



Proceeding, First International Conference on Radiation Sciences and Applications

Enhancing the production of astaxanthin pigment by *Phaffia rhodozyma* strains using some physical and chemical agents

A. A. M. Shahin¹; M. Z. El-Fouly¹; H. M. Gebreel²; Kh. A. Youssef²; H. A. El-Bialy¹.

¹ Microbiology Dept. National Center for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt.

² Microbiology Dept. Faculty of Science, Ain Shams University, Cairo Egypt.

ABSTRACT

Only one out of thirteen pigmented yeast isolates locally isolated from different sources was able to produce total carotenoids higher than the standard strains of *Phaffia rhodozyma* ATTC-24202 & NRRL Y-10922 (symbolized A and Y respectively), but it could not able to synthesis astaxanthin pigment, while the astaxanthin percentages of the standard strains A&Y were 84.2 and 92.8% of the produced carotenoids in the same order. Accordingly the standard strains were used for further studies. Application of rose bengal and benomyl fungicide as a mutagenic agents could not create mutants producing carotenoids higher than the wild strains, while successive exposure of *Phaffia rhodozyma* A & Y strains to UV radiation for three cycles succeeded to create a variant able to produce the carotenoids three fold than the wild strain A. Dose level of 3 kGy of gamma radiation increased the carotenoids production from *Phaffia* A&Y strains by about 75 and 55 % respectively. A Rapsd-PCR technique showed that the increase or decrease in carotenoid production by *Phaffia* variants (hyper producer AU₉ selected after successive treatment by UV radiation or hypo-producer YG₁₅ obtained after exposure to 4.5 kGy dose of gamma radiation) was accompanied by genetic variation compared to standard *Phaffia* strains. Fungal elicitor prepared from *Fusarium graminearum* increased the carotenoid production by more than five folds and fungal elicitors prepared from *Aspergillus oryzae* and *Aspergillus flavus* enhanced the carotenoids production by more than three folds while fungal elicitor prepared from *Fusarium chlamydosporum* increased carotenoid production of both *Phaffia* strains A&Y by two folds.

KEYWORDS: Radiation Grafting; Hydrophilic Monomers; Dyeing Properties

INTRODUCTION

Many microorganisms produce carotenoids (alone or associated with another pigments especially chlorophyll) either naturally or under man-control. Astaxanthin (3, 3'- dihydroxy- 13-13-carotene- 4, 4'-dione) is a carotenoid pigment that provides the natural orange-red pigmentation of marine invertebrates (lobsters, crabs and shrimps), fish (salmon and trout) and birds (flamingoes)⁽¹⁾. Since, these animals are unable to synthesis astaxanthin, they have to incorporate it from their diets including shrimps shell, krill and pigmented algae⁽²⁾. In the aquaculture industry, fish feed must supplemented with astaxanthin to acquire the appropriate degree of color for consumer appeal and itself or its precursor carotenoids may also contribute to the distinctive flavor of baked salmon⁽³⁾. The US Food and Drug Administration permits astaxanthin use as a pigment for salmon fish up to a limit of 8 mg/kg finished feed⁽⁴⁾. In the past, astaxanthin was isolated from the carapaces of certain crustaceans, but this process is very expensive. The kilogram cost of chemically synthetic pigments is high, and it has been reported that their use in fish feed contributes approximately 10% of the cost of the feed⁽⁵⁾.

However, because of the increasingly strict regulations concerning the safety of chemicals as food additives and the poor adsorption of synthetic carotenoids compared with biological sources, natural

carotenoid products 'may be given preference over synthetic chemical pigments as color enhancers. Astaxanthin biosynthesis is limited to only a few species of microorganisms including the yeast *Phaffia rhodozyma*⁽⁶⁾, the microalgae *Haematococcus pluvialis*⁽⁷⁾ and the marine bacterium *Agrobacterium aurantiacum* and *Alcaligenes sp.* strain PC-i⁽⁸⁾. There are two main applications, for microbial astaxanthin, firstly aquaculture⁽⁹⁾ and poultry feed, since both egg production and yolk coloration are increased⁽¹⁰⁾ and secondly orthomolecular medicine.

The most important orthomolecular functions of astaxanthin, which serves as a provitamin A, tumorigenesis suppressor (i.e. reduced: incidence of cancers), enhancement of immune system⁽¹¹⁾ and active oxygen species scavenger and/or quencher⁽¹²⁾. Carotenoids are well recognized as being able to detoxify singlet oxygen and a single 13carotene molecule can detoxify 250-100' molecules of singlet oxygen. Carotenoids differ in their capacity to detoxify 102 lycopene and astaxanthin are more effective than 13-carotene⁽¹³⁾. Astaxanthin may have a role in delaying or preventing degenerative diseases in human and animals⁽¹⁴⁾.

Phaffia rhodozyma considered the most important astaxanthin producer. The natural habitat of the orange-red heterobasidiomyceteous yeast *P. rhodozyma* is slime fluxes of deciduous trees including birch, beech, alder, aspen and dogwood in mountainous regions of Japan, Pacific Northwest, and Alaska. It was originally designated *Rhodozyma rontanae* but its unusual characteristics and the lack of a latin description allowed the change of the genus to *Phaffia rhodozyma* in honor of Herman Jan Phaff and recognition of the lifelong contributions to the biology of yeasts⁽¹⁵⁾.

The aim of the present investigation was directed towards: **1)** isolation of microorganisms able to produce carotenoid pigment from different sources. **2)** selection of the hyper-carotenoid producers compared to two standard strains of *Phaffia rhodozyma* (ATCC-24202 & NRRL Y-10922). **3)** production of mutations by ionizing and non-ionizing radiation and chemical agents (illuminated photosensitizer or mutagenic fungicide). **4)** studying the interaction between the astaxanthin-producer yeast and other fungal elicitors on astaxanthin production. **5)** using a molecular method to differentiate between variants created by physical agents .

MATERIALS AND METHODS

Microorganisms:

Pigmented yeasts were isolated from different samples (Carrot, Grape, Kaka, Orange, Apple, Strawberry, Kinri and Pineapple juice) besides two standard strains of pigmented yeast *Phaffia rhodozyma* ; ATCC-24202 (Symbol A) were obtained from MERCEN-Faculty of Agriculture, Ain shams university, Cairo and NRRL-Y 10922 (Symbol Y, mutant produced by ultra-violet radiation) was kindly provided by Dr/Kurtzman, Microbial genomics and bioprocess research unit, National Center for Agricultural Utilization Research, USA.

Isolation of pigmented yeasts:

Samples:

Rotten fruits and vegetables were chosen to isolate the target microorganisms (yeast). They were apple, banana, carrot, grape, kaka, kiwi, lemon, orange, strawberry, tomato and watermelon. In addition, ten different samples of juices including apple, banana, carrot, grape, guava, mango, orange, pineapple, strawberry sugarcane, molasses (Beet origin), Potato washing-water, red beet syrup and tomato wastewater were collected from different sources in local markets.

Isolation process:

Pigmented yeasts were isolated from previous samples according to⁽²⁾.

Estimation of carotenoid production and growth yield for selected yeast strains:

Total carotenoids were extracted and measured by milligrams per liter while specific carotenoid concentration was measured by micrograms per one gram of yeast cell according to⁽⁵⁾.

HPLC analysis:

Astaxanthin was analysed by HPLC (Perkin Elmer apparatus located in Microanalytical center, Faculty of Science, Cairo University) using a stainless steel C₁₈ reversed phase column (3.9 mm x 150 mm). The mobile phase was a solvent of methanol: acetone:triethylamine (9:1 v/v) at 1 ml min⁻¹. Authentic astaxanthin (sigma) was used as external standard for comparison.

Modes for enhancing astaxanthin production by *Phaffia rhodozyma*:

Photo-activation by rose Bengal:

Cells of *Phaffia rhodozyma* strains were exposed to chemically generated ¹O₂ produced by photoactivation of rose bengal with 550 nm light from zero to ten μM⁽¹⁶⁾.

