenerated in the VY/2 high osmosis regenerative medium. The regenerative morphology of the colonies is shown in Figure 4. The regeneration ratio of protoplasts changed little in short time, while with the enzymolysis time prolonged, the regeneration ratio decreased. Therefore, considering the forming ratio of protoplasts, 0.5 hours were selected as enzymolysis time in this study in order to obtain higher the regeneration ratio of protoplasts.

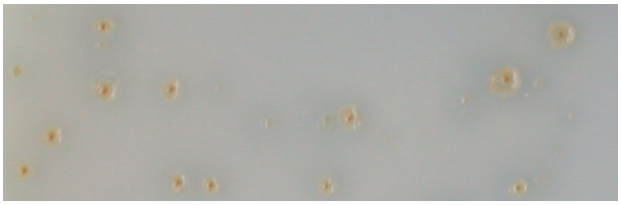

Fig.4 The regenerative morphology of the colonies of protoplast on the VY/2 high osmosis plate

DISCUSSION

Myxobacteria is among the best producers of secondary metabolites [10], and also among the bacteria that are difficult to manipulate [17]. The preparation of protoplasts and the regeneration of protoplasts for myxobacteria have not been reported.

The myxobacterium produces rich extracellular polysaccharides, and tends to aggregate, which greatly hampered the enzymatic digestion of the peptidoglycan layer of the cell wall for producing protoplasts. The preparation of protoplasts and the regeneration of protoplasts for myxobacteria were very difficult. We were able to partly solve this problem by using the cells in the early exponential growth stage and gently homogenizing the harvested cells with glass beads. Long periods of digestion with lysozymes decreased the regeneration of myxobacterial protoplasts. Before the digestion, treating the cells with EDTA for short time (10min) significantly decreased the time period of digestion with lysozymes, but still allowed efficient preparation and regeneration of protoplasts. Although high concentrations of sucrose, mannitol or salts such as $CaCl_2$ and $MgCl_2$ were able to stabilize the protoplasts, most of them were toxic for myxobacteial growth, even at rather low concentrations. At the concentration of 0.3M, only mannitol stabilized the protoplasts and allowed the cells to grow in limited colony size on agar. The limited colony size is, however, more applicable for further screening of the fusants. Using the protocol provided in this research, the efficiency for preparation of protoplasts reached nearly 100% (Fig. 3) and the regeneration ratio was more than 60% (Table 4).

Considering the effect of snailase, lysozyme and cellulase on the forming ratio of protoplasts, it is found that the lysozyme will obtain higher the forming ratio of protoplast than others. In addition, prolonging of the enzymolysis time will be helpful to raise the forming ratio of the protoplasts; while the enzymolysis time is too long, the forming ratio protoplasts and the regeneration ratio of protoplasts will decrease, this may be because of breaking of the protoplasts or enzymolysis too much, causing the loss of regenerative primers, and the reduction of protoplast activity.

The pH value is also an effective factor that indirectly affects the preparation of protoplasts because it has effect on enzyme activity. The results showed that when the pH value closed to the optimum of the compound enzyme

systems (pH 6.5), it was the most beneficial for the enzymes to digest. In addition, it is obviously that the enzymolysis temperature can also affect the enzyme activity. The temperature that cells lose their cell wall by enzymolysis is about 30℃.

The osmotic pressure stabilizers can keep the balance of interior and exterior osmotic pressure of the protoplasts, which have lost the protection of cell wall, and can prevent the protoplasts from being broken and are benefit to improve enzyme activities. So to select the optimal osmotic pressure stabilizer is very important. Up to now, for myxobacteria, there is no reasonable explanation about that a kind of chemical reagent is more suitable to be an osmotic pressure stabilizer than another.

The function of pretreatment changes the structure of cell wall by artificial control, which can increase cell wall more sensitive to enzyme and further raise the forming ratio of protoplasts.

The regeneration ratio of protoplasts is often a restricted factor in the application of protoplast technique. If the regeneration ratio of protoplasts is too lower or the protoplast can't regenerate, it will lead to be lack of materials for experiment to select, and it is very difficult to count. In conclusion, this study illustrates that it is fundamentally difficult to establish a universal method of the regeneration of protoplast for epothilone-producing myxobacteia, mainly due to differences in their requirement for medium in the regeneration of protoplast. In this study, several kinds of regeneration media containing different substances were developed. These media should enable efficient protoplast regeneration for myxobacteria described in this study, especially the regeneration ratio of protoplasts obtained on VY/2 medium was highest. Therefore, this study should provide a basis for the genetic improvement of myxobacteria with protoplast fusion.

In addition, effects of enzymolysis time on the regeneration of protoplast were studied in the paper. This study showed that with the prolonging of enzymolysis time, the regeneration ratio protoplast decreased, and much too high concentrations of enzyme can also affect the regeneration ratio of protoplasts. This may be because when the cell wall was removed too much, the primers for synthesizing cell walls will lost during regenerating, and when the concentration of the enzyme is too high, the impurities enzymes will affect the activities and the regeneration ratio of the protoplasts.

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