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Optimization and Stability of Total Pigments Production of Fusan from Protoplast Fusion of Microalgae *Dunaliella* and *Chlorella in vivo*: Attempts on Production of Sustainable Aquaculture Natural Food

Hermin Pancasakti Kusumaningrum[1)] and Muhammad Zainuri[2)]

[1)] Genetics Laboratory, Faculty of Mathematics and Natural Sciences, Diponegoro University,
[2)] Marine Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University,
Jl. Prof. Soedarto SH. UNDIP, Tembalang, Semarang. 50275
Email: gandasakti@yahoo.com; muhammad.zainuri@yahoo.co.id

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ABSTRACT

Diversification of feed rich in natural carotenoids is needed in aquaculture because they will increase survival and body weight of animal farming. Different types of important carotenoids such as β-carotene, zeaxanthine can be combined through a process of protoplast fusion making it cheaper and faster. 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 fusants 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 the fusant from *D. salina* and *C. vulgaris* maintain their stability in growth and increased carotenoid production during four periods of subculturing. This result suggesting the potency of fusant to be used as food supplement.

Key words: Carotenoid, Protoplast fusion, *Dunaliella, Chlorella*

INTRODUCTION

Demand of natural carotenoid pigments from microalgae had spurred interest in its production. A green microalga *Dunaliella salina* produced in large numbers of β-carotene carotenoid about 0.3% (Ben-Amotzs and Avron, 1990; Guedes *et al*., 2011). *Chlorella vulgaris* is known as microalgae that are often used as a food supplement for shrimp larvae due to large amount of its lutein. Astaxanthin is also one of important carotenoids compounds from *C. vulgaris* that act as antioxidants (Pisal *et al*., 2005; Cha *et al*., 2008). Despite its tremendous potential as main producer of carotenoids however, *Dunaliella* and *Chlorella* also has some limitation. Growth of the microalgae are very dependent on natural cycles which made their carotenoid production and stability are very limited and not available any time. The objectives of this research is to analyze carotenoid production and stability from fusant microalgae producing by protoplast fusion of *D. salina* and *C. vulgaris*, to be optimized as feed supplement for shrimp larvae. Protoplast fusion techniques intensively selected to enhance the ability of microalgae in producing carotenoids because of so it is not possible to hybridize the different species naturally. Genetic manipulations techniques such as protoplast fusion and DNA-mediated transformation are considered to be more effective comparing with mutation and selection techniques which are conventionally used for strain improvement (Lu *et al.*, 2011).

Hopefully, this study will develop natural food aquaculture rich of carotenoid from protoplast fusion of the microalgae *Dunaliella* and *Chlorella* which has the stability in their carotenoid production. This research also have scientific implications because incorporation of carotenoid of two different species through such techniques will broader to its salinity growth from fresh into saline water, increase carotenoid production and also improve possibilities to diverse types of carotenoids. Early stage of the resesarch was performed by protoplast fusion of *Dunaliella* and *Chlorella* followed by analysis on fusant stability and carotenoid production.

MATERIAL AND METHOD

Strains and Culture Conditions

Dunaliella salina was obtained from BBPAP (Bizcleiswater Aquaculture Research and Development Center) Jepara. The Walne medium was used for culturing *D. salina* modified from Bidwell and Spotte (1983). The medium consist of EDTA 45 g L^{-1}, $FeCl_3.6H_2O$ 1.3 mg L^{-1}, H_3BO_3 33.6 g L^{-1}, $MnCl_2.4H_2O$ 0.36 g L^{-1}, NH_4NO_3 100 g L^{-1}, Na_2PO_4 20 g L^{-1}, 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 1 L 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 (Rabbani *et al*., 1998).

Isolation of protoplast

Protoplast isolated using modified methods of Tjahyono *et al.*, (1994). Cells with a density of 10^7 soaked in a solution of sodium succinate buffer (pH 4.5), 0.7 M $(NH_4)2SO_4$, 0.6 M KCl and 0.1 M 2-mercaptoethanol. Protoplast is obtained by adding 2-3 mg mL^{-1} lysozyme for 2-3 hours.

