Acta Oceanol. Sin., 2014, Vol. 33, No. 10, P. 114–123
DOI: 10.1007/s13131-014-0546-y
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Studies on the isolation and culture of protoplasts from *Kappaphycus alvarezii*

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Received 16 April 2013; accepted 12 November 2013

Abstract

In this study, protoplasts were successfully isolated from *Kappaphycus alvarezii* using snail enzymes, abalone enzymes and cellulase. The optimum enzymic ratio was fixed to be 20% of abalone enzyme, 12% of cellulase and the osmotic stabilizer was 2.0 mol/L glucose. The optimum enzymic hydrolysis conditions were found to be dark enzymolysis at 30°C continuing for 4.0 h. The resultant density and yield of protoplasts achieved 32.60×10^4 mL^{-1}, 65.20×10^4 g^{-1} tissue for *Kappaphycus alvarezii*. Finally, under the temperature of 20°C, light intensity of 1 500–2 000 lx and photoperiod of 12 h/d, two developmental pathways were investigated: (1) callus-like cell mass and regenerated plantlet occurred on protoplast; (2) young shoots and callus-like cell mass occurred in tissue blocks after enzymolysis.

Key words: *Kappaphycus alvarezii*, protoplast, regenerated plantlet, callus-like, young shoots

Citation: Zhang Si, Liu Cui, Jin Yuemei, Chi Shan, Tang Xianming, Chen Fuxiao, Fang Xu, Liu Tao. 2014. Studies on the isolation and culture of protoplasts from *Kappaphycus alvarezii*. Acta Oceanologica Sinica, 33(10): 114–123, doi: 10.1007/s13131-014-0546-y

1 Introduction

Kappaphycus alvarezii belongs to Rhodopyta, Florideae, Gigartinales, Solieriaceae and *Kappaphycus*. It is widely distributed in tropical and subtropical sea areas. *Kappaphycus* seaweed is the main raw material to produce carrageenan, thus it is widely used in textiles and food. In addition, owing to its high pharmaceutical value (Jiang et al., 2008), economic value as well as ecological significance (Tseng, 1983; Zeng, 2001), there is a great demand for *Kappaphycus* both in the domestic and foreign markets.

As a crucial biological material for research purpose, protoplast without cell wall obstacles can be widely used in the study of cell biology, somatic cell crossbreeding, genetic transformation and gene positioning (Wang and Yao, 2009). Meanwhile, the culture of protoplasts is an important plant somatic cell breeding technology. By now, the photoplasts isolation and cultivation have been performed on more than 30 algae including Rhodophyta, Chlorophyta and Phaeophyta (Wang et al., 1996; Wu, 1988; Chu et al., 1998; Wang et al., 2007; Zhang, 1983; Hu, 2006; Araki and Morishita, 1990; Ma et al., 1998; Chen and Chiang, 1994; Chen, 1993; Reddy and Fujita, 1991; Della et al., 1991; Reddy et al., 1992; Xie and Ma, 2004; Kim et al., 2002; Zha and Kloareg, 1996; Zhang, 1991). Some regeneration plantlets of seaweeds such as *Porphyra yezoensis* (Saga and Sakai, 1984; Dai et al., 1988), *Porphyra suboriculat* (Tang, 1982), *Caloglossa leprierii* (Chen, 1993), *Ulva linza* (Zhang, 1983), *Monostroma angicava* (Zhang, 1983) have been obtained. Guo et al. (2008) proposed a regression model for enzymic degradation of *Eueheuma striatum* with respect to pH, temperature and concentration. Salvador and Serrano (2005) carried out the isolation of protoplasts from tissue fragments of Philippine cultivars of *Kappaphycus alvarezii* with yield of 8.20×10^3 g^{-1} tissue at 48 h. However, they did not investigate the ways of development and differentiation of protoplasts. This study carries out isolation and cultivation of protoplast from *Kappaphycus alvarezii*. In particular, we attempts to improve the preparation conditions of protoplast to further enhance the yield of protoplast and also probe into its development and differentiation. This will lay an important foundation for the research and application of *Kappaphycus alvarezii* cell in terms of materials and methods.

2 Materials and methods

2.1 *Materials*

Experimental materials in this study were provided by the Germplasm Resources Nursery of the Laboratory of Genetics and Breeding of Marine Organism in Ocean University of China, Qingdao. *Kappaphycus alvarezii* were harvested in May 2009 in Lingshui of Hainan Province, numbered as 200905062 in the database.

Hydrolytic enzymes which are produced by our laboratory mainly contain snail enzyme, abalone enzyme, cellulose enzymes and the osmotic pressure stabilizer. The extraction methods of snail enzyme follow Xu et al. (2007) and Liu et al.

Foundation item: The National Science Foundation Project under contract No. 2007FY210500; the National Department Public Benefit Research Foundation of China under contract No. 200805075; the Province Science and Technology in the Guangdong Project under contract Nos 2010B060200010 and 2010B020201015; the Science Expenditure in the Hainan Project under contract No. 11-20410-0015; the National Natural Science Foundation of China under contract Nos 41206106 and 41222038.
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(2002), which is described as follows. First clean the conch and temporarily cultivate it as hunger in the disinfected seawater of 2–3 d. Then we have it shelled to obtain its digestive glands, which is grinded into pasty status and followed with freeze centrifugation (9 590 *g* for 30 min). The proenzyme liquid is obtained by three times of centrifugation removing the supernatant each time. The temperature for preservation is –20°C. When it is used, the concentration of proenzyme liquid is diluted to 20% with seawater. The extraction of abalone enzyme is achieved by smashing the digestive glands of abalone and mixed with disinfected seawater by volume ratio of 1:1 and keep it still over the night at 4°C. Then supernatant is absorbed the next day for further refrigerated centrifugation at 2 160 *g* at 4°C. Collect the supernatant repeatedly and centrifuge several times to get the proenzyme liquid which is diluted to 20% with seawater when used. The cellulose is extracted by is dissolving it with 50 mmol/L citric acid or acetic acid buffer solution (pH 4.8–5.0) with concentration of 12%. The acetic acid buffer solution is produced by sterile seawater. Enzyme osmotic pressure stabilizer is 2.0 mol/L glucose after sterilization.