Mutagenesis by benomyl fungicide:

Phaffia rhodozyma cells which could tolerate the highest benomyl concentration (50 mg/ml) in the growth medium were cultivated on Yeast Malt agar medium (YM) containing this concentration to select hypercarotenoid producers

U.V. radiation:

Both *Phaffia rhodozyma* strains were irradiated with UV light from a germicidal lamp (254 nm) placed 11 cm above the surface of the liquid samples. Irradiated cells were sampled at appropriate times and plated on YM agar medium. All manipulations and the incubation were carried out in the dark to avoid photoactivation⁽¹⁷⁾. The selected yeast strains were estimated for growth and carotenoid production individually as mentioned before.

Gamma irradiation:

Yeast suspensions of *Phaffia* strains were irradiated at room temperature using Cobalt-60 model Russian gamma cell (Issledovate) located at the National Center for Radiation Research and Technology, Atomic Energy Authority of Egypt. The dose rate at the time of experiment was 4.519 kGy/hour and the total activity of the source was 16134 Ci. Different doses of gamma radiation (0, 1, 2, 3, 3.5, 4, 4.5, 5.5, 6, 7 kGy) were used.

Fungal elicitors:

A number of twenty-seven fungal isolates were grown in potato dextrose broth for 7 days at 30°C under shaking conditions (150 rpm). After broth cultures had been filtered, mycelial mats were resuspended in water and refiltered. A total of 40 grams (Fresh weight) of mycelial mats were homogenized in 0.1 M sodium acetate buffer (pH 6.5) for 10 min and then blended with 120 ml of ethylacetate in a mixture at room temperature overnight. Mixtures were filtered with a Whatman 1 filter and the filtrates containing lipids were discarded. The remains were collected and suspended in 100 ml deionized water. The pH of later suspensions was adjusted at 2 with 1 molar HCl and then they were autoclaved for one hour at 121°C. The autoclaved suspensions were filtered and the filtrates were adjusted to pH 5.8 with 0.5 M NaOH and used as fungal elicitors⁽¹⁸⁾. The carbohydrate content of fungal elicitors was determined by phenol-sulfuric acid method. Each fungal elicitor was added into the yeast proliferation media (Yeast malt broth) at a concentration 40 mg/l carbohydrate equivalent. All yeast malt broth media portions were sterilized by autoclaving for 20 min at 121°C. After inoculation of sterilized media by *Phaffia* yeast strains (A&Y), all flasks were incubated for five

days at 22°C. After the incubation period, the yeast growth and total carotenoids were determined as described before. The fungi that their elicitors achieved the best results were identified according to ⁽¹⁹⁾.

Effect of gamma irradiation on carotenoid pigment production

To study the effect of increasing doses of gamma radiation on the production of carotenoid pigment via the cell wall of *Phaffia rhodozyma*, cells of both *Phaffia* strains were exposed to gamma radiation at dose levels of (1, 2, 3, 4, 5 kGy) and cultured for 24 hour on broth synthetic medium.

Genetic analysis:

Phaffia rhodozyma variants obtained after exposure to ultraviolet and gamma radiations were genetically analysed using randomly amplified polymorphic DNAs (RAPDs-PCR) according to ⁽²⁰⁾.

Statistic analysis:

Statistic analysis was done according to ⁽²¹⁾.

RESULTS AND DISCUSSION

Isolation and Screening of pigmented yeast strains for growth and carotenoid production.

Rotten fruits and vegetables samples beside fermented juices were chosen for the selection of the pigmented isolates. According to morphological characteristics, a number of one hundred and thirty three yeast isolates were selected; thirteen of them were pigmented (Table 1). Similarly ⁽²²⁾ isolated spoilage yeasts from fermented pineapple juice and guava fruit nectar. The fresh products of fruits or vegetables will be covered by a complex mixture of bacteria, fungi, and yeast that are very characteristic for the product. He added that apple has a relatively large number of yeast and factors that caused injure or weaken the plant tissue may be expected to encourage microbial growth ⁽²³⁾.

The total of fifteen yeast strains, thirteen isolated from different samples (Symbolized C₁, C₂, G₁, K₁, K₂, K₃, K₄, O₁, P₁, S₁, S₂, W₁ & J₁) and two standard strains of *Phaffia rhodozyma* (A&Y) were assayed for carotenoids production. Also, the growth yield of tested yeasts was determined gravimetrically as cell dry weight.

Table (1): Screening of pigmented isolates and standard yeasts for growth and carotenoids production.

Samples	Symbol of pigmented yeast isolates	Growth yield (Dry weight) (g/l)	Total carotenoids (mg/l)	Carotenoid concentration (µg/g)
Carrot	C1	11.02*	2.36	214.81*
	C2	12.48*	2.57	205.94*
Grape	G1	13.24*	2.27	171.45*
Kaka	K1	11.24*	2.35	209.20*
	K2	12.14*	2.72	224.05*
	K3	10.94*	2.81	256.86*
	K4	13.07*	2.29	175.21*
Orange	O1	12.88*	2.35	182.45*
Apple	P1	12.16*	2.62	215.46*
Strawberry	S1	11.15*	4.09	366.82*
	S2	12.84*	2.62	204.05*
Kiwi	W1	16.31*	2.87	175.97*
Pineapple juice	J1	11.10*	2.31	208.11*
ATTC-24202	A	11.26	3.34	296.63
NRRL-Y-10922	Y	10.32	3.86	374.03

*The mean difference is significant at the 0.5 level.

It is clear from table (1) that the yeast strain (S₁) selected from rotten strawberry was the most hyper producing pigment among the screened strains either isolated or the standards. It produced 4.09 mg/l followed by the standard *Phaffia* strains A and Y which produced 3.34 and 3.86 mg/l respectively. The total carotenoid of the rest yeast strains was ranged from 2.27 to 2.87 mg/l. Data from table (1) also confirmed the lack of positive relationship between the production of carotenoids and growth yield of yeast strains. This result was in agreement with that of ⁽²⁴⁾ who observed that the carotenoid production retarded the yeast growth. ⁽²⁵⁾ explained the decrease in the final growth yield of *Phaffia rhodozyma* accomplished with increase in the astaxanthin productivity. They stated that, during the primary metabolism, the high initial concentration of carbon source is directed to amino acid synthesis (protein synthesis).

The carotenoid pigment produced by yeast strain S₁ and the biological astaxanthin produced by both standard strains of *Phaffia rhodozyma* (A&Y) were analyzed by HPLC. Unfortunately chart (1) proved that the carotenoid pigment produced by S₁ wasn't astaxanthin. Chart (1) also indicated that the percentage of biological astaxanthin produced by *Phaffia* strains (A&Y) were 84.2 and 92.8% respectively. Similarly, ⁽²⁶⁾ found that the yeast *Phaffia rhodozyma* produced astaxanthin as the major carotenoid and it represented more than 85% of the produced carotenoids. They added that the percentage of astaxanthin depends on the type of strain either wild or mutant. In addition, this chromatogram showed that the retention time of astaxanthin was at 6.913 minute. HPLC chromatogram of carotenoids extracted from *Phaffia rhodozyma* ATCC A-24224 cells by ⁽²⁷⁾ showed that astaxanthin was the principle carotenoid produced under illumination, astaxanthin accounted 83.4% of carotenoids and other carotenoids were 3-hydroxyechinenone (7.1%) and canthaxanthin (9.5%).

It is evident from the present experiment that the two standard strains of *Phaffia rhodozyma* were the highest astaxanthin pigment producer among the studied yeasts. It is also well known that astaxanthin is the most expensive pigment among the group of carotenoids (more than \$3000/Kg for stabilized dispersible powder containing 5-10% active ingredient). Also, it plays an important role in the aquaculture industry ⁽⁴⁾, poultry industry ⁽¹⁰⁾ and orthomolecular medicine including pro-vitamin A, tumor-inhibiting agents ⁽²⁸⁾ and singlet oxygen scavenger ⁽²⁹⁾. So, the two standard strains of *Phaffia rhodozyma* were chosen for further studies.