Protoplast fusion of D. salina (Modified from Tjahjono et al. 1994; Uppalati and Fujita 2002)

Early growth phase cells (approx.10^7-10^8 cells mL^{-1}) 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^{-1} of lysozyme on 35 °C for 20 minutes. The protoplast was mixed and kept in Walne medium containing sea water, 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 $CaCl_2$.

Protoplast regeneration

Protoplast regeneration were made by growing the recombinanat on Walne medium using sea water containing 5 mM glycine and 10 mM $CaCl_2$ and incubated for 5-7 days.

Analysis of pigment total product from fusion recombinant

Total pigment extracted according to Iwasaki and An (1991); Estevez *et al.* (2001). Cultures of 2 mL Eppendorf tube and then sentrifugated for 10 minutes at 4,500 rpm and then discarded the supernatantt. Pellets were washed with distilled water and supplemented with 0.1 M sodium phosphate pH 7 and 1 ml dimethyl sulfoxide (DMSO 1 ml which had been heated at a temperature of 55 °C. Mixture shaken for 15 minutes and then added 2 mL of organic solvent (diethyl ether) and then shaken back for centrifuged. Pigments contained in the upper phase was taken, then evaporated to remove organic solvent. When dry add the organic solvent (methanol by volume in accordance with the amount of pigment produced. The measurement of total pigment made according to the method of Iwasaki and An (1991); Britton (1995). Pigments totally determined by the coefficient extinction 1% ($E_{1cm1\%}$ = 1,600).

Growth analysis

Growth analysis was done gravimetrically using 1.0 mL culture on micro centrifuge flasks after measuring the cell dry weight. The culture was centrifuged for 15 minutes at 4,500 rpm. Pellet was dried using oven 80 °C until reaching constant weight.

Test the stability of fusant and parents was done by transfering microalgae on medium subculture from first untill fourth generation, as suggested by Panaiotov *et al.*, (2009).

Data analysis

Units experiments performed with parents as a control and fusant from *Dunaliella* and *Chlorella*. Stability and carotenoid production data obtained using three repetition times and analyzed using the Test of Independent Samples T Test Two at the level of 95%. The number of generations and the stability of the fusant from *Dunaliella* and *Chlorella* results were analyzed by linear regression method.

RESULT AND DISCUSSION

Microalgae *Chlorella* and *Dunaliella* are known to produce large amounts of carotenoids. The stability of fusant cells is an important factor in the development and improvement of carotenoid production (Kusumaningrum, 2003). *C. vulgaris* total pigment production after growing for seven days reached about 95 μg g^{-1} dcw, *D. salina* 102 μg g^{-1} dcw and highest total pigment production from fusant of *D. salina* and *C. vulgaris* reach 112 μg g^{-1} dcw. The β-carotene analysis performed on this research yielding concentration for fusant, *D. salina* and *C. vulgaris* about 2.8952 μg g^{-1} dcw; 4.3626 μg g^{-1} dcw; and 0.2728 μg g^{-1} dcw, respectively.

Johnson and An (1991) stated that the production of carotenoid pigments is influenced by several environmental factors, including light, aeration, nutrients, and other factors. Some of this effect is not specific and changes depending on the overall cellular metabolism .Carotenoids in *Chlorella* is produced in the form of primary and secondary carotenoids. Primary carotenoid in photosynthetic microalgae are generally produced during growth include lutein, zeaxanthin, α-carotene, β-carotene, violaxanthin and neoxanthin (Goodwin and Britton, 1988). The carotenoids analysis in *C. pyrenoidosa* showed that in normal growth, 93.1% of the total carotenoids produced in the form of lutein and its isomer, 2.6% in the form of α-carotene and its isomers, and a 2.6% α-carotene and its isomer; 1.3% zeaxanthin, 0.2% epoxy compound and 0.2% cryptoxanthin (Gouivea *et al*., 1996; Inbaraj *et al*., 2006). Lutein compounds were found in large numbers in determining the viability of microalgae. Unfortunately, *Chlorella* carotenoid concentrations are very affected on environmental conditions.

