I J Biotech., June (2003) 627-633

# Improvement of astaxanthin production from *Phaffia rhodozyma* by protoplast fusion

**Hermin Pancasakti K*, Endang Kusdiyantini, Widjanarka**

1) Microbiogenetics Laboratory, Department of Biology, Mathematics and Natural Sciences Faculty, Diponegoro University, Semarang, INDONESIA

## *Abstract*

Protoplast fusion between intrastrain of *P. rhodozyma* has been generated in order to increase astaxanthin production. The best protoplast was obtained under osmotic stabilizer using buffer phosphate with the addition of 0.1 M $CaCl_2$. Higher frequency of fusion and regeneration were obtained under 8 mg/ml lysozyme and 35 % PEG 4000 treatment. Frequency of regeneration was 36 – 97%, frequency of fusion was 86.5 %. Several hybrids showed two-fold increase levels of ploidy, while yields of carotenoid pigments increased up to 2.03 times than the levels of wild type. Carotenoids accumulated to a level as high as 2. 529 μg /g dcw with the estimation of astaxanthin production reached the value of 488.75 μg/g dcw.

Keywords: astaxanthin – *Phaffia rhodozyma* – protoplast fusion

## Introduction

Carotenoids, some of which are pro-vitamin A, have a range of diverse biological function and action. Carotenoids are used commercially as food colorants; animal feed supplements and, more recently, as nutra-ceuticals for cosmetic and pharmaceutical purposes. The demand and market for carotenoids is anticipated to change drastically with the discovery that caro-tenoids exhibit significant anti-carcinogenic activity and play an important role in the prevention of chronic diseases (Lee and Schmidt-Dannert, 2002).

Carotenoids occur universally in photo-synthetic organisms but sporadically in non-photosynthetic bacteria and eukaryotes. Although animals do not synthesize caro-tenoids, they are known for their important role as light harvesting accessories pigments and in protecting against damage by singlet oxygen and preventing chronic diseases in humans. 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 crusta-ceans, animals and birds. Recent studies

Mailing address: Jl. Prof. Sudharto, Pedalangan Tembalang Semarang 50275 Telp. 62-24-7474754, Fax. 62-24-7460039,
*) Contact person: e-mail gandasakti@yahoo.com

have suggested that carotenoids have health benefit in humans and animals by preventing or delaying some chronic diseases including cancer, arteriosclerosis, cataracts, and other maladies (Iwasaki and Murakoshi, 1992).

Astaxanthin synthesis from *P.rhodozyma* offers a promising method for production of carotenoids because a primary contributing factor to the increased interest in natural carotenoids is the current trend of avoiding food additives and synthetic chemical in foods. Due to low quantities of astaxanthin in natural isolates of *P. rhodozyma*, strain improvement is needed in order to increase astaxanthin production. Protoplast fusion has been used to bypass natural barriers of intra- or inter- spesific hybridization because *P.rhodozyma* is a imperfect, polyploidy or aneuploidy and has pedogamic sexuality that it has not been possible to perform conventional crosses, mutagenesis and genetic analyses (Johnson and Schroeder, 1996). Inter-species hybrids significantly increased astaxanthin production up to 30% the levels of wild type (Chun *et al.*, 1992). So far, intra-species hybridization of protoplast fusion in *P.rhodozyma* has never been done yet, although it is easier in analyzing the hybrids than inter-species ones.

## Materials and methods

### Yeast strains and culture conditions

*Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*, Golubev) was obtained from BCCM (Belgian Co-Coordinated Collections of Microorganism). The yeast was maintained in YEPD medium (glucose 10 g/l, peptone 5 mg/l, yeast extract 3 g/l, agar 20 g/l). The yeast was grown at 30°C in 200 ml Erlenmeyer flasks containing 100 ml of medium under continuous shaking. Cells were harvested by centrifugation at 500 g for 10 minutes.

### Protoplast fusion (modified from Chun *et al.*, 1992)

Early growth phase cells (approx. $10^7$-$10^8$ cells/ml) were washed with distilled water followed by suspension in sodium acetate buffer containing 0.7 M $(NH_4)SO_4$ and 0.6 M KCl and 0.1 M 2-mercaptoethanol. The cells were treated with 2, 4, 8, 60 mg/ml of lysozyme for 2-3 h. The protoplast was mixed and kept in 0.1 M phosphate buffer (pH 6) containing 35% polyethylene glycol (Mr. 4000; Sigma) and 0.1 M $CaCl_2$ for 45 min. The process was followed by agar overlay method. After 3-4 days, colonies on YEPD agar plates were examined.