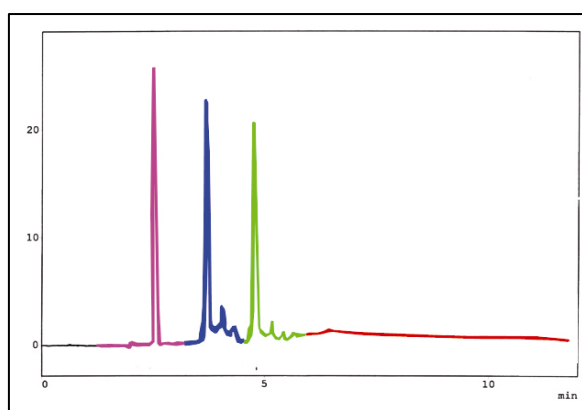


Chart (1): HPLC–biological astaxanthin profiles of intra-cellular extracts from *Phaffia rhodozyma* yeast strains grown in yeast malt broth medium compared with chemically synthesized astaxanthin.

Enhancing astaxanthin production by *Phaffia rhodozyma*

In the present study, several methods were used to increase the astaxanthin production by *Phaffia rhodozyma* i.e. induction of mutants or variants either by chemical or physical agents, using agro-industrial wastes as the main components of the fermentation media and optimization of culture conditions.

Induction of hyper-carotenoid producers by using chemicals

A. Rose bengal

Rose bengal (Disodium, 4, 5, 6, 7-tetra chloro-2, 3, 5, 7-tetrafluoresin) is a photoreactive pigment that used as efficient sensitizer in biological, medical and chemical studies⁽³⁰⁾. In this study, photo activation of rose bengal to generate singlet oxygen (1O_2) was used to enhance the production of astaxanthin by the selected *Phaffia* strains as a defense mechanism. Illumination of rose bengal with 550 nm light, produced singlet oxygen by photo catalyzed conversion of ground state (Triplet oxygen) to excited state (Singlet oxygen).

Figure (1) showed that the maximum growth yield of both *Phaffia rhodozyma* strains was achieved at 4 μ M concentration of rose bengal with illumination (550nm). At this concentration, *Phaffia* A and Y strains yielded 11.85 and 10.79 g/l as dry growth respectively compared with 11.12 and 10.20 g/l in medium free of rose bengal. After 4 μ M concentration of rose bengal, the growth yield of both strains decreased gradually. The role of singlet oxygen (Generated by photo oxidation of rose bengal) in retarding the radial growth of filamentous fungi is well known⁽³¹⁾. The decrease in both *Phaffia* growth after exposure to 6 μ M concentration of rose bengal or higher could be explained by⁽³²⁾ who demonstrated that the photosensitization of rose bengal produces either energy-transfer reactions leads to singlet oxygen generation specifically or a combination of energy-transfer and electron-transfer reactions, providing both singlet oxygen and reduced forms of oxygen such as super oxide anion and hydrogen peroxide. These free radicals react directly or indirectly with cell components and enhanced lethality.

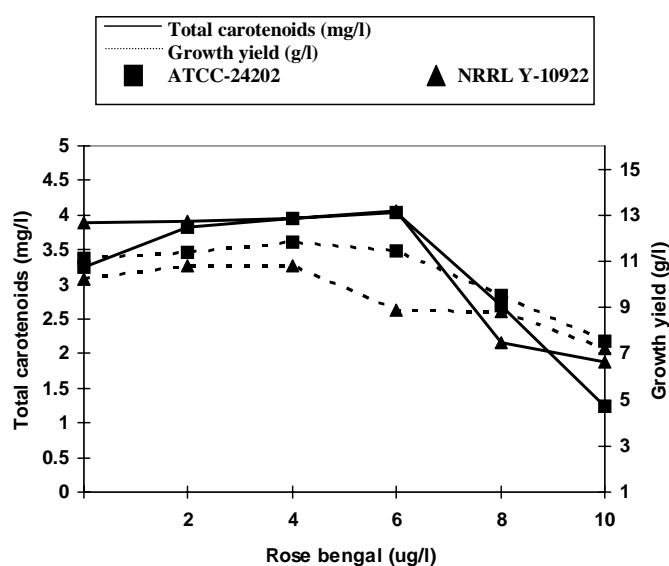


Figure (1): Effect of different concentrations of rose bengal on both *Phaffia rhodozyma* strains growth and carotenoids production.

Figure (1) also conducted that the different concentrations of rose bengal with illumination (550 nm) stimulated the carotenoid biosynthesis for both *Phaffia* strains until 6 μ M concentration. Nearly, four milligram per liter was the maximum total carotenoids produced by both *Phaffia* strains at this concentration of rose bengal although 6 μ M rose bengal decreased the growth of *Phaffia* Y strain. At the same trend,⁽¹⁶⁾ reported that the increase in the carotenoid pigment production by *P. rhodozyma* due to addition of rose bengal to the culture media did not usually associated with the increase in yeast growth.

Eight isolates derived from *Phaffia* A strain and seven isolates originated from *Phaffia* Y strain were selected visually (Deep orange-red color) from 6 μ M concentration of rose bengal and assayed individually for growth and carotenoids production, but unfortunately no successful hyper-carotenoid producers can be obtained by using photosensitization of rose bengal.

B. Benomyl

Benomyl [methyl-1-(butyl carbamoyl)-2-benzimidazole-carbamate] is a systemic fungicide that is selectively toxic to microorganisms and to invertebrates, especially earthworms. It is used against a wide range of fungal diseases of field crops, fruits, ornamentals and mushrooms⁽³³⁾. Benomyl showed a low acute toxicity to mammals that it has been impossible or practical to administer doses large enough to firmly establish a LD₅₀⁽³⁴⁾. Its mode of action on fungal cells was discussed by many investigators,^(35,36).

Figure (2) revealed that the growth of both *Phaffia* strains gradually decreased by increasing benomyl concentration up to 0.1 mg/l, thereafter sharply decreased and completely inhibited at 5 mg/l. Similarly,⁽³⁷⁾ inoculated different strains of *Phaffia rhodozyma* onto petri-dishes containing benomyl concentrations varying from 0.01 to 100 mg/l. They observed that *Phaffia* cells grew when the benomyl concentration was equal to or less than 1.0 mg/l. The effect of several fungicides including benomyl on some yeasts was investigated by⁽³⁸⁾. They found that *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* were the most resistant yeasts where *Rhodotorula glutinis* (Pigmented yeast) was the most susceptible. The adaptation of *Saccharomyces cerevisiae* to grow in the presence of antimitotic fungicide benomyl involves the dramatic activation at gene level especially transcription process (Protein synthesis)⁽³⁹⁾.

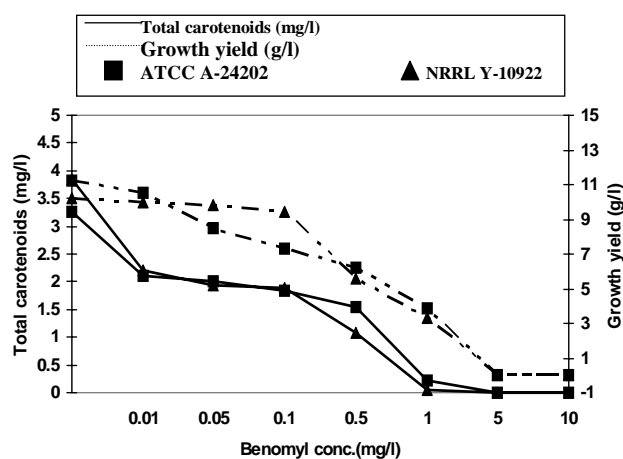


Figure (2): Effect of different concentrations of benomyl on *Phaffia rhodozyma* growth and carotenoids production.

As observed in fig. (2), the total carotenoids decreased sharply with increasing benomyl concentration and reached the minimum level 0.21 and 0.04 mg/l at 1.0 mg/l benomyl concentration for *Phaffia* A and Y strains respectively. The pattern of total carotenoids decrease for *Phaffia* A&Y strains were quite similar to growth yield decrease.

Induction of hyper-carotenoid producers by using physical agents:

A. Ultra Violet Radiation:

Ultraviolet radiation has been reported to suppress a number of photo chemical and photo biological processes in a wide variety of organisms. However, certain microorganisms when exposed to ultraviolet radiation in their natural environment have developed mechanisms to counteract the damaging effects of UV radiation especially on DNA by production of detoxifying enzymes or radical quenchers. Carotenoids are the important mechanism by which microorganisms prevent UV-induced damage⁽⁴⁰⁾. The fixation and maintenance in nature of genotype whose phenotype accumulates carotenoids is probably due to the protective action of carotenoids. The aim of the present experiment was directed towards isolation of *Phaffia rhodozyma* strains with significantly higher yield of astaxanthin⁽¹³⁾.