Inbaraj *et al.* (2006) showed that in normal growth, *Chlorella* were faill in producing secondary carotenoid like astaxanthin, cantaxanthin and echinnone. This result was in contrast with Gouivea *et al*. (1996) in gaining all of the primary and secondary carotenoid after applying environmental stress condition such as nitrogen depletion. It also showed that β-carotene production tend to decrease during periods of incubation and reached about 0.15 μg g^{-1} dcw on the seventh day of incubation and from the research result we can see that the amount of β-carotene production from *Chlorella* was almost the same although it was in salinity stress condition. Salinity have a stimulus effect on carotenoid production of microalgae as a positive response due to the influence of cations on the stimulation of the enzyme synthesizing carotenoids, as reported recently (Goodwin, 1993). Salinity in sea water medium can stimulate the growth of *Dunaliella* cells and increased content of β-carotene.

Light and temperature are also major factor that determines the formation of carotenoid pigment (Borowitzka and Borowitzka, 1988; Borowitzka, 1992). If the amount of light available was sufficient to inhibit the synthesis of chlorophyll. Chlorophyll and carotenoids will be synthesized in a balanced manner in the chloroplast. At the moment the balance is changing as a result of the increase in carotenoids, the plastids structure will change and as a result will be degraded chlorophyll but not carotenoids.

Light has an important role in the regulation of carotenogenesis in several organisms. Light will acts as an

inducer of carotenogenesis (Borowitzka, 1992). Temperature also the important environmental factors affecting the growth and development of living organisms that can cause changes in many biosynthetic pathways, including carotenoid biosynthesis. Temperature can regulate the concentration of an enzyme involved in the production of carotenoids as well as changes in enzyme concentration and ultimately regulate the level of carotenoids (Siero *et al*., 2003).

The β-carotene pigment in *C. pyrenoidosa* is the primary metabolite that formed simultaneously during their growth. The pigment will increase in log phase but will be less during the stationary phase (Gouviea *et al*., 1996; Gouviea and Emphis, 2002). Microalgae *D. salina* has two kinds of β-carotene which is all trans β-carotene and 9-cis-isomers of β-carotene. All trans β-carotene synthesized simultaneously with the formation of chlorophyll on the same time with β-carotene of *C. pyrenoidosa*. The second type of β-carotene in *D. salina* is 9-cis-isomer-β-carotene that synthesized in the logarithmic phase but will be accumulated in the lipid layer of the chloroplast for dealing with environmental stresses. This character has made *D. salina* more resistant to salinity and high light intensity.

Ben-Amots dan Avron (1990) stated that β-carotene production from *D. salina* reach about 0.3% from their total carotenoids, but it concentration depending upon nutrient deficiency, salinity stress and stronger light intensity (Mendoza *et al*., 2008; Jesus and Filho, 2010). The research result showed that that concentrations of β-carotene from *D. salina* was high enough. Nevertheles, the β-carotene concentration of fusant were lower comparing with *D. salina* but almost ten times higher from *C. vulgaris*. The fusant also showed the stability on carotenoid production from one generation to the fourth generation, the amount of carotenoid production showed a steady increase in the production of carotenoids in reaching untill 15% for each generation. Presumably, these character come from both parents. It is therefore plausible, that both pathway operate in given the relatively early accumulation of β-carotene from *C. vulgaris* will be followed by secondary β-carotene from *D. salina*. The *C. vulgaris* producing not only primary carotenoids but also secondary carotenoid while *D. salina* carotenoid production increased along with the stages towards cell death. This indicates that protoplast fusion was able to optimize and increase the amount of carotenoids started from log phase to stationary phase on fusant growth. The increase in carotenoid pigments during stationary phase because the pigment produced is used for cell survival. *D. salina* is a microalgae producer of natural carotenoids, especially β-carotene and zeaxantin, in accordance with *C. vulgaris* in nitrogen depleted condition. The formation of secondary metabolites mainly carotenoids, is not always directly proportional to the growth or multiplication of cell. Rate of pigment formation during cell growth will increases entering the stationary phase or nutrient deprivation.

Fusant obtained from fusion protoplast process in this research, instead of improved stability of the carotenoid pigment production, also increased fusant ability to live on a wider range of salinity. The condition was predicted to affect the growth of fusants that tend to decrease in their cell number from the first generation to the fourth. The growth rates of the fusants were faster than both parent *C. vulgaris* and *D. salina* and they were more osmotolerant at a salinity of 0.5-32 ppt. At the extreme salinities, fusan grew very slowly and decrease their β-carotene production below *D. salina*. This indicated dominancy of *D. salina* character on growth but also *C. vulgaris* in its cell structure (Fig.1.)