2.2 *Isolation, cultivation and detection methods of protoplasts*

Select unrotten and eugonic frond with normal color and clean sludge, miscellaneous algae and other attached impurity on the surface of frond with hairbrush. Use ultrasonic waves crush instrument to function every other 5 s and treatment time last for 5 s with total time of 1 min. Observe it under microscope until there is no akinete on the surface. Wash it clean at benchtop with sterile seawater and select healthy material. Treat it with 0.01% $HgCl_2$ solution for disinfection for 15 s and then wash it clean at benchtop with sterile seawater. Finally cut the injury of connection of material section with reagents and put the remaining parts in conical flask to recover and cultivate it under cultivation temperature of 20°C, light intensity of 1 500–2 000 lx and photoperiod of 12 h/d.

Select about 2 g top tissue of well-conditioned and asepsis-treated frond branch and then dry the frond with filter paper. Cut it into small pieces and put it in a small beaker filled with 20 mL enzyme liquid. Put it in the constant temperature table to conduct dark enzymolysis and promote cell dissociation. Perform microscopic examination every half hour to observe dissociation of protoplast and take photographs. When cells are loose, they can be separated under light pressure. When there is a lot of monoplast or protoplast dissociation, filtering-centrifugation methods can be adopted to separate monoplast or protoplast. Bolting-silk of 300 sections can be used to filter cell-mixed liquor; and non-degestive cells, cell rolls and fragments shall be eliminated; get centrifugation of supernatant and centrifuge for 10 min at 800 r/min to make monoplast or protoplast sink and cell debris remain in the supernatant. Abandon supernatant and wash it with disinfection seawater containing glucose of 1.0 mol/L, centrifuge 2 or 3 times and renew suspending to 4 mL after collection sediment. Then protoplast comes into being. Three parallel classes shall be set for each experiment.

Protoplast cultivation selects PES medium and formula shall be referred to Bold and Wynne (1978). Each 20 mL PES medium mother liquor is added with sterile seawater to reach constant volume of 1 000 mL and 1 mL GeO_2 (0.5%, w/v) is added as culture solution at the same time. Protoplasts of *Kappaphycus alvarezii* and tissue blocks after enzymic hydrolysis shall be cultivated in the petri dish with the temperature of 20°C. They will be cultivated in darkness for 2 d, after which they will receive lighting cultivation with photoperiod of 12 h/d and light intensity of 1 500–2 000 lx. Every 5 d cultivation solution will be replaced and photos shall be taken by microscopic examination.

The decoration method of fluorescent brightener staining shall be adopted to identify cells got by enzymolysis (Wang and Xia, 1981). Fluorescent decoration methods select VBL type fluorescent brightener staining which shall be matched to be 0.1% staining solution and stain samples shall be stained for 5 min. Observe them with a fluorescent microscope and exciting light wavelength is 370 nm UV-light. Lepocyte has green fluorescent to show the existence of cellulose while protoplast sends out the red due to chloroplast, showing the non-existence of cell walls. Blood counting chamber is used to calculate the density measurement of protoplast which is converted to yield according to the raw weight of enzymolysis organization lump. We adopts two ways to measure the survival rate of protoplast. One is to put the cells (protoplast) into the lower osmotic solution as well as the higher. The living cells' volume can expand in the former situation and shrink in the latter. Those cells whose volumes remain the same are dead (Wang, 1994). Another method is to adopt Evans Blue decoration methods to stain protoplast and count under the microscope. Living protoplast appears bright yellow and the dead is dark blue.

2.3 *Determination of enzymic hydrolysis conditions for Kappaphycus alvarezii*

2.3.1 *Evaluation of wall-breaking effects of different compound hydrolytic enzymes on Kappaphycus alvarezii*

In the experiment of Section 2.2, at room temperature, hydrolytic enzyme of different types and combinations are added to conduct the experiment. Protoplast density at 3.0 h is calculated to determine the best compound hydrolytic enzymes.

2.3.2 *Impacts of different concentrations of the osmotic stabilizer on protoplast formation of Kappaphycus alvarezii*

In the experiment of Section 2.3.1, after the best compound hydrolytic enzyme is determined, in the experiment of enzymic hydrolysis effect of *Kappaphycus alvarezii* with different concentrations of the osmotic stabilizer, enzymic hydrolysis is conducted for 3.0 h at room temperature in Section 2.2. Using Glucose as the osmotic stabilizer, its concentrations are respectively set to be 1.6 mol/L, 1.8 mol/L, 2.0 mol/L, 2.2 mol/L, and 2.4 mol/L to determine the best one.

2.3.3 *Impacts of different enzymic hydrolysis time on protoplast production of Kappaphycus alvarezii*

The steps are similar to those taken in experiment of Section 2.2. The optimal compound hydrolytic enzyme and concentration of the osmotic stabilizer are used to conduct enzymic hydrolysis. Experiments conducted respectively for 2.0 h, 2.5 h, 3.0 h, 3.5 h, 4.0 h, 4.5 h test the density and the survival rate of protoplasts.

2.3.4 *Impacts of different temperature on protoplast production of Kappaphycus alvarezii*

The steps are similar to those taken in the experiment of Section 2.2. The optimal compound hydrolytic enzyme, concentration of osmotic pressure modifier, and more appropriate time

for enzymic hydrolysis are applied to conduct experiments respectively under 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C to measure the density and survival rate of protoplasts.

