Application of Aquaculture Natural Food Produce by Protoplast Fusion Process of *Dunaliella salina* and *Phaffia rhodozyma*

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Abstrak

Selama ini pendapatan petani tambak cenderung statis sehingga diperlukan suatu upaya secara tepat dan efisien salah satunya melalui diversifikasi pakan. Sejauh ini usaha untuk mengamati pola introduksi suatu pakan baru, aplikasi dan dampaknya terhadap budidaya dan pendapatan petani tambak belum pernah terukur. Diversifikasi pakan mengggunakan pakan kaya karotenoid alami sangat dibutuhkan dalam budidaya perikanan karena terbukti dapat meningkatkan keloloshidupan dan menambah bobot hewan budidaya. Kedua jenis karotenoid β-karoten dan astaxantin dapat digabungkan melalui proses fusi protoplasma sehingga lebih murah, cepat dan efisien untuk diversifikasi dan pengembangan pakan kaya karotenoid. Tujuan khusus penelitian ini adalah pengembangan usaha budidaya untuk meningkatkan pendapatan petani tambak melalui diversifikasi pakan akuakultur dengan kandungan karotenoid tinggi hasil fusi protoplasma alga Dunaliella salina *dan khamir* Phaffia rhodozyma*. Tahap awal dalam penelitian ini akan melakukan kultivasi pakan rekombinan* Dunaliella salina *dan* Phaffia rhodozyma *diikuti analisis produksi karotenoid secara kuantitatif dibandingkan dengan kontrol. Tahap kedua adalah diversifikasi pakan dengan perbedaan konsentrasi fusan dan perbandingan dengan jenis pakan lain diikuti dengan analisis untung rugi.*

***Kata kunci** : karotenoid, fusi protoplas, D. salina, P. rhodozyma, rekombinan*

Abstract

Recently, fishponder income tend to be static so that required an effort to accurately and efficiently one of them through aquacultures food diversification. So far, efforts to observe the pattern of introduction of a new feed, applications and their impact on aquaculture and fish farmers income has never been measured. Diversification of feed use protoplast fusion process from D. salina *and* P. rhodozyma *which rich in natural carotenoids is needed in aquaculture because they will increase survival and body weight of animal farming. Different types of carotenoids can be combined through a process of protoplasm fusion making it cheaper, faster and efficient for diversification and development of carotenoid-rich diet. The specific objectives of this research is the development of natural food aquaculture with high carotenoid content using protoplasm fusion from the microalgae D. salina and yeast P. rhodozyma. The early stage in this research will cultivate recombinant followed by quantitative analysis of carotenoid production compared with controls. The second stage is the food diversification with different concentrations of recombinant and comparisons with commercial food followed by cost-benefit analysis. The research results have been obtained which most carotenoid-rich natural food resulted from recombinant fusion of* D. salina *and* P. rhodozyma*. Its application in vitro have increased body weight of shrimp comparing to artificial feed. In addition, recombinant food showing stable growth in both freshwater and salt water, can breed naturally and are safe for animal aquaculture consumption and also the environment.*

***Key words** : carotenoid, protoplast fusion, D. salina, P. rhodozyma, recombinant*

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Introduction

One of the most important operational functions in shrimp culture is the provision of adequate food supply to ensure that the cultured animals attained the desired harvesting size within the targetted time frame. Feeds is among the largest operational cost of shrimp farming and every efforts should be made to ensure efficient utilization of feeds for growth (FAO, 1991). Introduction of a new technology such as protoplasm fusion technique to produce the aquatic animal feed has a high potency which is more cheaper, easier and safer than other technologies. Achievements obtained are also economically advantageous because the production of carotenoids using these methods has increased two to three times (Kusumaningrum, 2003).

Carotenoids occur universally in photosynthetic organisms but animals do not synthesize carotenoids. Carotenoids are required as feed supplements in the poultry industry and in aquaculture of fishes and crustaceans. In addition to providing nutrition and possibly disease resistance, carotenoids give brilliant pigmentation and esthetic value to crustaceans (Iwasaki & Murakoshi, 1992). *Dunaliella salina* contains a certain type of beta-carotene called 9-cis-β-carotene, which is up to ten times stronger at preventing cancer than ordinary beta-carotene. More recent studies show that *D. salina* also contains another carotenoid called zeaxanthin, a valuable antioxidant with ability to both help prevent and treat this debilitating condition that causes progressive vision loss. For every gram of dry *D. salina*, 6 mg of zeaxanthin is produced, compared to only 0.2mg of zeaxanthin found in ordinary plants. *D. salina* is a green algae-producing β-carotene carotenoids in large numbers up to 8000 ug/g. *P. rhodozyma* is the largest manufacturer of astaxantin about 500 βg total carotenoids per gram dry weight of yeast cells. Astaxantin (3.3 '-dihydroxy-β, β -carotene-4 ,4-Dione) is a carotenoid pigment of the most widely used and needed lately. Strain improvement is needed in order to increase carotenoid production by inter-species hybrids between *D. salina* and *P. rhodozyma*. So far, inter-species recombinant from protoplast fusion had never been used as natural food aquaculture although it was found potentially useful as source of carotenoids in food additives or as food supplement in fish farming.