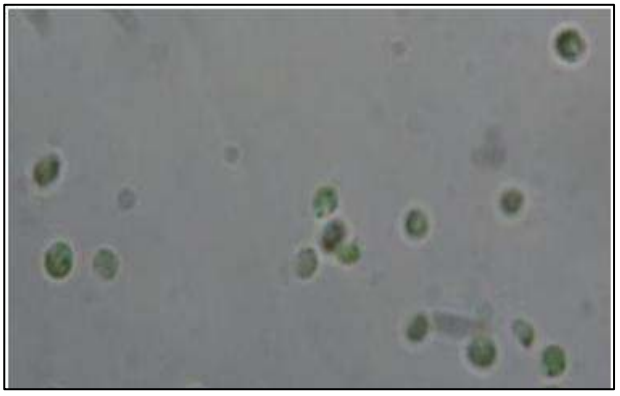

Figure 1. Fusant of *D. salina* and *C. Vulgaris*

C. vulgaris has specific environmental requirements for growth. Temperature play a role in the metabolic processes with optimum temperature range is 25-30 °C for *Chlorella* and also optimum salinity at 10-20 ppt. From the research result on carotenoid production and stabiliy, we can hope to find new combination from both microalgae in having the advantages on both microalgae character. This process, as sugested by Gerken *et al*. (2012) was facilitated by enzymatic digestion of the algal cell walls by lysozyme which has the potential to facilitate genetic transformation between two microalgae *D. salina* and *C. vulgaris*. Degradation of the cell wall may have great effects of the cell membrane as well, which could lead to an altered permeability barrier. Similar work has been done by Lee and Tan (1988) to fuse between red algar *Porphyridium cruentum* and *Dunaliella* spp. A comparison of the different macromolecular contents of parental and fusant cells is shown that some fusants in resembled *P. cruentum* in DNA and carbohydrate contents, its glycerol and protein contents were closer to those of *D. salina,* while others showing that the DNA content was intermediate between that of the parental strains and the contents of glycerol, protein and carbohydrate were decreased. The phycoerythrin to chlorophyll ***a*** ratios of most fusants were, however, similar to that of *P. cruentum*.

The research result also indicating that fusion protoplast giving the opportunities for somatic hybridisation enables nuclear and cytoplasmic genomes from both parent to be combined, fully or partially, at the interspecific and intergeneric levels to circumvent naturally occurring incompatibility barriers between two different species of microalgae. When protoplasts fused it will merged the contents of cells including DNA and cell nuclei between two or more cells that produce organisms with new properties result of mixing the two characters especially their carotenoid production.

Based on the results it may be concluded that the stability of carotenoids present in fusant of *D. salina* and *C. vulgaris* can be optimized in broader salinity spectrum as feed supplement. In particular this fusant can be tested to be used in brackish water pools for freshwater prawn farming, because brackish water is hostile to the growth of most terrestrial plant species.

Correlating with the used for feed supplement on shrimp and larvae, Darachai *et al.* (1998) and Boonyaratpalin *et al.* (2001) showing that supplementation feeding on *Penaeus*

monodon (Fabricius) with algae *Dunaliella salina* contains natural β-carotene and *Haematococcus pluvialis* with its astaxanthin, exhibiting significant effect on growth, weight, survival and immune response in high and low salinity. Combination of both natural astaxanthin and β-carotene from fusant of *D. salina* and *C. vulgaris* offering more promising positive effect as feed supplement for aquaculture animal.

The research implications also showing that protoplast fusion method developed can be used as a concise method to improve the carotenoid production in microalgae and fusants. Further research is warranted to appplication of fusants in managing shrimp larvae diseases. Furthermore, stability of carotenoids and fusant under different applications conditions and organisms will be investigated.

CONCLUSION

Carotenoid production of fusant are stable from the first generation untill the fourth. Production of carotenoid of fusant are greater 15% in average comparing with parental microalgae *D. salina* and *C. vulgaris* and can be optimized in broader range of salinity. The research result has improved production of carotenoid and potentially applicable to be used as feed supplement.

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