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Low-Dose Gamma Irradiation as Means of Isolating Carotenoid-Hyperproducing Yeast Mutant

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Abstract In order to isolate carotenoid-hyperproducing yeast, low-dose gamma irradiation was used as means of mutagenesis. *Phaffia rhodozyma* was treated by gamma irradiation of less than 10 kGy, which is considered to be a wholesome irradiation condition established by the Food and Drug Administration. Through repeated rounds of gamma irradiation and visual screening, mutant 3A4-8 was obtained. It produced a 3,824 μg carotenoid/g yeast, 69% higher content than 2,265 μg/g yeast of the unirradiated one. This result indicates that low-dose gamma irradiation could be used as means of mutagenesis to obtain carotenoid-hyperproducing strain of *Phaffia rhodozyma*, since only carotenoid-hyperproducing yeast survived gamma irradiation by scavenging oxygen radicals generated by radiolysis of water.

Key words: Carotenoid, γ-irradiation, mutant

Astaxanthin (3,3-dihydroxy-β-,β-carotene-4,4-dione) is not only an important pigment used in aquaculture industry, but also a strong antioxidant [3, 4, 7, 11, 14]. Astaxanthin is known to play a role in delaying or preventing degenerative diseases [1, 20]. *Phaffia rhodozyma* is the only yeast that produces astaxanthin, and there has been considerable interest in its commercial use [2, 13, 16, 20]. However, since wildtype strain produces only low quantity of astaxanthin [3, 20], there have been many studies on strain improvement, using antimycin, nitrosoguanidine (NTG), UV, H_2O_2, β-ionone, and duroquinone [4, 6, 9, 10, 18, 19, 20].

Astaxanthin has been known to protect *P. rhodozyma* against reactive singlet oxygen molecules generated in the natural environment [1, 18, 19]. Thus, a carotenoid-hyperproducing strain can survive longer than a wildtype strain under singlet oxygen-rich environment. Gamma-irradiation produces oxygen radicals generated by radiolysis of water [5, 17, 21] and can induce mutation of *P. rhodozyma* through a chromosomal rearrangement [13, 15]. Therefore, the objective of this study was to use γ-irradiation as a selection method of carotenoid-hyperproducing strain.

P. rhodozyma strains used were wildtype 67-385 and antimycin-NTG induced mutant 2A2N [1, 4]. Yeasts were grown as described elsewhere [1, 4, 18]. When *P. rhodozyma* was grown to log phase, the cells were harvested and re-suspended in distilled water and washed twice. The initial cell concentration of strain 67-385 and 2A2N were 5.3×10^7 and 4.4×10^7, respectively. The suspension was transferred to a sterile tube and irradiated at room temperature with 0, 1, 2, 3, 3.5, 4, 4.5, 5, 5.5, 6, and 7 kGy, using ^{60}Co gamma ray irradiator Type IR-79 (Nordion International Inc., Ontario, Canada) under air. Preliminary experiments were performed with up to 10 kGy. However, the yeast did not grow at 10 kGy. The irradiated cells were diluted with distilled water and plated on YM agar and incubated for 20

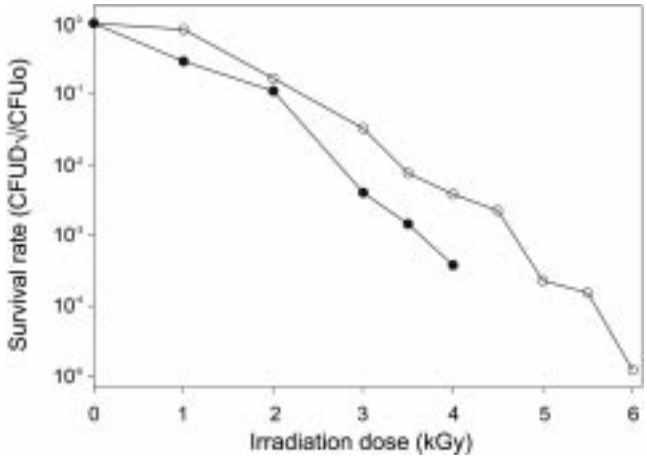

Fig. 1. Survival rate of *P. rhodozyma* by γ-irradiation.
CFUγ means colony-forming unit after irradiation and CFUo means colony-forming unit without irradiation. —●—, 67-385; —○—, 2A2N.

