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Interspecies Protoplast Fusion Process of *Dunaliella Salina* and *Chlorella Vulgaris* to Produce Rich Carotenoid Natural Food Supplement for *Penaeus Monodon fab.* Larvae

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ABSTRACT

Natural pigment carotenoids from Dunaliella salina and Chlorella vulgaris was β-karoten and zeaxanthin. Crustaceans can not synthetize carotenoid de novo and they need it to provide nutrition and possibly disease resistance, pigmentation and esthetic value. Green microalgae produce carotenoids and can be manipulated easily by protoplast fusion. The research was conducted to obtain some recombinants from interspesies protoplast fusion of D. salina and C. vulgaris. Interspecies protoplas fusion was carried out by protoplast isolation, protoplast fusion and protoplast regeneration. Microscopic and cell analysis will used to confirm positive regenerate protoplast. Analysis of the obtained fusants is limited to morphological description due to the complexity and variability of fusant. The stabilities of fusants obtained were examined by successive subcultures. The result revealed that conversion of the cells of to protoplasts was about 80% . The fusant maintain their stability during three periods of subculturing. This result suggesting the potency of fusant to be used as food supplement in liquid form. The regeneration of the protoplast was almost 100% with some of them having diploids formation. Most colonies of the recombinant having faster growth suggesting the positive result of potential strain.

Keywords : *carotenoid, protoplat fusion, Dunaliella, Chlorella,*

1. INTRODUCTION

Microalgae have a great potential for various applications including the production of compounds for food, feed and aquaculture, of higher value products for pharmaceutical and cosmetic industries [1][2]. Carotenoids from microalgae have been proposed as cancer prevention agents, life extenders, and the inhibitors of ulcer, heart attack and coronary artery disease. Naturally occuring lutein from microalgae like *Chlorella vulgaris* is not only one of the most prominent has been successfully applied to the analysis and carotenoids in human serum and foods, but also the representative of α,β-carotenoids [3]. Crustaceans need carotenoids to provide nutrition and possibly disease resistance, give brilliant pigmentation and esthetic value [4] [5]. *Dunaliella salina* and *Chlorella vulgaris* , was found potentially useful as source of carotenoids in food supplement in aquaculture. Under stress condition such as high light intensity, *D. salina* cells turn orange due to massive β–carotene formation under high light intensities. *Dunaliella* contains 9-cis-beta-carotene, which is up to ten times stronger at preventing cancer than ordinary β–carotene and can absorb far higher amounts of harmful ultraviolet radiation. Strains unable to accumulate β-carotene die when exposed to high irradiation, while the β-carotene-rich *Dunaliella* strains flourish [6]. *Dunaliella* also contains carotenoid zeaxanthin, a valuable antioxidant with ability to prevent progressive vision loss. For every gram of dry *Dunaliella*, 6 mg of zeaxanthin is produced, compared to only 0.2 mg of zeaxanthin found in ordinary plants.

Increased interest in natural carotenoids is the current trend of avoiding food additives and synthetic chemical in foods. Strain improvement of *D. salina* and *C. vulgaris* is needed in order to increase carotenoid production. Protoplast fusion has been used to bypass natural barriers of intra- or inter spesific hybridization when it has not been possible to perform conventional crosses, mutagenesis and genetic analyses [7]. The induced fusion of microalgal protoplasts (i.e. cells completely deprived of the wall) will produce complementation of auxotrophic mutants. Interspecies hybrids of *Phaffia rhodozyma* significantly increased b-karoten production up to 30% the levels of wild type [8] [9]. The protoplast fusion technique as a tool in microbial genetic contributed to the development of microbial genetics and the clarification of the mechanisms of microbial breeding. So far, intraspecies hybridization of protoplast fusion in *D. salina* and *C. vulgaris* had never been done yet.

2. MATERIALS AND METHODS

2.1. Microalgae strains and Culture Conditions

D. salina and *C. vulgaris* was obtained from BBPAP (Balai Besar Pengembangan Budidaya Air Payau) Jepara. The Walne medium was used for culturing *D. salina* and *C. vulgaris* was modified from [10]. The medium consist of EDTA 45 g/L, $FeCl_3.6H_2O$ 1.3 mg/L, H_3BO_3 33.6 g/L, $MnCl_2.4H_2O$ 0.36 g/L, NH_4NO_3 100 g/L, Na_2PO_4 20 g/L, 3 % Sodium thiosulfate, B_{12} vitamin 0.001 ppm, distilled water until 1 L. Sterilization was done by autoclaving at 15 lb/in^2 (103 kPa and 120°C). The medium was using by adding 0.5 ml solution to each 1L of seawater. For induction of β-carotene synthesis, cells were grown in a sulfate-depleted media ($MgCl_2$ instead of $MgSO_4$), under intense illumination conditions 600 lux and with 2 – 4 ppm O_2 passing to the liquid[2].