### DNA isolation techniques

Standard techniques for isolation of *P.rhodozyma* DNA (Sambrook *et al.*, 1989) were applied.

### Astaxanthin production assay

*Growth curve and pigment production.* Pigment production was done using YM broth (glucose 10 g/l, peptone 5 g/l, yeast extract 3 g/l, malt extract 3 g/l, pH 5). The media was autoclaved for 20 min, at 115 °C. *P. rhodozyma* was grown in 100 ml media in flasks, inoculated with 5% v/v starter ($10^7$-$10^8$ cell/ml) and incubated for 5 days at 28 °C using continuous shaking at 180 rpm (Kusdiyantini *et al.*, 2001). Growth and pigment production was analyzed during 12 hours of incubation.

*Growth analysis.* Growth analysis was done gravimetrically using 1.0 ml culture in micro centrifuge flasks after measuring the cell dry weight. The culture was centrifuged for 15 min, 4500 rpm (Vazques *et al.*, 1998). Pellet was dried using oven 80°C until reaching constant weight.

*Analysis of pigment production.* Total

pigment was extracted according to Sedmak *et al.* (1990). Analysis of total pigment was done using the method of An *et al.* (1989).

## Results and Discussion

### Formation of protoplast

Treatment of different osmotic buffer to the protoplast of *P. rhodozyma* has shown that the protoplast was highly sensitive to the osmotic support medium. Modification of Chun et *al.* methods (1992) in the formation of the protoplast using acetate buffer caused degradation of peptidoglycan cell wall by lysozyme. The buffer will enter to the cell and increase the cell size to spherical. However, acetate buffer cannot stabilize the extracellular medium optimally, due to burst of the cell. These maybe caused by harmful effects of acetate on several steps in cell metabolism and growth. According to Fowke and Constabel (185), 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 *P.rhodozyma* 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. We may therefore anticipate such condition with the use of phosphate buffer. The addition of $MgSO_4.7H_2O$ in the 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 (0.1 M $CaCl_2$), in addition to stabilizes the membranes of the cell under treatment of enzymes degradation, also increases the frequency of fusion.

To determine the optimum concentration of lysozyme, which allows the formation of protoplast, cells were incubated in different concentration of enzyme solution according to the reference (Fig. 1). After 48 hours incubation at 30°C, the colony emerged and demonstrated active growth in the regeneration medium, suggested that the protoplast had the ability to form the new cell wall and regenerate.

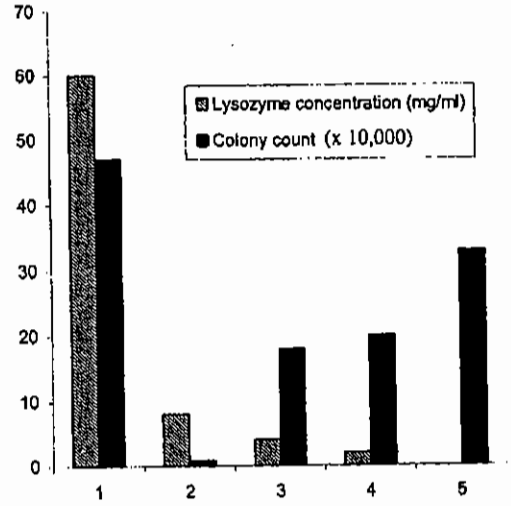


Figure 1. Effect of different lysozyme concentration on regeneration of protoplast (expressed as colony count)

The results demonstrated that the lower the lysozyme concentration, ranging from 2, 4, 8 mg/ml, the higher the number of *P. rhodozyma* colonies. High concentration of lysozyme (60 mg/ml) neither cause degradation of cell, nor affect the stability of protoplast. Protoplasts did not loose the ability to regenerate and grew well on the medium as compared to control without lysozyme treatment. Although treatment using 60-mg/ml lysozyme resulted in the highest number of colonies and frequency of regeneration, it did not result in a good hybrid according to DNA content and pigment production (Fig 2.).