Astaxanthin is the main carotenoid found in the integument of shrimp. It is important for shrimp health, since it is involved in intra-cellular protection by stabilizing cell membranes and improving health and immunology through the removal of oxygen free radicals (Roche News, 1993 *in* Darachai, 1998).

Materials and Methods

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, $FeCl_3.6H_2O$ 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 (Rabbani *et al*., 1998).

P. rhodozyma obtained from the BCCM (Belgian Co-ordinated Collections of Microorganism). Yeast were grown and stored in the medium with the following composition: glucose 10 g/L, peptone 5 mg/L, yeast extract 3 g/L and for 20 g/L. Storage temperature 4°C (Chun *et al*., 1992). Starter/inoculum was grown in 250 ml erlenmeyer containing medium with the following composition: glucose 10 g/L, peptone 5 g/L, yeast extract 3 g/L malt extract 3 g/L at pH 5 and temperature of the room. Cultures were incubated for 18-24 hours on a rotary shaker with a speed of 180 rpm.

Isolation of protoplasm

Protoplasm isolated using modified methods of Chun *et al*., (1992). Cells with a density of 107 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. Protoplasm is obtained by adding 2-3 mg/ ml lysozyme for 2-3 hours.

Protoplast fusion of P. rhodozyma

Protoplasm two cells were fused using method of Chun et al., (1992) by means mixed in phosphate buffer solution (pH 6) containing 35% polyethylene glycol 0.1 CaCl2 then incubated for 45 minutes.

Protoplast fusion of D. salina

Protoplast fusion of *D. salina* was carried out using modified method of Tjahjono *et al*. (1994) and Uppalati & Fujita (2002) as follows. Early growth phase cells (approx. 10^7-10^8 cells/ml) were washed

withpotassium 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 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 fusion of D. salina and P. rhodozyma

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 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 & An (1991); Estevez *et al.* (2001) . Cultures of 2 ml Eppendorf tube and then sentrifugated for 10 minutes at 4500 rpm and then discarded the supernatant. 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% ($E_{1cm1\%}$ = 1600).

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 min at 4500 rpm. Pellet was dried using oven 80°C until reaching constant weight.

Aquaculture food production

Composition of artificial diets as follows: Flour swordfish / tuna: 66.8%, Wheat flour: 18%, Fish oil: 5% Bone Meal: 5%, Aquamix: 5% , Fusion recombinant (concentration: 40, 60, 80 and 100 mg / 100 g feed).

Maintenance of test animals tiger prawn (Penaeus monodon Fabricius)

This study use d 60-day-old shrimp (PL-60), weighing between 0.2 to 0.5 grams. Food that is used in this study there are several types consisting of natural food *D. salina*, recombinant food, in the form of pellets with different fusion recombinant cells and commercial food as a control. Natural food *D. salina* and recombinant food using a density of 2700 individuals per ml. Natural food *D. salina* is shrimp feed formulation, and recombinant food is artificial food with addition of various concentration of recombinant. Biota tested were tiger shrimp PL-60 with initial weight from 0.2 to 0.5 grams. Maintenance is done in an aquarium measuring 25 x 60 x 35 cm, with a volume of 10 liters of water. The density of each aquarium is 5 tail. The food is some 5% of body weight, and given twice a day to feed. Natural food remains with the density of 2700 individuals per ml. Maintenance carried out for five weeks. Observations on the quality and safety of natural food prefetch on this research conducted by recombinant feeding of shrimp larvae in *in vitro* scale. Every two days the body weight of shrimp larvae were weighed in the laboratory to see the development and increase in weight of shrimp as an effect of the provision of food diversification protoplast fusion results.