3 Results

3.1 *Isolation, identification and regeneration cultivation*

Kappaphycus alvarezii would proceed enzymic hydrolysis in darkness under 25°C. After 3.0 h, some cells of *Kappaphycus alvarezii* became loose. After 3.5 h, most parts of tissues were at a unicellular free state. After 4.0 h, a great number of cells of *Kappaphycus alvarezii* were in a free state. Cells under enzymic hydrolysis were rounded with uniform cytoplasm. The sizes of cells vary from each other due to the different locations of tissues of *Kappaphycus alvarezii*. After being stained by VBL fluorescent brightener, it could be seen that cells presented bright jacinth fluorescence (Fig. 1a) under fluorescent microscope, not covered by green presented by cell walls, which indicated that cell walls were removed. Under the same view, protoplasts was observed uniform (Fig. 1b) in a light field. From fluorescence detection, it could be seen that protoplast presented jacinth fluorescence (Fig. 1c) due to the existence of chloroplast, and fragments of cell walls presented green (Fig. 1c). Besides, we proceeded fluorescent staining to supernatant after centrifugation. Protoplasts were not found to present jacinth, clearly proving that fragments of cell walls presented green fluorescence (Figs 1d and e). After staining of Evans Blue, the surviving protoplasts presented light yellow while the dead cells turned into deep blue. The results could be seen in Fig. 1f. During experiment, statistics were conducted respectively for the number of protoplasts during different processes of enzymic hydrolysis, after which data regarding to density and the survival rate of protoplasts were obtained. Meanwhile, the independent samples *t*-test was used for the data to carry out pairwise comparison.

After being cultivated for 2 d, protoplasts of *Kappaphycus alvarezii* would produce cell walls. After 22 d of cultivation, fluorescent staining was conducted for cells of *Kappaphycus alvarezii* again. The results showed that the cells present dark green fluorescence, which was different from that in the stage of protoplasts, and contour of round cells could be seen (Figs 2a and b). By comparison with protoplast figures (Figs 1b and c), it indicated that cell walls formed and it once again testified that cells obtained from enzymic hydrolysis were protoplasts. In terms of development of protoplasts, cytoplasm was well distributed after 5 d of cultivation (Fig. 2c). After 10 d of cultivation, regular shape of cells could be observed and cytoplasm was uniform with normal color (Fig. 2d). After 25 d of cultivation, contour of cells was clear with regular shape and their color presented ivory (Fig. 2e). As the cultivation continued, there were two approaches to develop protoplasts. The first approach was to generate somatic cells. In other words, cell division occurred when cultivation lasted for 40 d (Fig. 2f); cells divided into larger cell clusters after 45 d (Fig. 2g); there was a condition in which many small cells surrounded a big cell (Fig. 2h); the big cell would suffer from division of cytoplasm, but there was no obvious division of cell walls during the observation under a light microscope, and cells would continuously divide into large structures of cell cluster on the 55th day (Fig. 2i). Another development approach for protoplasts was direct regeneration of the plant. When cultivation was conducted for 65 d, regenerated plantlet grew (Fig. 2j).

The growth condition of tissue blocks after enzymic hydrolysis of *Kappaphycus alvarezii* was observed every 5 d. The observation results showed that the growth was in a good

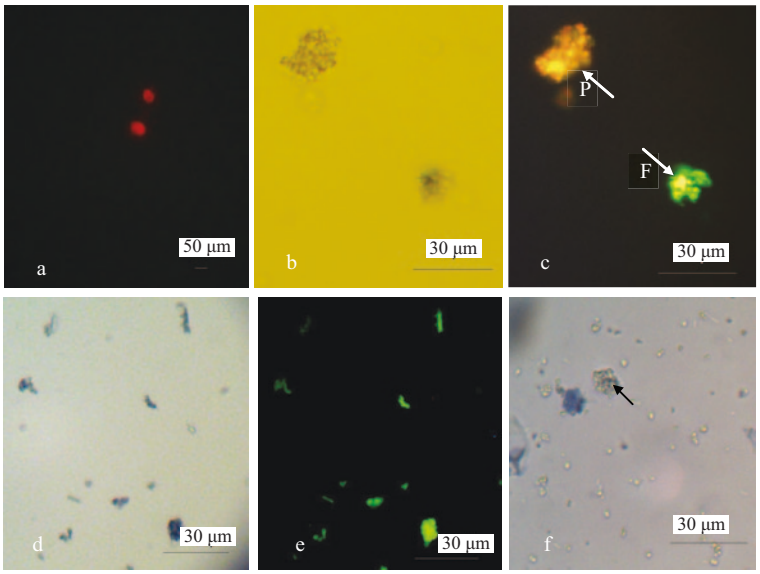

Fig.1. Different identification results of protoplasts. a. Protoplasts were observed under a fluorescent microscope and presented bright jacinth fluorescence; b and c. protoplasts were observed under the bright field and fluorescent microscope and then we could easily distinguish between the jacinth protoplast and the green fragment of cell wall; d and e. supernatant after centrifugation was observed under the bright field and fluorescent microscope and we could only observe the green fragments of cell wall; and f. protoplasts were observed by the Evans Blue staining: the bright yellow cell was alive (arrows) and the blue cell was dead. P represents protoplast and F fragment.