### Protoplast fusion process

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. 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 neither be 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.

Experiment using 35 % PEG as fusogenic agents revealed that the fusion protoplast frequency reached $8{,}65.10^{-1}$. Data as shown on Table 1 was used to calculate the frequency of fusion based on the difference of colony count that grew from protoplast without fusion process or by adding PEG, compared to growing hybrid colonies on the disks. The protoplast fusion frequency obtained was:

$$\frac{(4410.\,10^2 - 296.10^2)}{(4410.\,10^2 + 296.10^2)} = 8{,}65.\,10^{-1}\ (86{,}5\ \%)$$

This indicates that almost all of the protoplast (86.5 %) were able to fuse under concentration of 35% PEG suggesting that PEG applied was optimal in inducing fusion. The observation was supported by microscopic features showing that hybrids tended to form a cell than to disperse as the parental cells, indicating that some hybrids had form a ploidy, as previously suggested by Chun *et al.* (1992) and Tjahjono *et al.* (1994).

Table 1. Colony count of hybrids on different concentration of lysozyme

| Concentration of lysozyme (mg/ml) | Colony count |
|---|---|
| 2 | $296.\,10^2$ |
| 4 | $411.\,10^2$ |
| Control without PEG | $4410.\,10^2$ |

**Protoplast regeneration**

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 2-4 incubation on 30 °C, some hybrids have been obtained. Regeneration of protoplast resulted in high frequency, reaching the value of 75% of all protoplasts that have been induced by PEG. Data on Table 2 was used to calculate regeneration frequency (Table 3), based on the different percentage of colony grew with addition of KCl compared with the number of colony grew without KCl. The addition of KCl was suggested to optimize the concentration osmotic stabilizer that allows the formation of osmotically-fragile cells.

Table 2. Colony counts of hybrids on different concentration of KCl

| | Lysozyme concentration (mg/ml) | | no lysozyme |
|---|---|---|---|
| | 60 | 8 | |
| Colony count with KCl | $47.10^4$ | $0.8.10^4$ | $33.10^4$ |
| Colony count without KCl | $0{,}6.10^4$ | $1{,}7.10^4$ | $4.10^4$ |

It was observed that the addition of KCl affected the cell ability in producing pigment. Some colonies of the fusant lost their red pigment and the color turned white. We presumed that it occurred through recombination and rearrangement of gene during kariogamy process.

Table 3. Frequency of regeneration of protoplast fusion amongst strain of *P.rhodozyma*

| Frequency of regeneration | Lysozyme concentration (mg/ml) | | Control without lysozyme |
|---|---|---|---|
| | 60 | 8 | |
| | 97 % | 36% | 78.3% |

**DNA content of *P. rhodozyma* and hybrids**

Several hybrids have been selected based on pigment color intensity. Analysis of DNA

contents was done to examine the ploidy characteristic of hybrids. The result as shown on Fig.2. revealed that some hybrids had a lower DNA content than their parents.

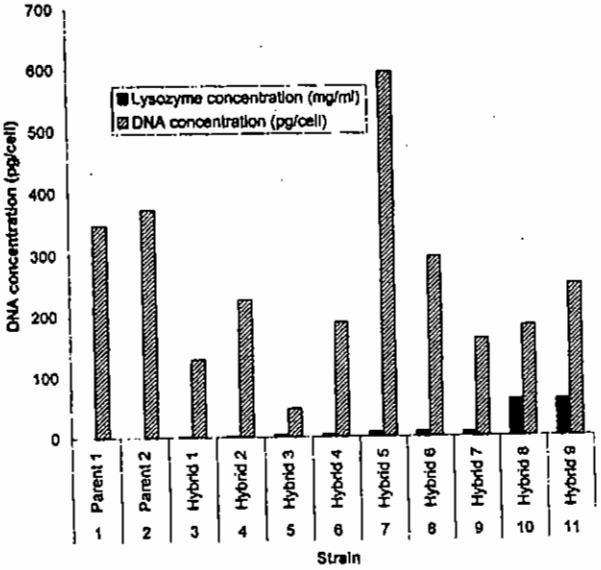


Figure 2. Effect of lysozyme treatment on DNA content of hybrids compare to parents

It was assumed that the lower DNA content maybe due to uncompleted recombination that decreased the ability of the hybrids to grow well on the medium. However, several hybrids had a higher DNA contents as compared to parents, especially hybrid 5, which had almost twice of DNA content, accompanied with high intensity of pigment color. This finding clearly indicates that some hybrids had successfully formed a ploidy as represented by a multiple concentration of DNA.