Figure (3) showed the effect of increasing doses of ultraviolet radiation on the viability of both *P. rhodozyma* strains when the cells were placed at distance 11cm below the source of radiation. The increasing doses of UV radiation decreased the total count of studied yeasts. The lethal dose for *Phaffia* A strain was

lower than that for *Phaffia* Y strain. The former strain showed no growth at dose equal to 6.833 KJ/m² where Y strain survived to dose 8.786 KJ/m². This difference could be attributed to the difference in origin between the two tested strains. After ten minutes of ultraviolet exposure (245 nm), the count of both *Phaffia* strains decreased by about one log cycle but after 20 minutes of exposure (3.905 KJ/m²), the count of *Phaffia* A strain decreased by 4.16 log cycles while the count of *Phaffia* Y strain decreased by 2.92 log cycles. At 4.393 KJ/m² dose of ultraviolet radiation (35 min), cells of *Phaffia* A strain were destroyed while *Phaffia* Y strain persisted up to 8.786 KJ/m² ultraviolet radiation dose (45 min). Similarly ⁽¹⁷⁾ demonstrated that the ultraviolet radiation effect on *Phaffia rhodozyma* viability depended on both culture growth stage and irradiated distance. There was no change in original total yeast count at 50 cm while the death rates reached 30-50% depending on the culture growth stage after five minutes at 11 cm distance.

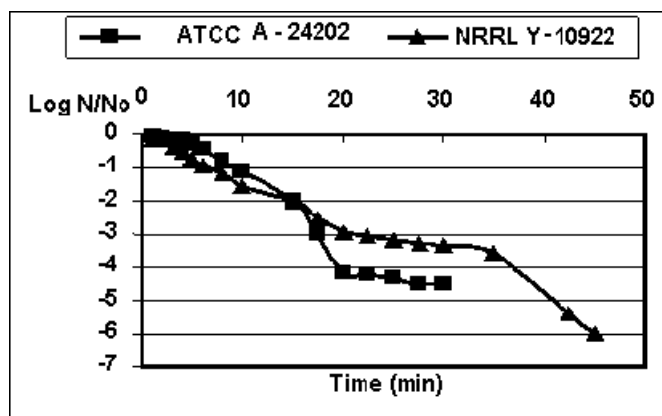


Figure (3): Effect of increasing doses of ultraviolet radiation on the viability of both *Phaffia rhodozyma* strains (ATCC A-24202 and NRRL Y-10922).

The decrease in survivor yeast count after UV radiation explained by ⁽⁴¹⁾ who reported that the oxidative damage to DNA is induced by UV-mediated photosensitizing process that mostly involved singlet oxygen together with smaller contribution of hydroxyl-radical-mediated reactions through initially generated superoxide radicals.

Majority of *Phaffia rhodozyma* cells of both strains, after exposed to UV radiation, did not change their phenotype (Their colors were orange-red as before exposure to UV radiation). On the other hand, few of them lost their colors completely (Bleached) or partially (The intensity of their colors decreased to pale orange, yellow or creamy) while the color of few others were increased clearly. Similarly ⁽⁴²⁾ isolated the unpigmented *Aspergillus fumigatus* mutant strain after UV-radiation.

To achieve the aim of this study (Selection of hyper-carotenoid producers), a total of twenty-six isolates from both *Phaffia* strains were selected at UV dose equal to 2.929 KJ/m² or higher by visual screening. Table (2) showed the growth yield and carotenoids production of twelve isolates-derived from *Phaffia* A strain. Results revealed that only two isolates (A₁ & A₅) showed a slight increase in growth yield compared to unirradiated *Phaffia* A strain, the increase did not exceed, 2%. All the rest of irradiated isolates showed a lower growth yield in comparison with the parent *Phaffia* A strain. Most UV-irradiated isolates also failed to produce higher carotenoids over than the parent *Phaffia* A strain except A₇ that produced 3.45 mg/l while the parent strain produced 3.37 mg/l only. Regarding to carotenoid concentration which depends on growth yield of tested yeast isolates, A₇, A₁₀, A₁₁ cleared up higher carotenoid concentration than unirradiated *Phaffia* A strain. It is interesting to mention that the increase in carotenoid concentration for A₇ & A₁₀ were attributed to decrease in growth yield while the increase for A₁₁ was only 5% compared to parent strain.

Data in table (2) also illustrated the carotenoids production and growth yield for fourteen irradiated isolates belong to *Phaffia* Y strain. All irradiated isolates failed to accomplish growth yield higher than or

equal to parent Y strain. Only Y₇ Y₈ and Y₁₁ expressed higher total carotenoids over unirradiated *Phaffia* Y strain. The increase in total carotenoids for these three irradiated isolates was only between 2-4%.

The UV radiation in generating pigmented mutants from *Phaffia rhodozyma*. Their attempts were not successful after screening of several thousand colonies following UV exposure. They found that the UV-generated mutants were very pale and reduced in astaxanthin content⁽⁴³⁾. Also, reported tha the ultraviolet mutagenesis was not effective to produce mutants with higher carotenoids content in *Phaffia rhodozyma* (UCD 67-3 85)⁽⁴⁴⁾.

Previous trials in this study failed to produce super hyper-carotenoid producers. So, the successive exposure to ultraviolet radiation was used in the following experiment. *Phaffia rhodozyma* cells of both strains (A&Y) were exposed to ultraviolet radiation (Dose = 3.417 KJ/m²) then cultured in broth medium for 48 hours. This step was repeated for three times before plating the irradiated yeast cells on yeast malt agar medium and incubated for 5 days at 22°C. The growing colonies were screened visually for hyper-carotenoid producers.

Table (2): Screening of ultraviolet irradiated isolates selected from *Phaffia rhodozyma* ATCC A-24202 for growth yield and carotenoids production.

Strain	Previous dose (KJ/m ²)	Selected yeast strains	Growth yield (Dry weight) (g/l)	Total carotenoids (mg/l)	Carotenoid concentration (µg/g)
ATCC A - 24202	0.000	A	11.41	3.37	295.35
	2.929	A1	11.63*	3.22	276.87*
		A2	10.26*	2.90	282.65*
		A3	11.37	3.17	278.80*
		A4	11.39	3.29	288.85*
	3.905	A5	11.46	2.75	239.97*
		A6	11.02*	3.14	284.94*
		A7	9.85*	3.45	350.25*
	5.857	A8	10.89*	2.65	243.34*
		A9	9.37*	2.56	273.21*
		A10	8.65*	2.98	344.51*
		A11	10.02*	3.12	311.38*
A12		11.32*	2.87	253.53*	
NRRL Y-10922	0.000	Y	10.28	3.85	374.51
	2.929	Y1	8.72*	3.35	384.17
	4.393	Y2	9.53*	3.70	388.25
		Y3	10.21	3.07	300.69*
		Y4	9.43*	2.90	307.53*
	7.322	Y5	10.01*	2.60	259.74*
		Y6	8.36*	3.71	443.78*
		Y7	9.45*	3.97	420.11*
		Y8	8.09*	4.00	494.44*
	8.786	Y9	8.31*	3.73	448.86*
		Y10	7.91*	2.96	374.21
		Y11	8.32*	3.94	473.56*
		Y12	10.06	2.81	279.32*
		Y13	8.65*	3.40	393.06
Y14		9.51*	3.52	370.14	

Results represented in table (3) cleared up that the successive exposure to UV radiation was a very successful procedure for the selection of hyper-carotenoid producing strains. Variant AU₉ that selected from *Phaffia* A strain produced 14.56 mg/l total carotenoids and its growth yield was nearly on the same level of unirradiated *Phaffia rhodozyma* A strain. So, its carotenoid concentration reached 1282.82 µg/g compared with 302.28 µg/g achieved by control. It is worth to mention that, since there was no decrease in growth yield,

the increase in carotenoid concentration for AU₉ irradiated isolate is a really increase. At the same trend, ⁽¹⁷⁾ recommended successive growth cycles between a liquid culture exposed to UV radiation and one which is unirradiated could enrich the population of highly pigmented cells allowing the selection of astaxanthin super-producers from *Phaffia rhodozyma*.