Early growth phase cells (approx. 10^7-10^8 cells/ml) were washed with potassium phosphate buffer as osmose solubilizing solution followed by suspension in 3 % sodium chlorida buffer, 1 mM $CaCl_2$ and 0.1 M 2-mercaptoethanol. The cells were treated with 1 % 10 mg/ml of lysozyme on 35°C for 20 minutes. The protoplast was mixed and kept in Walne medium containing 60 mM polyethylene glycol (Mr. 6000; Sigma), 5 mM glycine and 10 mM $CaCl_2$ for 45 min. The process was followed by serial washing with suspension containing 5 mM glycine and 10 mM $CaCl_2$. Microalgal colony of recombinant were growing on Walne media. Growth curve for 7 days were examined followed by three periods of subcultures.

2.2. Protoplast fusion

Protoplast isolation. Microalga protoplasm were isolated using a modified method of [10][11]. Cell density were 10^6 cells. Release of cell wall was induced using 3 % NaCl, 1 mM $CaCl_2$, 1 % lisozyme for 20 minutes on 35°C. Protoplast fusion were induced using 60 mM PEG 6000, 10 mM $CaCl_2$ and 5 mM glisin, mixed with Walne medium and incubated for 15 minutes on 30°C. Cell were washed twice using sorbitol/manitol, 10 mM $CaCl_2$ and 5 mM glisin adding with Walne Medium and incubated 15 minutes on 30°C. Recombinant were grown on Walne Medium. Protoplast Regeneration will done after incubation for 5-7 days. Analysis of the obtained fusants is limited to morphological description due to the complexity and variability of fusant. The stabilities of fusants obtained were examined by successive subcultures.

3. RESULTS AND DISCUSSION

The most exciting possibilities in working with protoplasts are their use in genetic transformation of macroalgae and in their application to somatic hybridization and breeding.

3.1. Formation of protoplast

Protoplasts are living cells devoid of cell walls. Protoplast is a viable cell whose wall and other materials external to the plasmalemma have been removed, but it retains all internal components [12]. Treatment of either cells or tissues with specific cell wall lytic enzymes results in total removal of their rigid and complex polysaccharide cell wall. Treatment of different osmotic buffer to the protoplast of *D. salina* has shown that the protoplast was highly sensitive to the osmotic support medium. Method modification of [9][10] in the formation of the protoplast using sodium chloride buffer caused degradation of peptidoglycan cell wall by lysozyme. The buffer will enter to the cell and increase the cell size to spherical. Sodium chloride buffer can stabilize the extracellular medium optimally. A condensation of DNA in cell nuclei and decreased of protein synthesis are two common effects of

osmotic stress on the cell. When the cell wall of *D. salina* was digested with lysozyme, totally or partially, a hypotonic shock can rupture the wall and allow protoplast to release, unless the extracellular medium is stabilized with buffer osmotic medium. The rupture of *D. salina* cel wall to release the spheroplast can be seen in Fig 1. We may therefore anticipate such condition with the use of potassium phosphate buffer. The use of potassium phosphate buffer offers more suitable agent to manage the strength of the cell wall and produce the best protoplast. The use of salt in low concentration (1 mM $CaCl_2$) in addition of stabilizes the membranes of the cell under treatment of enzymes degradation, also increase the frequency of fusion. The result of *D. salina* protoplast showed that 80 percent or almost all of the colony can construct the protoplast.

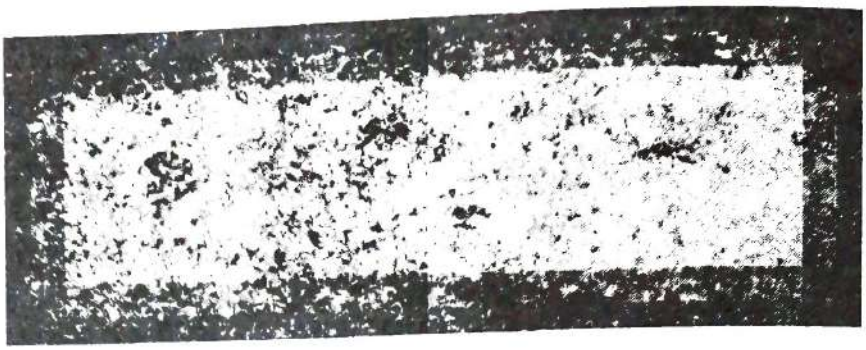

Figure 1. Spheroplast of *C. vulgaris* (left) and *D. salina* (right) (1000x)