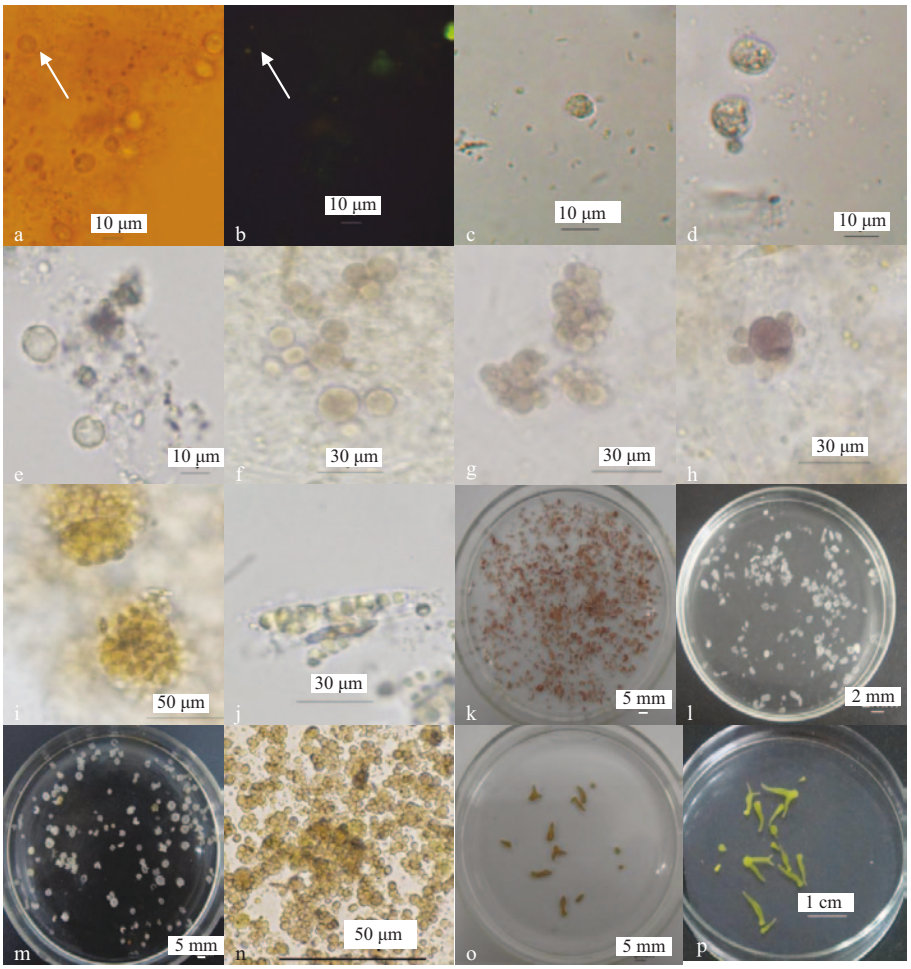

Fig.2. Regeneration of protoplasts and tissue blocks. a and b. Contour of round cells after 22 days' culture were observed under the bright field and fluorescent microscope which presented dark green fluorescence (arrows); c. cells after 5 days' culture; d. cells after 10 days' culture; e. cells after 25 days' culture; f. cells after 40 days' culture; g and h. cells after 45 days' culture; i. cells after 55 days' culture; j. cells after 65 days' culture; k. tissue blocks after 5 days' culture; l. tissue blocks after 15 days' culture; m. tissue blocks after 60 days' culture; n. tissue blocks after 75 days' culture; o. tissue blocks were induced to produce young shoots after 65 days' culture; and p. young shoots after 100 days' culture.

condition. After 5 d of cultivation, some parts of tissue blocks (Fig. 2k) would turn to white from the original brown. The diameter of the tissue blocks, which were compact in structure and free from deformation under pressure, was about 0.20–0.30 cm. After 15 d of cultivation, tissue blocks (Fig. 2l) would all turn into white. The diameter of the tissue blocks was about 0.30–0.40 cm. The tissue blocks became soft and suffered from deformation under pressure, but not dispersed. After 60 d, diameter of tissue blocks (Fig. 2m) did not change obviously. They were transparent with a soft structure. As the cultivation continued, there were two approaches for the development of tissue blocks. The first approach was young shoots (Fig. 2o) growing from tissue blocks after enzymic hydrolysis, which occurred when the cultivation reached 65 d. The length of young buds was about 0.20–0.80 cm. The buds would be separately cultivated under a good condition and ways of budding should be observed. Buds grew from upper medulla of tissue blocks. As the buds grew, their roots of shoots would be gradually constricted. During the growth of new shoots, secondary buds could be generated by roots of shoots to form a branch structure. After 100 d of cultivation, those shoots was well developed (Fig. 2p).The total length of shoots could reach 0.40–1.00 cm. Another development approach was the formation of cell mass. After 75 d of cultivation, external cells of tissue blocks divided into cell mass with chromatophore (Fig. 2n).

3.2 Determination of enzymic hydrolysis conditions for *Kappaphycus alvarezii*

3.2.1 *Comparative analysis of isolation effect with hydrolytic enzymes*

Category and combination of hydrolytic enzymes was the principal factor considering the effect of enzymic hydrolysis. Experiment results of enzymic hydrolysis effect of hydrolytic enzyme on *Kappaphycus alvarezii* after enzymic hydrolysis up to 3 h could be seen in Table 1. The results showed that under single function of cellulose, snail enzymes and abalone

Table 1. Effect of hydrolytic enzyme on the cell wall of *Kappaphycus alvarezii*

Hydrolytic enzyme	Density of protoplasts /10^4 mL^{-1}	Hydrolytic enzyme	Density of protoplasts /10^4 mL^{-1}
Cellulase	2.50±0.80*	Cellulase+Snail enzymes	9.00±0.50*
Snail enzymes	0.21±0.05*	Cellulase+Abalone enzymes	10.40±1.00*
Abalone enzymes	0.85±0.11*	Snail enzymes+Abalone enzymes	4.60±1.20*

Note: * p<0.05.

enzymes, *Kappaphycus alvarezii* could be hydrolyzed. Adding snail enzymes or abalone enzymes to cellulose could facilitate hydrolysis. Protoplasts of *Kappaphycus alvarezii* obtained from combined enzymes of cellulose and abalone enzymes had the highest density, which could reach (10.4±1.0)×10^4 mL^{-1}. *t*-test method was applied to compare density datas of protoplasts in *Kappaphycus alvarezii* that had different duration of enzymic hydrolysis and inspection level was α=0.05. The results also showed that difference among density data of protoplasts of *Kappaphycus alvarezii* with different category and combination of hydrolytic enzymes was statistically significant (P<0.05).