Table (3): Screening of hyper-carotenoid producers of both *Phaffia rhodozyma* strains (ATCC A-24202 & NRRL Y-10922) by successive ultraviolet irradiation

Strain	Selected yeast strains	Growth yield (g/l)	Total carotenoids (mg/l)	Carotenoid concentration (µg/g)
ATCC A-24202	A	11.38	3.44	302.28
	AU1	10.20*	1.78	174.51*
	AU2	11.46	3.37	294.07*
	AU3	12.24*	3.80	310.46*
	AU4	11.88*	3.22	271.04*
	AU5	10.96*	3.37	307.48*
	AU6	13.72*	4.89	356.41*
	AU7	11.34	2.18	192.24*
	AU8	11.74*	3.98	339.01*
	AU9	11.35	14.56	1282.82*
	AU10	12.04*	3.58	297.34*
AU11	12.20*	3.50	286.89*	
NRRL Y-10922	Y	10.25	3.83	373.66
	YU1	11.02*	3.25	294.92*
	YU2	11.14*	4.22	378.82*
	YU3	11.54*	3.38	292.89*
	YU4	11.31*	3.35	296.20*
	YU5	10.52*	4.42	420.15*
	YU6	11.83*	3.88	327.98*
	YU7	10.80*	2.98	275.93*
	YU8	11.26*	2.65	235.35*
	YU9	11.40*	0.73	64.04*
	YU10	10.38*	3.77	363.20*
YU11	12.14*	3.04	250.41*	

*The mean difference is significant at the 0.5 level.

Attempts for hyper-carotenoid producers from *Phaffia* Y strain were failed completely. The carotenoid production capability of irradiated isolate YU₉ was dropped off. It produced only 0.73 mg/l as total carotenoids while its growth yield was similar to parent *Phaffia* Y strain. The rest results of irradiated isolates belong to *Phaffia* Y strain were not interesting. This failure explained by ⁽⁴⁵⁾ who demonstrated that the variants are more susceptible to photosensitization than the wild type yeast (*Phaffia rhodozyma*) owing to low super oxide dimutase activity.

Recent studies on the production of carotenoids suggested that, in order to make the process of carotenoid production industrially feasible, yeast strain have to be stable ⁽⁴⁶⁾. The stability of AU₉ variant was studied over a period of six months.

B. Gamma Irradiation:

The two strains of *P. rhodozyma* were exposed to increasing doses of gamma irradiation. The result presented in figure (4) indicated that the dose response curves of both *Phaffia* strains were belonged to non-exponential dose survival curve that poses an initial shoulder, followed by exponential rate death. The increase in radiation dose after shoulder part was paralleled to the decrease in the number of survival yeast cells.

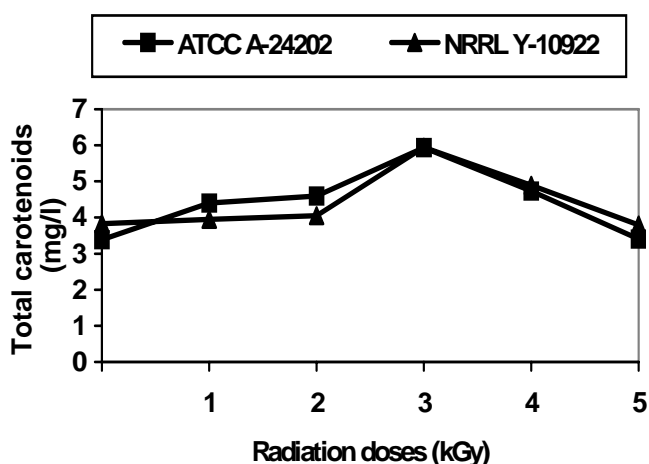


Figure (4): Gamma radiation dose response curves of both *Phaffia rhodozyma* strains (ATCC A-24202 and NRRL Y-10922).

Both tested *Phaffia* strains were able to survive to 5 kGy, it is clear that the decreasing rate of both strains are almost the same although the number of survivor in *Phaffia* A strain was higher than the number of survivor in *Phaffia* Y strain after 0.5 kGy. The D_{10} values for *Phaffia* (A&Y) strains were 0.87 and 0.9 kGy respectively. The relationship between gamma resistance (high D_{10} value) and carotenoids production by several yeasts was reviewed by ⁽⁴⁷⁾. The viability of wild strain of *P. rhodozyma* (67-385) decreased obviously in comparison with 2A2N strain (Mutant derived from antimycin treatment) when they were exposed to 3 kGy of gamma radiation. Also, they observed that the mutant 2A2N survived up 6 kGy, while the wild strain (67-385) didn't survive up 4.5 kGy. They added that the mutant produced five and half times more carotenoids than the wild type was more resistant to free radicals generated by gamma radiation ⁽¹⁴⁾.

From visual screening thousands of *P. rhodozyma* colonies survived gamma radiation, only twenty-nine isolates having a deep orange-red pigment compared to parent strains. These irradiated isolates were picked-up, purified and kept in slants until analysis. The growth yield and carotenoids production of gamma-irradiated isolates belong to *Phaffia* A&Y strains were studied individually and recorded in table (4). All gamma-irradiated isolates belongs to *Phaffia* A strain attained a lower growth yield compared to control (unirradiated parent strain). On the other hand, all gamma-irradiated isolates derived from *Phaffia* Y strain except one (YG₁₄) exhibited a higher growth yield compared to parent strain. The maximum increase in growth yield (Nearly 24%) was achieved by YG₉ isolate irradiated previously by 3 kGy. All previous results confirmed the haphazard action of gamma irradiation ⁽⁴⁸⁾. The *P. rhodozyma* mutants isolated by gamma irradiation had a different growth pattern compared to parent strain. They explained the change in growth yield between the irradiated isolates and unirradiated parent strain by the direct effect of gamma radiation on the protein synthesis ⁽¹⁴⁾. Gamma-irradiated isolates AG₄, AG₅, AG₁₀, AG₁₁, AG₁₃ & YG₁₁ achieved a high carotenoid productivity were selected from 1, 4 and 4.5 kGy. Gamma irradiated isolates selected after exposure to 0.5 kGy were not able to produce the same level of carotenoids obtained by the former isolates. This confirmed the phenomena of ⁽⁴⁹⁾ who reported that the hypersensitivity at low doses of gamma radiation was not found in yeast cells.

Table (4): Screening of gamma irradiated isolates selected from *Phaffia rhodozyma* ATCC A-24202 & NRRL Y-10922 for growth yield and carotenoids production.

Strain	Previous dose (kGy)	Irradiated isolates	Growth yield (Dry weight) (g/l)	Total carotenoids (mg/l)	Carotenoid concentration (µg/g)
ATCC A-24202	0.0	A	11.26	3.38	300.18
	0.5	AG1	10.65*	3.80	356.81*
		AG2	9.54*	3.55	372.12*
		AG3	10.43*	4.03	386.39*
	1.0	AG4	10.06*	4.45	442.35*
		AG5	10.05*	4.48	445.77*
	2.0	AG6	9.73*	3.98	409.04*
		AG7	10.55*	3.40	322.27*
	3.0	AG8	10.45*	3.83	366.51*
		AG9	10.49*	3.49	332.70*
	4.0	AG10	10.32*	4.74	459.30*
		AG11	9.82*	4.46	454.18*
		AG12	10.59*	3.47	327.67*
	4.5	AG13	10.41*	4.61	442.84*
		AG14	10.20*	3.05	299.02*
5.0	AG15	10.47*	3.04	290.35*	
NRRL Y-10922	0.0	Y	10.26	3.85	375.24
	0.5	YG1	11.38*	2.56	224.97*
		YG2	11.48*	3.13	272.65*
	1.0	YG3	11.95*	2.71	226.78*
		YG4	11.40*	3.05	267.54*
	2.0	YG5	11.18*	3.50	313.06*
		YG6	12.24*	2.66	217.32*
		YG7	11.76*	3.44	292.52*
	3.0	YG8	11.89*	3.74	314.55*
		YG9	12.72*	2.81	220.91*
	4.0	YG10	11.08*	1.60	144.40*
		YG11	11.76*	4.74	403.06*
		YG12	10.98*	3.76	342.44*
	4.5	YG13	11.31*	2.86	252.87*
YG14		10.21*	2.87	281.10*	

*The mean difference is significant at the 0.5 level.