Result and Discussion

New feed production using fusion protoplasm technique has high economic potential because it is easier and safer than other technologies as well as economically profitable. Research in the laboratory in vitro have been carried out to see the production of carotenoids. Protoplasm fusion has been done separately on both *D. salina* and *P. rhodozyma* and also their parent as a control. Protoplasm fusion recombinant also has been employed to see its effect on growth, survival and body weight of shrimp. The research was conducted on a small scale.

The initial phase of research were carried out by cultivation of recombinant feed *D. salina* and *P. rhodozyma* in larger quantities. This is necessary in order to perform quantitative analysis of carotenoid production compared with controls. The second stage is the diversification of feed with different concentrations of recombinant and comparisons with other diets. Stage of research will also look at analyzing the effect of the use of recombinant as feed for aquaculture animals compared with both parent. In the final stage, will be formulated diet rich in carotenoids which has superior production.

***P. rhodozyma* cultivation and pigment production**

P. rhodozyma is a natural astaxantin-producing yeast as the main pigment of red orange. Astaxantin production from wild type *P. rhodozyma* <500 □g total carotenoids per gram DCW of yeast cells (Johnson & Schroeder, 1996). Production of total pigment from fusion protoplast recombinant of *P. rhodozyma* is approximately 1.2412 □g/g DCW. Cultivation of the yeast showed life cycle of *P. rhodozyma* is 24-80 hours at room temperature.

Cultivation of D. salina and pigment production

Cultivation of *D. salina* were performed on liquid media. Optimum growth of *D. salina* at temperature of 26°C under illumination and aeration treatment. The optimum growth of microalgae was at pH 7.2 and salinity 32 ppm. This result was in accordance with Borowitzka & Borowitzka (1988) and Orset & Young (2000) in showing that *D. salina* will accumulate high □-carotene under growth-limiting conditions such as illumination/irradiation, temperature and salinity.

Under limiting cultivation condition *D. salina* showing a life cycle of seven days. Cells on the bright green logarithmic phase and stationary phase changed into yellowish green color associated with the formation of carotenoids. The highest cell density reached on day 3 and began to decline on day 4. The highest total pigment production reached 111.16 □g /g or equivalent to 3.3 to 15.56 □ g/ g □-carotene.

Protoplast fusion D. salina and P. rhodozyma

The addition of certain chemical compounds such as PEG has stimulated incorporation of protoplast cells of *D. salina* and *P. rhodozyma*. Combined protoplast between *D. salina* and *P. rhodozyma* will form a new gene combinations. The research revealed that the fusion process had produce three types of recombinant consist of intraspecies recombinant fusion between *D. salina*, intraspecies recombinant fusion between *P. rhodozyma* and interspecies recombinant fusion of *D. salina* and *P. rhodozyma*. According to Santiago (1985) fusion process occurs by the existence of a close attachment mechanism between the protoplast membrane of touch, with the dissolution of the barrier in the form of a wall at the meeting point of the constituent parts of the cytoplasm and then mixed. Finally, two or more protoplasts will join the dots that contain two or more stem cell nucleus. Nucleus in karion may fuse before, during or after mitosis. According to two genetically different protoplast isolated from the somatic cells and are experimentally fused to obtain parasexual hybrid protoplasts. The hybrid protoplast contained heteroplasoic cytoplasm and two fused parent nuclei (Verma *et al.*, 2004).

Recombinant Fusion Protoplast Analysis

Microscopic observations of the recombinant fusion results show that fusion has produced organisms capable of performing ploidi more than one cell either in a single species or different species. Unification of more than one cell will often change the appearance of the shape of microscopic organisms. The appearance that often arises is recombinant