3.2.2 *Comparative analysis of isolation effect with osmotic stabilizer*

Cell wall is a structure to protect cell. During the process of enzymic hydrolysis, after cell walls were removed, if the osmotic pressure within cell and that of external hydrolytic enzymes were greatly different, protoplasts might crack or contract. Therefore, the osmotic stabilizer of certain concentration was added to hydrolytic enzymes to maintain phase balance of the osmotic pressure of solution and that within cell. In this experiment, combined hydrolytic enzyme of cellulose and abalone enzyme was applied and glucose was used as the osmotic stabilizer so as to study the effect of enzymic hydrolysis of the osmotic stabilizer in different concentrations (1.6 mol/L, 1.8 mol/L, 2.0 mol/L, 2.2 mol/L, and 2.4 mol/L). Figure 3 showed that with glucose penetrant of different concentrations, there was little difference in density of protoplasts isolated by *Kappaphycus alvarezii* after 3.0 h of enzymic hydrolysis at room temperature and there was no obvious difference (P>0.05) among data, with density of protoplasts coming within (4.40±0.80)×10^4 mL^{-1}, (5.10±1.0)×10^4 mL^{-1}, (6.40±0.8)×10^4 mL^{-1}, (6.00±0.9)×10^4 mL^{-1}, (5.80±1.40)×10^4 mL^{-1}, but when the concentration of glucose was 2.0 mol/L, density of protoplasts of *Kappaphycus alvarezii* was relatively higher. Therefore, it was appropriate to select 2.0 mol/L as the concentration of the osmotic stabilizer in the experiment (Fig. 3).

3.2.3 *Comparative analysis of isolation effect at enzymic hydrolysis time*

Enzyme combined with 12% cellulose and 20% abalone enzyme was applied in enzymic hydrolysis at room temperature. Results of enzymic hydrolysis time for the isolation experiment on protoplasts of *Kappaphycus alvarezii* could be seen in Fig. 4. In the experiment, statistics were conducted for the number of protoplasts at different enzymic hydrolysis time (2.0 h, 2.5 h, 3.0 h, 3.5 h, 4.0 h, and 4.5 h) to obtain density data of protoplasts, which were (1.00±0.14)×10^4 mL^{-1}, (3.30±0.15)×10^4 mL^{-1}, (5.10±0.21)×10^4 mL^{-1}, (18.20±1.20)×10^4 mL^{-1}, (35.10±1.00)×10^4 mL^{-1} and (47.50±1.10)×10^4 mL^{-1} respectively. According to survival protoplasts in statistics of results of Evans Blue staining, data of the survival rate of protoplasts was obtained. Density of surviving protoplasts obtained after 2.0 h, 2.5 h, 3.0 h, 3.5 h, 4.0 h and 4.5 h of enzymic hydrolysis were (7.10±0.01)×10^3 mL^{-1}, (21.6±0.01)×10^3 mL^{-1}, (30.8±0.01)×10^3 mL^{-1}, (9.57±0.02)×10^4 mL^{-1}, (16.97±0.01)×10^4 mL^{-1} and (16.69±0.03)×10^4 mL^{-1} respectively. Statistical inspection was conducted for the yield and survival rate data of protoplasts of *Kappaphycus alvarezii* at different enzymic hydrolysis time. *t*-test method was applied and inspection level was α=0.05. The results showed that difference among the survival rate data of protoplasts of *Kappaphycus alvarezii* at different enzymic hydrolysis time (2.0 h, 2.5 h, 3.0 h, 3.5 h, 4.0 h and 4.5 h) was statistically significant (P<0.05).

It could be seen from density and survival rate data of protoplasts in Fig. 4 that the survival rate would decrease with the growth in time within the given range of enzymic hydrolysis time. This indicated that longer enzymic hydrolysis time might do more harm to cells. Meanwhile, it could be seen from observation that yield of protoplasts increased when enzymic hydrolysis time extended. Seen from density of surviving protoplasts

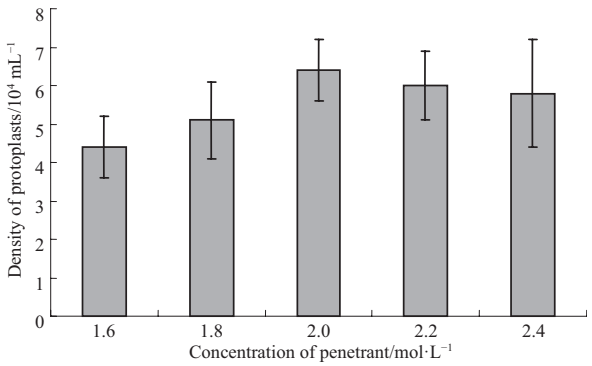

Fig.3. Protoplasts formation of *Kappaphycus alvarezii* on different concentration of glucose.

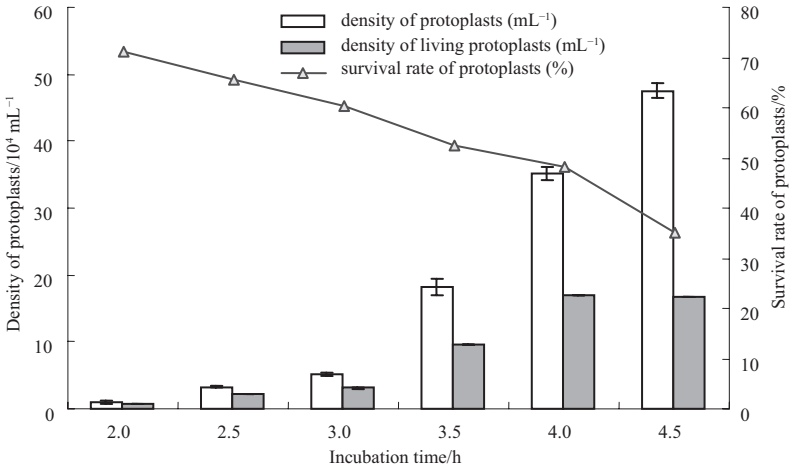

Fig.4. The effect of incubation time on the protoplasts production from *Kappaphycus alvarezii.*

in Fig. 4, it was optimal when enzymic hydrolysis time for *Kappaphycus alvarezii* was 4.0 h.