Fungal elicitors

Results in tables (5) showed that fungal elicitors had different effects on *Phaffia* proliferation and its carotenoid production. For *Phaffia* A strain, only seven fungal elicitors had a negative effect on the growth, whereas the rest of fungal elicitors (Twenty) slightly enhanced the growth. Similarly, the majority of tested fungal elicitors enhanced the growth of *Phaffia* Y strain but the enhancement level was less than that observed for *Phaffia* A strain.

Regarding to carotenoids production, five fungal elicitors achieved a huge increment for both *Phaffia* strains. Fungal elicitor (F₄) increased the total carotenoids by 670 and 531% for *Phaffia* A and Y strains respectively (i.e. 6.7 and 5.3 folds over than the control), followed by F₂₁ (470 and 397) %, F₂₀ (443 and 368) %, F₅ (260 and 215)% and F₆ (133 and 113)% respectively. Fungal elicitors; F₄, F₂₁, F₂₀, F₅ and F₆ were identified as *Fusarium graminearum*, *Aspergillus flavus*, *Aspergillus oryzae*, *Fusarium chlamydosporum* and *Paecilomyces varrotii* in the same order. The data in tables (5) also indicated that another four fungal elicitors out of the rest (22), increased the total carotenoids by different levels (Ranged from 8% to 80%); 13 of the

tested fungal elicitors gave negative results for pigment production and the percentage of decrease was ranged between 8 and 80% and the rest fungal elicitors had no effect on carotenoid production by both tested *Phaffia* strains. It worth to mention that, all the fungal elicitors have the same positive, negative or neutral effect on carotenoid production by both *Phaffia* strains but in different level, this revealed the same chemical effect was stressed on the *Phaffia* metabolism. At the same trend, ^(50, 51) found that the contaminant of the fungus *Epicoccum nigrum* affected carotenogenesis in various strains of *Phaffia*. The astaxanthin and total carotenoids yields were increased by nearly 40% in the wild *Phaffia rhodozyma* UCD 67-385, and they examined the effect of six fungal elicitors prepared from *Rhodotorula rubra*, *R. glutinis*, *Panus conchatus*, *Coriolus versicolor*, *Mucor mucedo* and *Mortiealla alpina* on the growth and total astaxanthin produced by *Xanthophyllomyces dendrorhous*. They found that *M. mucedo* elicitor at concentration 30mg/l promoted the biomass and the total carotenoids by nearly 70 and 78% in compared with control. Similarly, ⁽⁵²⁾ reported that the flavonoid pigment was increased in cell cultures of *Ruta graveolens* L. after treatment with fungal elicitors.

Table (5): Effect of different fungal elicitors on proliferation of *Phaffia rhodozyma* ATCC A-24202 and NRRL Y-10922 and their carotenoids production.

Fungal elicitors	Growth yield (Dry weight) (g/l)		Total carotenoids (mg/l)		Change in Total carotenoids (%)		Carotenoid concentration (µg/g)	
	(A)	(Y)	(A)	(Y)	(A)	(Y)	(A)	(Y)
Control	11.30	10.35	3.37	3.88	-	-	298.23	374.88
F1	11.94*	10.56*	0.64	0.49	-81.01	-87.37	53.60*	46.40*
F2	11.76*	10.71*	3.10	3.07	-8.01	-20.88	263.61*	286.65*
F3	12.16*	11.21*	0.95	1.25	-71.81	-67.78	78.13*	111.51*
F4	12.14*	11.08*	25.98	24.49	+670.92	+531.19	2140.03*	2210.29*
F5	11.82*	10.81*	12.13	12.22	+259.94	+214.95	1026.23*	1130.43*
F6	12.10*	11.15*	7.85	8.28	+132.94	+113.40	648.76*	742.60*
F7	11.60*	10.50*	3.10	3.44	-8.01	-11.34	257.24*	327.62*
F8	11.08*	10.27*	3.40	3.82	+0.89	-1.55	306.86	371.96
F9	11.84*	10.58*	2.54	2.44	-24.63	-37.11	214.53*	230.62*
F10	12.10*	10.52*	3.37	3.87	0	-0.26	278.51*	367.87
F11	11.78*	10.35	2.75	2.87	-18.40	-26.03	233.45*	277.29*
F12	11.34*	10.32	1.48	1.25	-56.08	-67.78	130.51*	121.12*
F13	11.58*	10.68*	2.56	2.45	-24.04	-36.86	221.07*	229.40*
F14	11.20*	10.38*	4.57	4.52	+35.61	+16.49	408.04*	435.45*
F15	11.36*	10.48*	3.79	4.21	+12.46	+8.51	333.63*	401.72*
F16	11.36*	10.35	1.95	2.95	-42.14	-23.97	171.65*	285.02*
F17	11.20*	10.61*	3.37	3.79	0	-2.32	300.89	357.21*
F18	11.64*	10.72*	4.43	4.57	+31.45	+17.78	380.58*	426.31*
F19	10.94*	10.00*	3.46	3.92	+2.67	+1.03	316.27*	392.00*
F20	11.16*	11.18*	18.33	18.18	+443.92	+368.56	1642.47*	1626.12*
F21	11.44*	10.35	19.23	19.31	+470.62	+397.68	1680.94*	1865.70*
F22	11.82*	10.48*	1.99	2.02	-40.95	-47.94	168.36*	192.758
F23	10.26*	10.16*	2.21	2.20	-34.42	-43.30	215.40*	216.54*
F24	11.98*	10.41*	N.D	N.D	-	-	-	-
F25	11.16*	10.38	6.21	6.20	+84.27	+59.79	556.45*	597.30*
F26	11.36*	10.52*	2.95	3.07	-12.46	-20.88	259.68*	291.83*
F27	11.32	10.61*	1.97	2.08	-41.54	-46.39	174.03*	196.04*

*The mean difference is significant at the 0.5 level.

In the present study, five fungal elicitors could enhance the carotenoid production by *Phaffia rhodozyma* vigorously, but the mechanism by which fungal elicitors stimulated carotenoid biosynthesis isn't known completely and need more studies. The yeast/fungus interaction in vivo to explain the stimulation of

astaxanthin synthesis in *Phaffia* was discussed by ^(50, 53, 51). They recorded that the component of elicitors prepared from fungal cell wall were very complicated including proteins, polysaccharides, glycoproteins, peptides, oligosaccharides and lipids, and they mentioned that these compounds could stimulate the growth or carotenogenesis of *Phaffia*.

Using gamma radiation as an alternative method to facilitate carotenoid pigment extraction.

Gamma radiation is known to exert different damaging effects on microorganisms; the damage of cell wall is one of them. The majority of carotenoid-containing yeasts belong to basidiomycetes and their cell walls are thought to be considerably more complex than other yeasts ⁽⁵⁴⁾. Because cell wall is believed to be the barrier which prevents the extraction of carotenoids, it is necessary to weak this barrier to allow solvent penetration and consequent carotenoid extraction. This hypothesis is supported by the fact that sphaeroplasts of yeast or mechanically disrupted cells are amenable to carotenoid extraction. This experiment was conducted to study the use of gamma radiation as a mode for pigment extraction and identify the suitable dose at which the maximum total carotenoids can be extracted without being damaged by radiation.

Figure (5) indicated that the extracted total carotenoids increased with increasing gamma radiation doses until 3kGy for both strains, then decreased and nearly reached the original level (Unirradiated cells) after exposure to 5 kGy. At 4kGy, the increase in total carotenoids percentage decreased by half compared to the increase at 3 kGy for both *Phaffia* strains. After this dose, there was a decrease in extracted carotenoid indicating the destruction of pigment at higher gamma radiation doses.