Protoplast isolation from macrophytic benthic marine algae was reported as early as 1970. Preparation of protoplasts and their subsequent applications for both basic and applied research of marine macroalgae remains largely under developed due to lack of development of reliable methods with consistent yields of viable protoplasts [11]. The success in producing large numbers of viable protoplasts became possible only through the development of an enzymatic methods. There are 16 species of marine green algae from which successful protoplast isolation and regeneration has been reported. Recently, report about simple method for mass isolation of protoplasts from species of *Monostroma*, *Ulva* and *Enteromorpha* was done using an enzyme mixture containing 2% each of Cellulase Onozuka R-10 and Macerozyme R-10 dissolved in 3% NaCl[10].

The research result showing high yields obtained by the method have been attributed to the high activities of the enzymes as a result of dissolution in a medium with a low NaCl content. As the NaCl content used in their method corresponds to that of seawater concentration, an effort was made to further modify their method by optimising several protoplast isolation parameters including enzyme constituents and NaCl concentration in enzyme mixture. Berliner (1981) stated that the induced protoplasts of fresh-water algae lose the mechanical barrier that maintains their internal osmotic pressure. They retain their integrity and viability in an osmotically protective medium of equal or greater tonicity than that of the normal internal cellular osmolarity.

3.2. Protoplast Fusion Process

A fusogenic agent, such as polyethylene glycol (PEG) could induce the fusion and transient hybrids or diploids formation. During this hybrid state, the genomes of chromosomes would reassort which lead to a genetic recombination. The result as shown on Fig 1., and microscopic examination, suggested that protoplast fusion did not occur in a single step, but rather through step-by-step reactions in which each step did not proceed at the same rate. Each cell had a different ability to make a fusion as illustrated in Fig. 2.. After becoming a hybrid, each hybrid did not have either the same ability to regenerate or grew well on the medium. In the medium, some possibilities may occur: (a) the cell could not neither able to withstand the lysozyme treatment, nor recombined completely, (b) protoplast could not grow as good as the perfect hybrids, (c) the protoplast could not fuse, (d) protoplast fused completely and made a perfect hybrids from two or more cells.

Figure. 2. Fusion protoplast of *D. salina* and *C. vulgaris*

The protoplast and recombinant viability can be assessed by the exclusion of vital dyes by living cells as illustrated on the fig 3 using crystal violet. The dyes most commonly used are trypan blue, crystal violet, neutral red, and fluorescein diacetate. Regeneration of an outer wall followed by cell division is the desired outcome of most protoplast experimental work. Berliner (1981) showing that protoplast formation and regeneration are theoretically ideal tools for the understanding of cell wall structure and formation. Research experiment using as fusogenic agents indicates that almost all of the protoplast (86.5 %) were able to fuse under concentration of 10mM PEG 6000 PEG suggesting that of PEG applied was optimal in inducing fusion. The observation was supported by microscopic features showing that hybrids tended to form a recombinant cell than to disperse as the parental cells, indicating that some hybrids had form a ploidy, as previously suggested by [10][11].