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Table 1. Carotenoid content of *P. rhodozyma.*

Strains	Carotenoid content (μg/g yeast)
67-385	428
2A2N	2,265
3A	2,668
3A4-8	3,824
3A5-3	2,866

days. Colonies grown on the plate were counted to determine survival rate. The number of colonies of strain 67-385 decreased more significantly with increasing irradiation dosage, compared with that of 2A2N (Fig. 1). Strain 2A2N survived up to 6 kGy irradiation, while strain 67-385 did not survive above 4.5 kGy. This result indicates that the mutant producing more carotenoid is more resistant to oxygen radicals than the wildtype strain by scavenging oxygen radicals. After the irradiation, the mutant 3A, having deep red pigment, was selected and irradiated again under the condition identical to the first irradiation. Two colonies having deep red pigment, 3A4-8 and 3A5-3, were finally selected and their carotenoid content was measured by the method described in the literature [1, 4].

The 3A4-8 and 3A5-3 mutants produced considerably more carotenoid content than the wildtype strain or the mutant 2A2N (Table 1). In particular, the mutant 3A4-8 produced the highest carotenoid content, 3,824 μg/g yeast, after 20 days of culture, compared with 2,265 μg/g yeast for strain 2A2N. The pattern of carotenoid production in mutant 3A4-8 during growth was different from that of strain 2A2N (Fig. 2). Production of carotenoid in strain 2A2N occurred during the early stationary phase and stopped after 15 days. However, mutant 3A4-8 still showed a significant increase in carotenoid production after 15 days, implying that mutant 3A4-8 kept on synthesizing caotenoid even after the growth of the mutant had ceased.

A primary function of carotenoid in *P. rhodozyma* is to protect cells from singlet oxygen, and it has been demonstrated to quench singlet oxygen [18]. Carotenoid content in the yeast depends on the level of intracellular activated oxygen species, which may induce or activate the enzymes involved in carotenoid biosynthesis [19]. Astaxanthin protects cells by removing reactive oxygen species and rapid synthesis of carotenoid through relief of end-product inhibition in the biosynthesis pathway [18]. Schroeder and Johnson [20] also suggested that singlet oxygen might induce carotenoid synthesis in *P. rhodozyma* by gene activation. Oxygen radicals have been known to cause changes in the molecular properties of proteins as well as enzyme activities [8, 12, 21]. Therefore, in this study, oxygen radicals generated by γ-irradiation might have modified the pathway in astaxanthin biosynthesis of *P. rhodozyma*, and increased carotenoid production of the mutant screened by γ-irradiation.

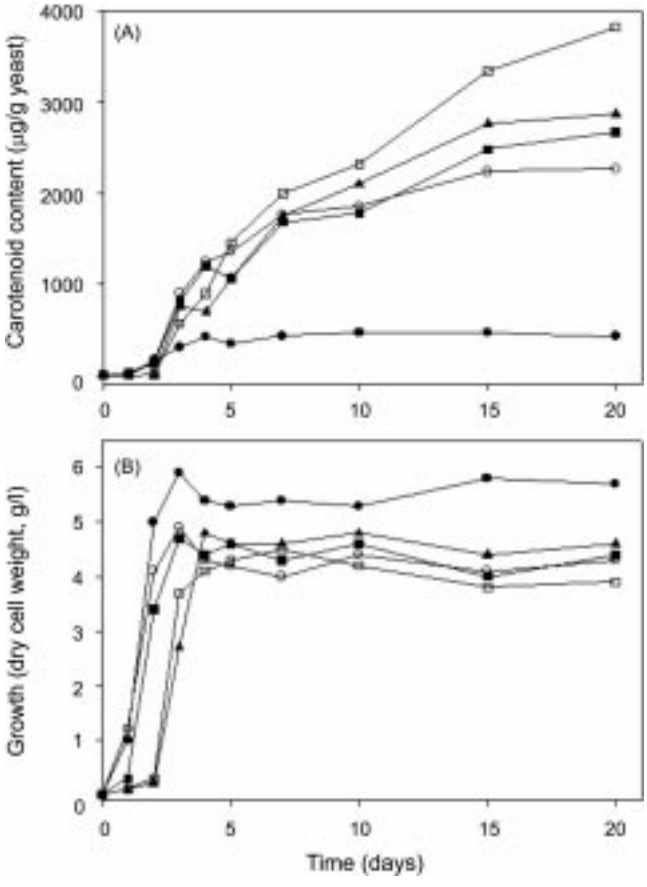

Fig. 2. Carotenoid content (A) and growth curve (B) of *P. rhodozyma*.
●, 67-385; ○, 2A2N; ■, 3A; □, 3A4-8; ▲, 3A5-3.

In conclusion, γ-irradiation could be an efficient method to obtain a carotenoid-hyperproducing strain of *P. rhodozyma* by providing a selection process due to oxygen radicals generated by γ-irradiation. The mutant 3A4-8, screened by consecutive low dose γ-irradiation, produced 69% more carotenoid than the parent strain. To elucidate the mechanism of carotenoid overproduction in the mutant, measurement of the activities of oxygen radical scavenging enzymes such as superoxide dismutase, catalase, and the enzymes involved in carotenoid biosynthesis are in need.

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