3.2.4 *Comparative analysis of isolation effect under enzymic hydrolysis temperature*

In the experiment, combined enzyme of 12% cellulose and 20% abalone enzyme was applied and 2.0 mol/L glucose was used as the osmotic stabilizer. Results of separation experiments under enzymic hydrolysis temperature (15°C, 20°C, 25°C, 30°C, 35°C and 40°C) on protoplasts of *Kappaphycus alvarezii* could be seen in Fig. 5. Density of protoplasts were $(16.40\pm2.30)\times10^4$ mL^{-1}, $(23.80\pm1.50)\times10^4$ mL^{-1}, $(29.00\pm3.30)\times10^4$ mL^{-1}, $(35.10\pm2.60)\times10^4$ mL^{-1}, $(40.10\pm2.30)\times10^4$ mL^{-1}, and $(42.50\pm1.20)\times10^4$ mL^{-1} respectively.

After 4.0 h of enzymic hydrolysis, when the temperature was 15–40°C, density of protoplast would increase with the growth of temperature. Seen from the results of Evans Blue staining, temperature had a certain effect on the survival rate of protoplasts of *Kappaphycus alvarezii*. As the temperature rose, the survival rate of protoplasts would decrease. Density of surviving protoplasts at 15°C, 20°C, 25°C, 30°C, 35°C and 40°C was $(12.83\pm0.04)\times10^4$ mL^{-1}, $(17.97\pm0.03)\times10^4$ mL^{-1}, $(19.55\pm0.03)\times10^4$ mL^{-1}, $(20.57\pm0.04)\times10^4$ mL^{-1}, $(19.30\pm0.03)\times10^4$ mL^{-1} and $(12.92\pm0.01)\times10^4$ mL^{-1} respectively. Statistical inspection was conducted for the density data of protoplasts of *Kappaphycus alvarezii* at different enzymic hydrolysis temperatures. *t*-test method was applied and inspection level was α=0.05. The results showed that difference among density data of protoplasts of *Kappaphycus alvarezii* at 15–30°C was statistically significant (P<0.05). When the temperature was over 30°C, there was no obvious difference among density data of protoplasts. The results also showed that difference among survival rate data of protoplasts of *Kappaphycus alvarezii* at 25–40°C was statistically significant (P<0.05). Seen from density of surviving protoplasts in Fig. 5, the effect of enzymic hydrolysis of *Kappaphycus alvarezii* was optimal when the temperature was below 30°C.

3.2.5 *Analysis on isolation effect of protoplasts of Kappaphycus alvarezii*

Using *Kappaphycus alvarezii* as the material, its protoplasts

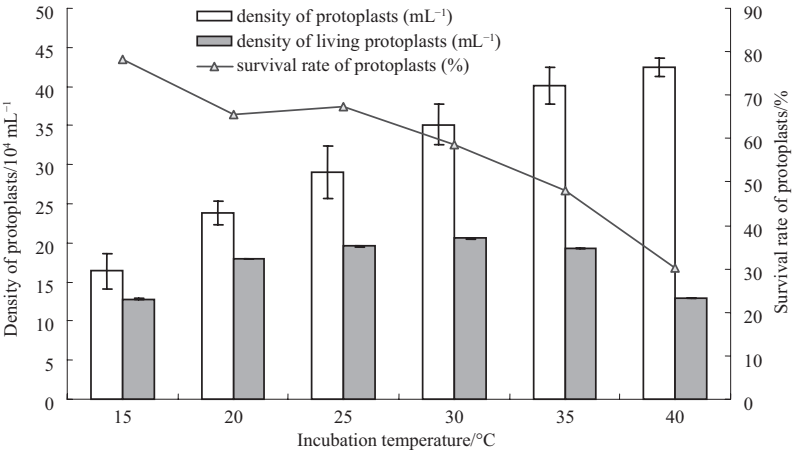

Fig.5. The effect of temperature on the protoplasts production from *Kappaphycus alvarezii*.

could be successfully prepared with 20% abalone enzyme, 12% cellulose and 2.0 mol/L glucose penetrant. After 3.0 h, 3.5 h and 4.0 h of enzymic hydrolysis in the darkness at 30°C, density of protoplasts of *Kappaphycus alvarezii* was (16.40±3.60)×10^4 mL^{-1}, (23.80±1.50)×10^4 mL^{-1} and (35.10±1.80)×10^4 mL^{-1} respectively. *t*-test method was also applied to compare density datas of protoplasts each other and inspection level was α=0.05. Seen from Table 2, with different enzymic hydrolysis time (3.0 h, 3.5 h and 4.0 h), difference among density data of protoplasts of *Kappaphycus alvarezii* was statistically significant (P<0.05).

4 Discussion and conclusions

Since hydrolytic enzyme of digestive glands is discovered in marine shellfish, great progress has been made in the isolation and culture technology of large algal protoplasts. The enzymic method is applied as the major isolation method for algal protoplasts. Enzymic hydrolysis for cell walls of algae is characterized by strong specificity and causes minor damage to cells due to its moderate conditions. Property, activity and purity are generally utilized to determine the category and effective combination of enzymes. Some existing researches have demonstrated that the enzymatic activity of each batch of crude enzyme of snail enzyme is usually different, which leads to a large difference in yields of protoplasts (Reddy and Fujita, 1991). *Chiton* sp. and *Patella* sp. are used to produce the combined hydrolytic enzyme more applicable to rhodophyta that can achieve better yields of protoplasts (Yu et al., 2000). Abalone enzyme is considered applicable to *Laminaria japonica* (Hu et al., 2000; Liu et al., 2002; Inoue et al., 2011), *Undaria pinnatifida* (Hu et al., 2000) and *Porphyra yezoensis* (Hu et al., 2000), etc. In the experiment, the single enzyme of cellulase, snail enzyme and abalone enzyme, the combined enzyme of snail enzyme and cellulase, the combined enzyme of cellulose and abalone enzymeare successively applied with enzymic hydrolysis, after which the Solieriaceae seaweed's protoplasts are successfully prepared.