**Growth and pigment production**

Carotenoid analysis was done further to check pigment production in several hybrids, which had high DNA content. Three hybrids suitable for this analysis were hybrids 2, 4 and 5 compared to the parental strain. Analysis of pigment production was done on hybrids and parental strain in of *P. rhodozyma* cultures for 5 days using agitation at 180 rpm based on the growth curve of parental strain *P. rhodozyma* and hybrids in coconut water media as shown on Fig. 3. Energy for growth came from oxidation of glucose as carbon source and pigment was produced in the stationary phase.

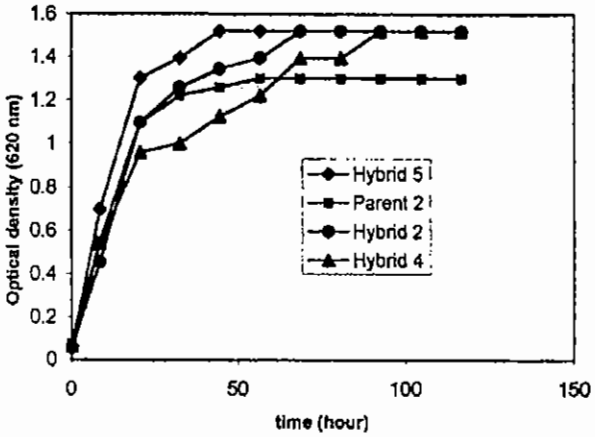


Figure 3. Growth curve of hybrids and parental strain of *P.rhodozyma*

The quantity of total pigment produced by the hybrids and parental strain of *P. rhodozyma* during stationary phase after 120 hours incubation is shown on Table 4. It was observed that optimum pigment production occurred during exponential to stationary phase.

Table 4. Pigment Production of *P.rhodozyma* and Hybrids

| No | Strain | Total Pigment (µg/g dcw) |
|---|---|---|
| 1. | Parent 2 | 1.2412 |
| 2. | Hybrid 2 | 0.8255 |
| 3. | Hybrid 4 | 1.6644 |
| 4. | Hybrid 5 | 2.529 |

Hybrid 5 showed the highest pigment production, in accordance with the DNA content (Fig. 2), which also showed the highest concentration. It can be concluded that hybrid 5 was diploid, based on carotenoid production, as compared to monoploidy of their parents. The hybrid 5

had 2.03 times higher of DNA content, due to the formation of diploid characteristic. In addition, hybrid 4 also exhibited almost the same characteristic pattern for DNA concentration as hybrid 5. This finding clearly indicates that the application of fusion protoplast techniques could improve *P.rhodozyma* ability in increasing pigment production. Based on these results, assuming that astaxanthin was the major carotenoid pigment in *P.rhodozyma* [comprises of 85 % of total carotenoid, Andrewes *et al.* (1976); Fang & Cheng (1993)] then it could be concluded that astaxanthin production of hybrid 5 was 203 % higher than the parental strain.

It was also observed that the optimum pH for increasing pigment production was pH 5.0. In addition, agitation on the cultures also provided good aeration for optimum production of pigment. Thus, oxygen availability was important in controlling growth rate and metabolite production.

## Acknowledgment

We thank Diponegoro University Semarang, in giving us opportunities and laboratory to do this research. This research was funded by Direktorat Jenderal Pendidikan Tinggi, Departemen Pendidikan Nasional Indonesia.

## References

Andrewes, A.G., Phaff, H.J. and Starr, M.O., 1976. Carotenoids of *Phaffia rhodozyma*, A red pigmented fermented Yeast. *Phytochemistry*, 15: 1003-1007

Calo P., de Miguel T., Velazquez, J.B., and Villa, T.G., 1995. Mevalonic acid increases trans-astaxanthin and carotenoid biosynthesis in *Phaffia rhodozyma*. *Biotechno. Lett.*, 6: 575 – 578.