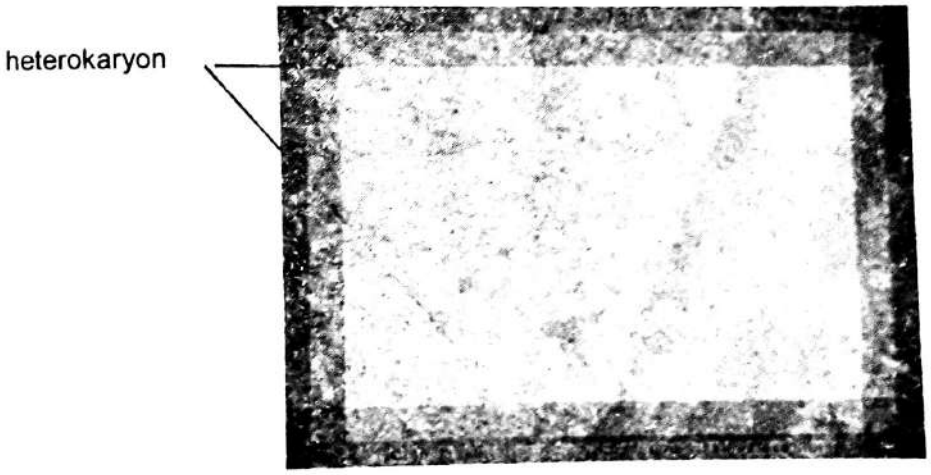

Figure 3. Viability of heterokaryon recombinat

3.3. Protoplast Regeneration

Under condition favoring early and efficient regeneration, such as regenerating on a complete or rich medium, the heterokaryon is induced to form a cell wall and divide before the chromosomes have been replicated, thus reducing the opportunity for genetic recombination to occur. Protoplasts are cell bounded only by cytoplasm membrane. After treatment with PEG to induced fusion, the protoplasts were able to generate cell wall and grew normally on the medium. After 7-10 days of incubation on 30 °C , some hybrids have been obtained. Regeneration of protoplast resulted in high frequency, reaching the value of 80% of all protoplasts that have been induced by PEG. Protoplasts in culture show rapid cytoplasmic streaming, decrease in size, and most of the cell organelles, in particular the chloroplasts, aggregate conspicuously around the nucleus. The result of the research shows that the fusion protoplast process gaining number of protoplasts formed and regeneration percentages was high enough although it was lower comparing to interspecies protoplast fusion of *Phaffia rhodozyma* [8] also other microalga [12]. Their overall regeneration rate of the protoplasts isolated was usually >

90% and showed normal morphogenesis. This result probably caused by different genus to be involved [13].

The rate and regularity of cell wall formation depend on the state of differentiation of the donor cells, conditions of isolation of protoplasts, and the plant species. The process of cell wall formation begins within few hours after isolation and maybe completed in two to several days. Protoplasts lose their characteristic spherical shape once the wall formation is complete. According observation from some researcher under a fluorescence microscope using calcofluor white showing that the freshly formed cell wall is composed of loosely arranged microfibrils; this requires an exogenous supply of a readily metabolized carbon source (sucrose) in the nutrient medium. Ionic osmotic stabilizers in the medium are reported to suppress the development of a proper wall. Soon after the formation of a wall around the protoplast the reconstituted cells show considerable increase in size and first divisions generally occur between 2 to 7 days. Subsequent divisions give rise to small cell colonies. A direct relationship exists between wall formation and cell divisions. Protoplasts capable to develop wall. Heterokaryon are more often observed in a protoplast culture. They form as a result of incomplete cytokinesis during first division which, results in spontaneous fusion. Such heterokaryon undergo continued growth and some, are capable of continued growth and differentiation.

Analyzing of heterokarion will be better when followed by analyses of recombination of genomic DNA. However, the analyses on the integrated genomic DNA in the cells as a result of protoplast fusion process have been puzzling researchers for decades for the complexity and variability of fusant DNA. Scientist had reviewed that quantitation of the dominant genome in the fusant culture is not possible. Often analysis of the obtained fusants is limited to morphological description and measurement of enzymatic activity. The stabilities of fusants obtained were examined by successive subcultures [13][15].

Although there has been an accumulation of significant amount of data regarding stability or segregation of the fusants, the genetic analysis is still incomplete. Fusants posses whole genome rearrangements which are not possible to be predicted. Even powerful and highly informative technique such as the DNA sequencing is non applicable due to absence of specific genetic markers for sequencing in the fusant cultures. More difficult is analysis of the genom. Random amplification polymorphic DNA (RAPD) and polymerase chain reaction (PCR) techniques have been used recently to investigate genetic similarities among fusants. RAPD technology can scan numerous loci in the genome through DNA amplification with several random primers, which makes it particularly attractive for analysis of genetic relationship between species or kingdoms. Unfortunately, this system has been found unreliable due to the fact that obtained profiles are subject to significant variations. Standardization is difficult, even not possible when the technique is applied in different laboratory settings. Other molecular typing techniques such as Multi Locus Enzyme Electrophoresis (MLEE), Pulse Field Gel Electrophoresis (PFGE), Ribotyping, Restriction Fragment Length Polymorphism (RFLP) and DNA sequencing are not reliable for various reasons [14] [15] [16] [17] [18] . The main of which is low typeability of the fusants. One of the most striking features was research on the pathogenic genus *Beauveria* on variability of pathogenicity among the hybrids of protoplast fusion, but no correlation between molecular pattern and pathogenicity was found [19]. Nevertheles, somatic hybridization via protoplast fusion provides an attractive method for the genetic improvement especially when dealing with different host ranges and involving species with isogamous species and vegetative incompatibility.

4. CONCLUSION

The protoplas fusion of *D. salina* and *C. vulgaris* tend to achieved high yields of protoplast and recombinant which almost 80%. The observation showing that hybrids tended to form a recombinant cell than to disperse as the parental cells, indicating that some hybrids had form a ploidy. These result indicated a potency of recombinant microalga to produce higher carotenoids comparing with their parents.

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