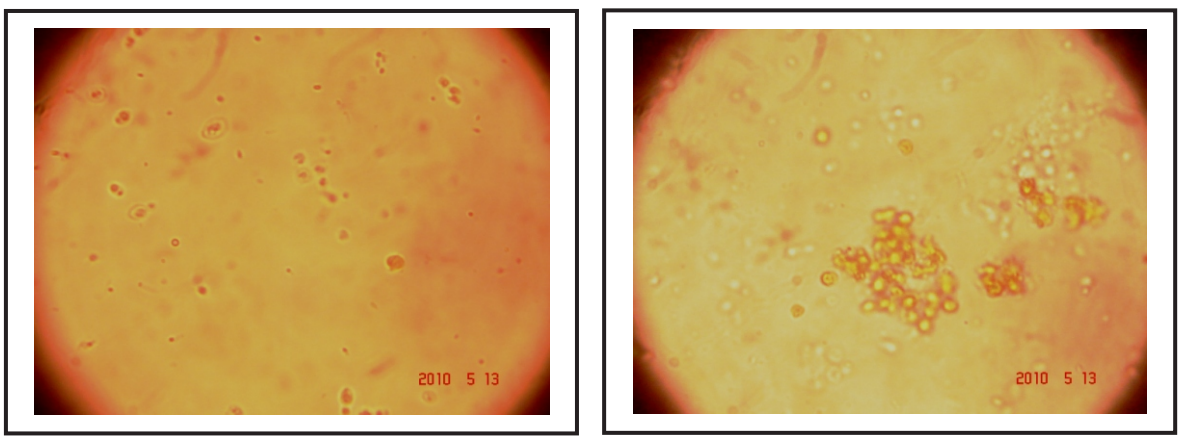

Figure 1. Poliploidi recombinant resulted from protoplast fusion process between D. salina and P. rhodozyma

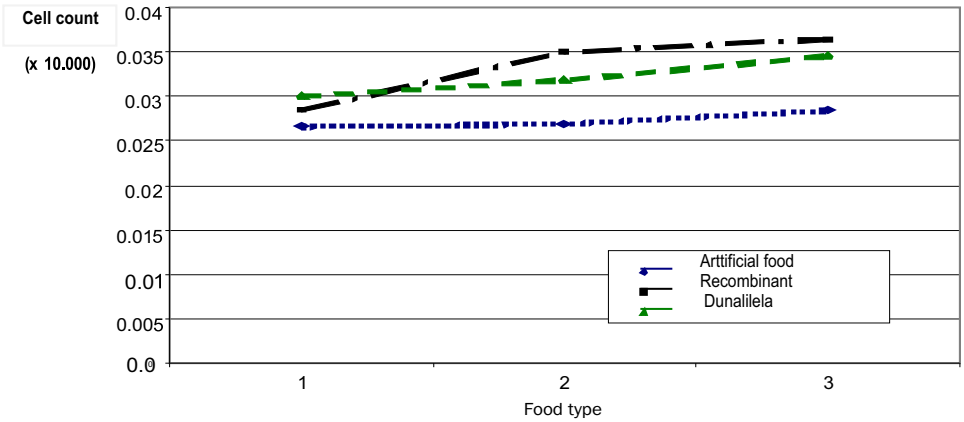

Figure 2. Weight gain of shrimp post-larvae after feeding with recombinant resulted from protoplast fusion comparing to artificial feed

poliploidi where one cell will have more than one cell mass and its contents. Recombinant poliploidi in *D. salina* and *P. rhodozyma* cells grew into a whole (as shown in Figure 1). If unification occurs in one species, the recombinant would have a better capability in terms of growth and secondary metabolite production. Protoplast fusion between *D. salina* and *P. rhodozyma* has produced cell and colony recombinant with different types such as recombinant form of *D. salina* but red like *P. rhodozyma*, recombinant form as *P. rhodozyma* but green like *D. salina*, recombinant shaped and colored like *P. rhodozyma* but able to move actively like *D. salina*, recombinant shaped and colored like a *D. salina* but non-motile as *P. rhodozyma*, shaped, colored and moving like a *D. salina* but able to live in freshwater, shaped, colored and non-motile as *P. rhodozyma* but able to live in salt water, shaped, colored, moving like *D. salina* and can live in freshwater but poliploidi, shaped, colored, moving like *P. rhodozyma* and can live in freshwater but poliploidi, and so forth. Various variations that appear indicated that they had gained a variety of types of recombinant favorable for use as a natural food source. Analysis of the obtained fusants usually limited to morphological description and measurement of metabolite production or enzym activity. The stabilities of fusants obtained were examined by successive subcultures (unpublished). The nature of character and metabolites combination in recombinant protoplast fusion can not be known for certain. Similarly, the percentage of the combined properties of both parents. Nevertheless, the parent which is more dominant in contributing in the offspring can be seen from the appearance of recombinants that arise. Furthermore, Panaiotov *et al.* (2009) found that analyses on the integrated genomic DNA in the cells due haploidization and non-disjunctional segregation of the heterozygous diploids can be done by amplified fragment length polymorphism (AFLP) because of the complexity and variability of fusant DNA.

D. salina and P. rhodozyma

Research results obtained a combination of two or more fusant cell nucleus between *D. salina* and *P. rhodozyma* that exhibited potency of protoplast fusion recombinant to produce combination of several carotenoids like □-carotene, zeaxantin and astaxantin better than their parent. The ability of recombinant to live in both fresh-water and saline water supported gaining of both characteristic from their parents. However, these recombinant characteristic still need to be analyzed in further research.