Chun S.B., Chin J.E., Suk Bai G.H., 1992. Strain Improvement of *P. rhodozyma* by Protoplast Fusion. *FEMS Microbiol. Lett.*, 93: 221-226.

Fang, T.J., and Cheng, Y.S., 1993. Improvement of astaxanthin production by *Phaffia rhodozyma* through mutation and optimization of culture conditions. J. *Ferment and Bioeng.*, 6: 466 – 469.

Javadekar, V.S., SivaRaman, H., and Gokhale, D., 1995. Industrial yeast strain Improvement: construction of highly flocculent yeast with a killer character by protoplast fusion. *Indst. Microbiology*, 15: 94-102.

Iwasaki, R., and.Murakoshi, M., 1992. Palm oil yields carotene for world markets. *Inform.*, 2: 210-217.

Johnson, E.A., and Lewis, M.J., 1979. Astaxanthin formation by the yeast *P. rhodozyma*. J. *General Microbiology*. 115: 173-183.

_____, Villa, T.G., and Lewis, M.J., 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. *Aquaculture*. 20: 123 – 143.

_____, and An, G.H., 1991. Astaxanthin from microbial source. *Crit. Rev. in Biotechnology*. 4: 297 – 326.

_____, and Schroeder, W.A., 1996. Advances in Biochemical Engineering/ Biotechnology. In: A. Fiechter (ed.) Microbial Carotenoids. 141-145., Springer-Verlag, Berlin

Kusdiyantini, E. P., Goma, G.G., and Blanc, P., 1998a. Growth kinetics and astaxanthin production of *Phaffia rhodozyma* on glycerol as a carbon source during batch fermentation. *Biotechnol. Lett.*, 10: 929 – 934.

_____ E. 1998b. Contribution a l'etude de la croissance et de la production d'astaxanthine chez *Phaffia rhodozyma* PR 190 (Contribution to the Study of the Growth and Astaxanthin Production by *Phaffia rhodozyma* PR 190). PhD Thesis. Institut National des Sciences Appliqués de Toulouse, France, 149 p.

_____ E, Widjanarko, Zainuri, M., Triwibowo, Y., Joedoro, S., 2001 (*in press*). Produksi astaxanthin oleh *Phaffia rhodozyma* dengan menggunakan molase sebagai sumber karbon selama fed –

batch culture.

Kusumaningum, H.P., 1993. Fusi Protoplas antara Bakteri *Escherichia coli* dan *Bacillus subtilis*. Skripsi. Biologi FMIPA UNDIP. Semarang.

Meyer, P.S., Du Preez, J.C., and Kilian, S.G., 1993. Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. *World J. Microbiol. & Biotechnol.*, 9: 514-520.

Nagy, I.Z., Palagyi, D., Vagvolgyi, C., and Ferenczy, L., 1994. Genomic Comparison among Wild-type and Mutant Strains of *P. rhodozyma*. *FEMS Microbiology. Lett.* 123 : 315-318.

Okagbue, R.N., and Lewis, M.J., 1984. Use of alfalfa residual juice as a substrate for propagation of the red yeast *Phaffia rhodozyma*. *Appl. Microbiol. Biotechnol.*20: 33 -39

Sandmann, G., 2001. Genetic Manipulation of Carotenoid Biosynthesis : Strategies, problems and achievements. *TRENDS in Plant Science* . 1: 14–17

Torrissen, O. J., and Naeval, G., 1984. Pigmentation of salmonids-genetical variation in carotenoid deposition in rainbow trout. *Aquaculture*, 38: 59-66.

Verdoes, J., 1997. Molecular genetics of Carotenoid biosynthesis in yeast: Improved production of Natural Pigments. *Gene.* 184: 89-97

Wery, J., 1997. Genetics of the yeast *P.rhodozyma*. *WAU dissertation.* 2334 : 1-4

Wurtzel, E.T., Valdez, G., Matthews, P.D., 1997. Variation in Expression of Carotenoid genes in Transformed *E.coli* Strains. *Bioresearch Jour.* 1: 1-11

Zainuri, M., E. Kusdiyantini and Widjanarko, 2001 (in *press*). The utilization of *Phaffia rhodozyma* as pigment source of tiger shrimp *Penaeus monodon* Fabricius feed. *Jour. Coast. Dev.*