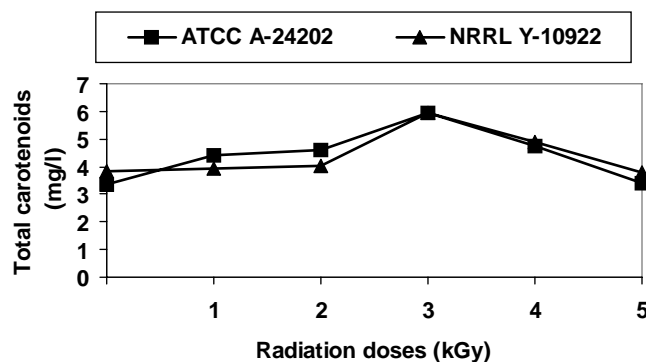


Figure (5): Effect of increasing doses of gamma radiation on carotenoid extraction by both strains of *Phaffia rhodozyma*.

Although, the use of gamma radiation to facilitate pigment extraction from microbial cells was not mentioned before, the total carotenoids levels in fresh raw coriander leaves remained unaffected by exposure to 1 kGy dose ⁽⁵⁵⁾. Also, they added that 1 kGy enhanced carotenoid extractability. Since, the dimethylsulphoxide caused the rupture of *P. rhodozyma* cell wall, increasing the amount of extracted astaxanthin from both *Phaffia* strains obtained after exposure to gamma radiation could be attained to break the binding of carotenoid pigment with another cell constituents as reviewed before ⁽⁵⁶⁾.

Differentiation between *Phaffia* variants induced by radiation treatment using RAPDs-PCR fingerprinting technique.

In the present study, successive ultraviolet radiation has been used to create carotenoid hyper producer strain (AU₉) that produce more than 1200 µg/g of dry yeast. However, genetic instability is a major drawback of using mutagenesis to create mutants. This over producer strain was kept for six months before its efficiency to produce astaxanthin was reexamined. It was found that the productivity of AU₉ (*Phaffia rhodozyma* variant) is as it is after the previous maintaining time. To exclude this waste time, a satisfactory mean to reliably distinguish between the different variants of *Phaffia* should be developed.

This study proposed to develop a rapid and reliable discriminatory method based on the use of molecular tools. Polymerase chain reaction PCR-based fingerprinting is therefore simple, rapid and can be

performed with very small quantities of microbial culture. Restriction fragment length polymorphisms (RFLPs) have been used extensively for detection and exploitation of naturally occurring DNA sequence variation during genetic studies. Randomly amplified polymorphic DNAs (RAPDs) has provided a quick alternative to RFLPs which doesn't necessitate the use of radioactivity and requires much smaller amounts of DNA ⁽²⁰⁾.

Chart (2) showed that the amplified DNA fragments of genomic DNA of both *Phaffia rhodozyma* standard strains (ATCC-24202 and NRRL Y-10922) as well as two variant strains previously exposed to radiation either ultraviolet radiation (hyperproducer AU₉) or gamma radiation (hypoproducer YG₁₅), a total 4 major bands were visible as well as some faint bands and ranged in size from 0.4 to 1.0 kb. *Phaffia rhodozyma* standard strains produced four DNA fragments [Chart (2) Lane (1&3)], approximately 0.4, 0.5, 0.9 and 1.0 kb in size. Lane (2) represented AU₉ variant that obtained after three cycles of ultraviolet radiation, showed a new 0.45 kb fragment but the 0.5 kb fragment was disappeared. The difference in RAPD fragments detected by using a single primer indicated the variant AU₉ was exposed to particular mutagenesis. This variant was characterized by an increasing in astaxanthin content (Up to 1200 µg/g) compared to about 300 µg/g produced by parent *Phaffia* A strain. This extraordinary band may be overexpression of one of cluster genes responsible for carotenogenesis in *Phaffia*. This suggestion was confirmed by [Chart (2) Lane (4)] that represented YG₁₅ which previously exposed to gamma radiation, lost the ability to produce pigment (Albino variant) and didn't yield any visible bands on amplification. i.e. the fortuitous primer may be specific to one of astaxanthin synthesis genes. Similarly, ⁽²⁰⁾ examined the DNA fingerprints of different *Phaffia rhodozyma* strains obtained by NTG mutagenesis. They observed 3 and 5 major DNA bands ranged in size from 0.3 to 2 kb, two fragments of 0.95 kb and 0.68 kb occurred in the parental strain and all mutants and the 1.17 kb fragment was present only in mutant M₄ (hyperproducer) which obtained after the first cycle of NTG mutation.

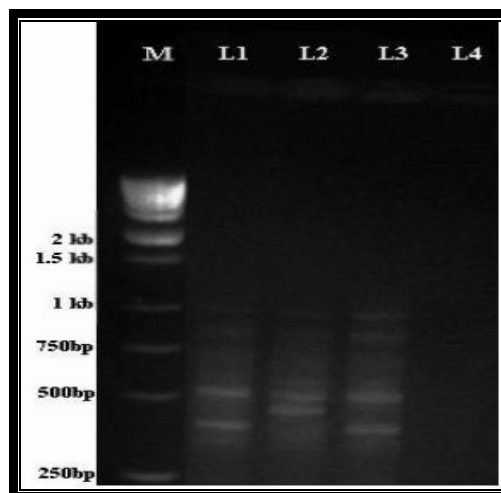


Chart (2): Amplified DNA fragments producing using the RAPD amplification with an arbitrary primer and genomic DNA isolated from different *Phaffia rhodozyma* strains. Lane 1, *Phaffia* A strain; Lane 2, *Phaffia* AU9 variant; Lane 3, *Phaffia* Y strain & Lane 4, *Phaffia* YG15 variant.

In conclusion, RAPDs could be a useful method for fingerprinting variants used in commercial astaxanthin production.

REFERENCES

- 1-Christophersen, C.; Anthoni, V.; Nielsen, P.H.; Jacobsen, N. and Tendal, O.S. (1989). Source of a nauseating stench from the marine sponge, *Halichondria Panicea*, collected at Clever Bank in the North sea. *Biochemical Systematics and Ecology*, 17(6): 459-461.

- 2-Fontana, J.D.; Guimarães, M.F.; Martins, N.T.; Fontana, C.A. and Baron, M. (1996). Culture of the astaxanthinogenic yeast *Phaffia rhodozyma* in low-cost media. *Applied Biochemistry and Biotechnology*, 57/58, 413-422.
- 3-Johnson, E.A. and Schroeder, W.A. (1995). Microbial carotenoids. *Advances in Biochemical Engineering Biotechnology*, 53, 119-178.
- 4-Vázquez, M. and Martín, A.M. (1998). Optimization of *Phaffia rhodozyma* continuous culture through response surface methodology. *Biotechnology and Bioengineering*, 57(3): 314-320.
- 5-Bon, J.A.; Leathers, T.D. and Jayaswal, R.K. (1997). Isolation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. *Biotechnology Letters*, 19(2): 109-112.
- 6-Flores-Cotera, L.B.; Martín, R. and Sanchez, S. (2001). Citrate, a possible precursor of astaxanthin in *Phaffia rhodozyma*: influence of varying levels of ammonium, phosphate and citrate in a chemically defined medium. *Applied Microbiology and Biotechnology*, 55(3): 341-347.
- 7-Kakizono, T.; Kobayashi, M. and Nagai, S. (1992). Effect of carbon/nitrogen ratio on the ecosystem accompanied with astaxanthin formation in a green algae, *Haematococcus pluvialis*. *Journal of Fermentation and Bioengineering*, 74, 403-405.
- 8-Yokoyama, A.; Izumida, H. and Miki, W. (1994). Production of astaxanthin and 4-ketozeaxanthin by a marine bacterium, *Agrobacterium aurantiacum*. *Bioscience Biotechnology and Biochemistry*, 58, 1842-1844.
- 9-Storebakken, T. and No, K.H. (1992). Pigmentation of rainbow trout. *Aquaculture*, 100, 209-229.
- 10-Gu, W.L.; An, G.H. and Johnson, E.A. (1997). Ethanol increase carotenoid production in *Phaffia rhodozyma*. *Journal of Industrial Microbiology and Biotechnology*, 19 (2): 114-117.
- 11-Lambert, C.R. (1995). Laser-Tissue Interaction VI. *Proc. SPIE-Int.Soc.Opt.Eng.*, 2391, 218-224.
- 12-Palágyi, Z.; Ferenczy, L. and Vagvolgyi, C. (2001). Carbon-sources assimilation pattern of the astaxanthin-producing yeast *Phaffia rhodozyma*. *World Journal of Microbiology and Biotechnology*, 17(1): 95-97.
- 13-Ducrey-Santopietro, L.M.; Spencer, J. F.T.; Spencer, D.M. and Siñeriz, F. (1998). Effects of oxidative stress on the production of carotenoid pigments by *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*). *Folia Microbiology*, 43(2):173-176.
- 14-Sun, N.; Lee, S. and Song, K.B. (2004). Characterization of a carotenoid-hyperproducing yeast mutant isolated by low-dose gamma radiation. *International Journal of Food Microbiology*, 94(3):263-267.
- 15-Miller, M.W.; Yoneyama, M. and Soneda, M. (1976). *Phaffia*, a new yeast genus in the Deutromycotina (Blastomycetes). *International Journal of Systematic Bacteriology*, 26, 286-291.
- 16-Schroeder, W.A. and Johnson, E.A. (1995). Singlet oxygen and peroxy radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. *Journal of Biological Chemistry*, 270(31): 18374-18379.
- 17-Adrio, J.L.; Veiga, M.; Casqueiro, J.; Lopes, M. and Fernandez, C. (1993). Isolation of *Phaffia rhodozyma* auxotrophic mutants by enrichment methods. *Journal of General Applied Microbiology*, 39(3): 303-312.
- 18-Yu, L.J.; Lan, W.Z.; Qin, W.M. and Xu, H.B. (2001). Effects of salicylic acid on fungal elicitor-induced membrane-lipid peroxidation and taxol production in cell suspension cultures of *Taxus chinensis*. *Process Biochemistry*, 37, 477-482.
- 19-Ulloa, M. and Hantin, R.T. (2000). *Illustrated dictionary in Mycology*, Academic Press. Inc., London. UK.
- 20-Meyer, P. S.; Wingfield, B. D. and Du-Preez, J. C. (1994). Genetic analysis of astaxanthin-overproducing mutants of *Phaffia rhodozyma* using RAPDs. *Biotechnology Letters*, 8 (1): 1-6.
- 21-SAS (1994). *SAS/STAT user. Guide Statistics, Version 6, Fourth edition*, SAS Institute, Inc. Cary, N.C.
- 22-Tchango, J. T.; Tailliez, R.; Eb, P.; Njine, T and Hornez, J. P. (1997). Heat resistance of the spoilage yeasts *Candida pelliculosa* and *Kloeckera apis* and pasteurization values for some tropical fruit juices and nectars. *Food Microbiology*, 14(1): 93-99.