Application of recombinant food rich in carotenoids for Penaeus monodon Fab.

Artificial feed for shrimp (*P. monodon*) as ruled by SNI 02-2724-2002 is a mixture of various feed raw materials, formulated with the content of certain nutrients in the form of crumbs and pellets to increase the growth of shrimp monodon are cultured, and do not contain contaminants that can cause health problems, and safe for the environment. The growth rate of *P. monodon* was higher in smaller such as post larva stage, than in larger shrimp. This condition is relating to the higher molting frequency observed in postlarvae (Kibria, 1993). As we can see in Figure 2, natural diets such as *Dunaliella* gave the better growth and survival rate in the young post larvae comparing to artificial food. But the most interesting is the result which showing that their recombinant from fusion protoplast gave best growth.

As the shrimp grew, the compound diets such as combination among artificial and natural feed became better than natural diets. Astaxanthin cannot be synthesised by animals and must be provided in the diet as is the case with other carotenoids. Some species such as crustaceans have a limited capacity to convert closely related dietary carotenoids into astaxanthin, although feeding astaxanthin directly to shrimp rather than precursors results in better

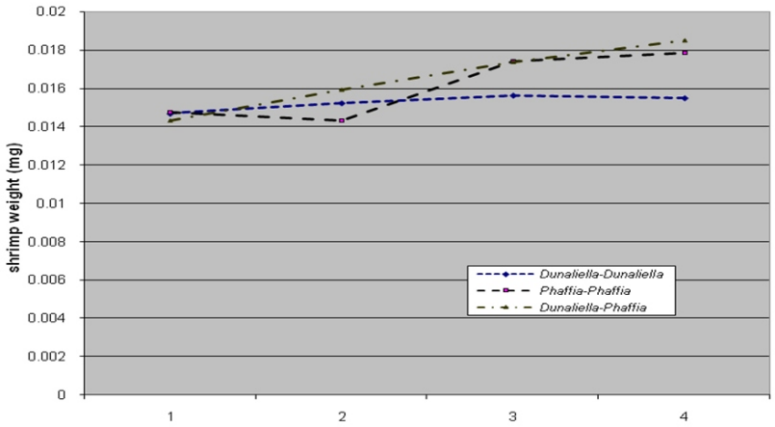

Figure 3. Weight gain of shrimp post-larvae after feeding with recombinant resulted from protoplast fusion of *D. salina* and *P. rhodozyma*

pigmentation due to conversion inefficiencies. Studies indicate that algal astaxanthin has a higher bio-efficacy than synthetic astaxanthin, especially when used in larval and postlarval shrimp feeds, resulting in improved survival (Aquaxan, 1999).

Feed diversification were applicated using various recombinant to the growth of shrimp larvae *P. monodon* Fab. Diversification in the manufacture of feed is done by adding artificial feed with each different type of recombinant fusion results from both intra and interspesies protoplast fusion of *D. salina* and *P. rhodozyma*. Aquaculture natural food were applicated by addition of artificial feed with recombinant fusion results intraspesies of *D. salina*, feed intraspesies of *P. rhodozyma* and inter-species feed microalgae *D. salina* and the yeast *P. rhodozyma*. Application of recombinant food rich in carotenoid for *P. monodon* Fab. was shown in Figure 3.

The research showed that shrimp weight measurement on the graph exhibited the overall weight of the shrimp tend to increase in the provision of various diversification feed protoplast fusion. The results obtained indicate superior food has gained rich carotenoids that can be used as a source of feed. In addition to the increased weight of shrimp, the feed also has been shown to improve survival of shrimp larvae.

The stability of growth is a very important stage in the development of superior natural food. The *in vitro* results form this research show that the natural food superior fusion results will breed independently when it is added in the waters, so that its availability will take place continuously without having to be added at certain times. *D. salina* and it's recombinant is an active photosynthetic microalgae so they will increase dissolved oxygen content in the waters. The pond waters which are rich in natural food from fusion results would help improve the fertility and quality of waters which will greatly support the growth of shrimp larvae.

Conclusion

Addition of artificial feed with natural food rich in carotenoids forming from recombinant fusion between *D. salina* and *P. rhodozyma* has improved increasing shrimp weight and survival compared to artificial feed and natural food only. They have stable growth in both freshwater and salt water, can breed naturally and are safe for animal aquaculture consumption and also the environment.

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