The enzyme and other factors play important roles on the effect of enzymic hydrolysis. Within the range of the given time, the density of protoplast is directly proportional to the time of enzymic hydrolysis. When the parameter (density of protoplast) is considered, the single enzyme of snail enzyme, abalone enzyme and cellulase exert a weak influence on the isolation of target algae while the density of protoplasts achieved by the combined enzyme of snail enzyme and cellulose and that of snail enzyme and abalone enzyme is relatively low. In this way, the ideal isolation effect cannot be obtained and the density required by following researches is not reached. Based on the experiment, the research discovers that snail enzyme does not present better selectivity towards target algae. The reason mainly lies in the narrow feeding habit of snail, which has limited the effect of snail enzyme in the preparation of protoplasts of Solieriaceae. The yield of protoplasts achieved by the combined enzyme of abalone enzyme and cellulose can exceed 10^5 g^{-1}. The combined enzyme presents a better applicability towards the target algae in the experiment and can fully satisfy the material density required by following experiments and researches.

In the process of preparing algal protoplasts by enzymic method, the isolation effect of the algal protoplasts will be influenced by many factors, such as the selection of materials, pretreatment of materials and the use of enzymic preparations. Healthy fronds play as the ideal materials for the preparation of algal protoplasts. The selection of ordinary materials incorporates the selection of genotype and physiological status of materials. The selection of genotype will show indirect impact on many aspects like the yield and vigour of protoplasts as well as the regeneration of plants, while the selection of physiological status of materials directly influences the isolating effect and survival rate of protoplasts (Yu et al., 2009). Normally the fresh and tender fronds or the fronds that grow vigorously in the selective chamber are chosen as enzymolysis materials. In all researches related to the preparation of protoplasts of *Sargassum fusiforme* (Wang et al., 1996), *Undaria pinnatifida* (Wu, 1988), *Gracilaria verrucosa* (Chu et al., 1998) and others, the tender parts of the frond top are explicitly utilized. Moreover, many researches have proved that the materials should be pretreated before the enzymolysis separation of algal protoplasts in order to change the physiological status of cell walls, improve the toughness of cell membranes, enhance the efficiency of enzymolysis, and maintain the integrity of protoplasts. However, not all the protoplasts isolation should be pretreated but specific situations should be considered. The composition and quality of enzyme as well as its purity and activity play as the key factors that influence the effect of enzymic hydrolysis. Different enzyme combination experiments can be carried out in order to confirm the best combination of hydrolytic enzyme. The enzyme concentration is often selected based on different enzymatic activities and the enzymatic activity of hydrolytic enzyme made in every batch is different (Yu et al., 2000), so preliminary experiments of optimum concentration should be conducted to isolate and make protoplasts with different batches of hydrolytic enzymes. After the enzymolysis of cell wall, protoplasts are likely to break or contract due to the change of the osmotic pressure of outside solution. Therefore, in order to protect the protoplast without the cell wall, the osmotic stabilizer should be added to the hydrolytic enzymes. Different types and concentrations of penetrants should be selected for different materials, such as glucose, cane sugar, mannitol, sorbitol, KCl and NaCl, etc. The algae often chooses seawater and sugar alcohol as the penetrant. Salvador and Serrano (2005) made an experiment on the isolation of protoplasts of *Kappaphycus alvarezii*, when the yield of protoplasts made in the condition of enzymolysis at 25°C for 48 h turned out to be 8.2×10^3 g^{-1} tissue. However, the density of protoplasts of *Kappaphycus alvarezii* made at 30°C with 4.0 h dark enzymic hydrolysis mentioned in this research reached (32.60±1.40)×10^4 mL^{-1}, with its yield as (65.2±2.80)×10^4 g^{-1} tissue. The preparation efficiency and yield in this research

Table 2. Effect of enzymatic hydrolysis on *Kappaphycus alvarezii*

	Incubation time/h		
	3.0	3.5	4.0
Density of protoplasts/×10^4 mL^{-1}	16.40±3.60*	23.80±1.50*	32.60±1.40*
Density of living protoplasts/×10^4 mL^{-1}	9.58±0.01*	12.04±0.01*	16.08±0.01*

Note: * p<0.05.

result have both been improved to a large extent. We consider the optimized enzymic hydrolysis system as the main contribution. The selection and grinding of materials in the enzymic hydrolysis process and the combination of 20% abalone enzyme and 12% cellulose both promote the idolation of protoplasts of *Kappaphycus alvarezii*.

The experiments have demonstrated that for the identification of protoplasts, the traditional swelling method can hardly identify the protoplasts due to the difficulty to control the mobility of the cell and the volume of the swelling liquid and to observe the swelling process of the cell. With the fluorescent brightener staining decoration method for identification, and the motivation by the UV-light with 370 nm wavelength, if the parietal cell is featured with blue-green fluorescence, it shows the existence of cellulose. But if the protoplast has jacinth fluorescence instead of blue-green fluorescence because of chloroplast, it shows that there is no cellulose. According to the protoplasts stained by Evans Blue staining method, the living protoplast is in bright yellow and dead protoplast is in dark blue, so we can accurately calculate the survival rate of protoplasts. The accuracy and clarity of fluorescence staining and Evans Blue staining will make the identification results of the protoplasts more distinct and exact and will also greatly improve the reliability.

In this research, experiments with different osmotic pressure concentration, different enzymic hydrolysis time and different enzymic hydrolysis temperature prove that the protoplasts of *Kappaphycus alvarezii* have a rather high separation rate with the condition of 2 mol/L glucose and 4.0 h enzymic hydrolysis at 30°C. The density measurement and the survival rate of protoplasts provide a basis for the follow-up experiments. The experiments demonstrate that within the enzymic hydrolysis time, the yield will be higher but the survival rate is on a declining curve if the time is longer. The reason probably lies in that mechanical factors in the enzymic hydrolysis conditions will damage the produce of the cells to an extent or the hydrolytic enzyme itself will be toxic to the cells result in the survival rate inversely proportional to the enzymic hydrolysis time. The enzymic hydrolysis time shows direct influence on the vigour of cells. Enzymic hydrolysis in short time is very important to improve the survival rate and keep its normal development. Therefore, in the process of preparing protoplasts, survival rate should be attached to with great importance.

The cultivation method of algae protoplasts is similar to cell cultivation, which consists of the shallow-layer liquid cultivation, solid cultivation and combined cultivation of liquid and solid. On the basis of these cultivation methods, micro hanging-drop cultivation, nurse cultivation, AGAR island cultivation and others emerge successively, among which the nurse cultivation is applicable to the cultivation of low-intensity protoplasts and screening of the hybrid cells. Different cultivation methods can be applied according to different algae and different cultivation aims (Zhao et al., 2009). During the cultivation of algae protoplasts, cultivation conditions such as light and temperature play a very important part. For green plants, sufficient light is the necessary condition for their normal growth and development. But during the early cultivation of protoplasts, protoplasts shall be placed in dark or weak light condition because growth and development of protoplasts will be restricted by light. After dark cultivation, protoplasts will be brought to normal light cultivation. It is discovered in research that the intensity of light is the determinative factor of sprout growing (Lillo, 1989). Temperature is another important factor for protoplasts cultivation, which should be different for specific specie of algae. In this experimental research, regeneration cultivation of protoplasts of *Kappaphycus alvarezii* should be completed in the cultivation temperature at 20°C, photoperiod of 12 h/d and intensity of light 1 500–2 000 lx. Under the cultivation condition, regeneration cultivation of protoplasts of *Kappaphycus alvarezii* is in good condition and obtains cell mass and regenerated plantlet. The occurrence rate of protoplast regeneration is usually very low, which may be related to the improper cultivation condition of protoplasts isolation. The low occurrence rate of algae protoplasts regeneration and the lack of organic organ formation potential might be explained by the variance diversity in natural population genes (Mussio and Rusig, 2009).

According to the report at home and abroad, the development and differentiation ways of algae protoplasts can be summarized into three pathways: callus-like, regeneration plantlet and germ cell pathway. Many kinds of Rhodophyta can be classified as the callus-like or regeneration plantlet (Chu et al., 1998; Wang et al., 2007; Zhang, 1983; Hu, 2006; Araki and Morishita, 1990; Chen, 1993; Zhang, 1991; Liu et al., 2007), but there are relatively fewer reports for protoplasts isolation and regeneration in Phaeophyta (Wang et al., 1996) and Chlorophyta (Reddy and Fujita, 1991; Della et al., 1991; Reddy et al., 1992). This research focuses on the regeneration cultivation research for protoplasts of *Kappaphycus alvarezii* and observed two developmental pathways, one of which refers to observe the cell division at 40 d during regeneration cultivation of *Kappaphycus alvarezii*. Following cultivation gets cell mass, where there exists protoplasts mass packed by a common exine, which means cytokinesis can occur in cells, but the new cell walls cannot be observed clearly. Besides, it is also observed in 65 d cultivation that protoplasts cell grows to be regenerated plantlet directly. These two developmental pathways including callus-like cell mass and regenerated plantlet occurred on different petri dishes and under the same regeneration cultivation condition. This result was discovered for the first time in my experiment without any information before. The reason why two developmental pathways happened may be cells, like cortex cells and pith cells, which came from different tissues, developed variously and then revealed different developmental pathways. With our experiment results and research reported in previous studies, we have a certain understanding of developmental pathways of protoplasts of *Kappaphycus alvarezii*. However, how these two pathways happened and where callus-like cell mass and regenerated plantlet came from are proposed to conduct further research.

During the algal tissues cultivation, it is a key step to create the sterile cultivation environment. One of the difficulties in early algal tissues cultivation research lies in that sterile explants are very difficult to be obtained. This research chooses 0.01% $HgCl_2$ as the disinfection reagent which is able to kill the bacterium on the surface of algae for 15 s with little harm to the algae itself. The experimental material processed by $HgCl_2$ can fully meet the requirement of follow-up sterile regeneration cultivation experiment of explants. During the cultivation of tissue pieces after enzymic hydrolysis of *Kappaphycus alvarezii*, there appear two developmental pathways of differentiation the same as protoplasts in tissue pieces. One is that tissue pieces show sprouting regenerated, which means young buds come out after 65 d cultivation, and for the other pathway, the cell

mass is observed to be emerged at 75 d. Anicia and his partners (Hurtado et al., 2009; Hurtado and Biter, 2007), Leila et al. (2008) have also observed the two above-mentioned separation developmental pathways. Among existing documents, algal explants of *Solieria* (Wang et al., 1985), *Gracilaria* (Zhao and Zhuang, 1999) and *Chondrus* (Chen, 1987) are also reported to grow with young shoots or regeneration plantlet, while Phaeophyta explants of *Laminaria*, *Laminaria* and *Macrocystis* (Wang, 1994) are more likely to be induced and become callus-like. Differences among protoplasts and tissues cultivation developmental pathways of Rhodophyta, Chlorophyta and Phaeophyta indicate the better developmental totipotency of cells and tissues of Rhodophyta seaweed.

In recent years, researches of protoplasts have permeated into biological sciences such as molecular genetics and cytobiology owing to some excellent properties inside protoplasts. Researches of plants protoplasts have attracted more and more attention. In this study, protoplasts were successfully isolated from *Kappaphycus alvarezii* with snail enzymes, abalone enzymes and cellulase. Optimum enzymic ratio was fixed to be 20% abalone enzyme and 12% cellulase and the osmotic stabilizer was 2.0 mol/L glucose. Optimum enzymic hydrolysis conditions were 30°C dark enzymolysis 4.0 h, as a result, for the density and yield of protoplasts, 32.60×10^4 mL^{-1}, 65.20×10^4 g^{-1} tissue were achieved for *Kappaphycus alvarezii*. On the basis of isolation method of protoplasts of *Kappaphycus alvarezii* established in this research, it will provide significant material basis for *Kappaphycus* seaweed somatic hybridization and genetic transformation by carrying out further and deeper study of timing order and differentiation method for protoplast single cell proliferation and improving efficiency of callus-like and regeneration plantlet of protoplast developmental differentiation.

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