- 23-Zagory, D. (1999). Effects of post-processing handling and packaging on microbial populations. *Postharvest Biology and Technology*, 15(3): 313-321.
- 24-Johnson, E.A. and An, G.H. (1991). Astaxanthin from microbial sources. Critical review of *Biotechnology*, 11(4):297-326.
- 25-Yamane, Y.I.; Higashida, K.; Nakashimada, Y.; Kakizono, T. and Nishio, N. (1997). Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: Kinetic and Stoichiometric analysis. *Applied and Environmental Microbiology*, 63(11): 4471-4478.
- 26-Nagy, Á.; Palágyi, Z.; Ferenczy, L. and Vágvölgyi, C. (1997). Radiation-induced chromosomal rearrangement as an aid to analysis of the genetic constitution of *Phaffia rhodozyma*. *FEMS Microbiology Letters*, 152, 249-254.
- 27-Vázquez, M. and Santos, V. (1998). 3-hydroxy-3', 4'-didehydro- β - Ψ -caroten-4-one (HDCO) from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) cultivated on xylose media. *Biotechnology Letters*, 20(2): 181-182.
- 28-Bendich, A. (1991). B-carotene and the immune response. *Proceedings of the Nutrition Society*, 50, 363-374.
- 29-Chan, H.Y. and Ho, K.P. (1999). Growth and carotenoid production by pH-stat cultures of *Phaffia rhodozyma*. *Biotechnology Letters*, 21, 953-958.
- 30-Kowakowska, M.; Kepczynski, M. and Szczubialka, K. (2003). New polymeric photosensitizers. *Pure Applied Chemistry*, 73(3): 491-495.
- 31-Bragulat, M.R.; Abarca, M.L.; Bruguera, M.T. and Cabanes, F.J. (1991). Dyes as fungal inhibitors: effect on colony diameter. *Applied and Environmental Microbiology*, 57, 2777-2780.
- 32-Camoriano, A.; Deflora, S. and Dahl, T.A. (1993). Genotoxicity of volatile and secondary reactive oxygen species generated by photosensitization. *Environmental Molecular Mutagen*, 21(3):219-228.
- 33-Baron, R.L. (1991). Carbamate insecticides. In *Hand book of Pesticide Toxicology*. Hayes, W.J. and Laws, E.R. Eds. Academic Press, New York.
- 34-McCarroll, N.E.; Protzel, A.; Ioannou, V.; Stack, H.F.; Jackson, M.A.; Waters, M.D. and Dearfield, K.L. (2002). A survey of EPA/OPP and open literature on selected pesticide chemicals III. Mutagenicity and carcinogenicity of benomyl and carbendazium. *Mutation Research/Reviews in Mutation Research*, 512(1): 1-35 Meyer, P.S.; Wingfield, B.D. and Du-Preez, J.C. (1994). Genetic analysis of astaxanthin-overproducing mutants of *Phaffia rhodozyma* using RAPDs. *Biotechnology Letters*, 8(1): 1-6.
- 35-Gualandi, G. and Bellincampi, D. (1981). Induced gene mutation and mitotic non-disjunctions in *A. nidulans*. *Toxicology letters*, 9 (4): 389-394.
- 36-Fournier, P.; Gaillardin, C.; Persuy, M.; Klootwijk, J. and Heerikhuizen, H. (1986). Heterogeneity in the ribosomal family of the yeast *Yarrowia lipolytica*: genomic organization and segregation studies. *Gene*, 42(3): 273-282.
- 37-Calo, P.; Velázquez, J.B.; Sieiro, C.; Blanco, P.; Longo, E. and Villa, T.G. (1995). Analysis of astaxanthin and other carotenoids from several *Phaffia rhodozyma* mutants. *Journal of Agricultural and Food Chemistry*, 43(5): 1396-1399.
- 38-Calhelha, R.C.; Andrade, J.V.; Ferreira, I.C. and Estevinho, L.M. (2005). Toxic effects of fungicide residues on the wine-producing process. *Food Microbiology*, 23(4): 393-398.
- 39-Tenreiro, S.; Fernandes, A.R. and Sá-Correia, I. (2001). Transcriptional Activation of FLR1 Gene during *Saccharomyces cerevisiae* Adaptation to Growth with Benomyl: Role of Yap1p and Pdr3p. *Biochemical and Biophys. Research Communications*, 280(1): 216-222.
- 40-Sinha, R. P.; Klisch, M.; Gröniger, A. and Häder, D.P. (1998). Ultraviolet-absorbing/screening substances in cyanobacteria, phytoplankton and macroalgae. *Journal of Photochemistry and Photobiology B: Biology*, 47(2-3): 83-94.

- 41-Cadet, J.; Sage, E. and Douki, T. (2005). Ultraviolet radiation-mediated damage to cellular DNA. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 571 (1-2): 3-17.
- 42-Brakhage, A.A.; Langfelder, K.; Schmidt, A.; Wanner, G.; Gehringer, H. ; Bhakdi, S. and Juhn, B. (1998). Identification of polyketide synthase gene (pks P) of *Aspergillus fumigatus* involved in conidial pigmented biosynthesis and virulence. 20th fungal genetic conference, Sexual and asexual differentiation posters.
- 43-An, G.H.; Schuman, D.B. and Johnson, E.A. (1989). Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Applied Environmental Microbiology, 55(1): 116-124.
- 44-Retamales, P.; León, R.; Martínez, C.; Hermosilla, G.; Pincheira, G. and Cifuentes, V. (1998). Complementation analysis with new genetic markers in *P. rhodozyma*. Antonie van Leeuwenhoek, 73 (3): 229-236.
- 45-An, G.H. (1997). Photosensitization of the yeast *Phaffia rhodozyma* at a low temperature for screening carotenoid hyperproducing mutants. Applied Biochemistry and Biotechnology, 66, 263-268.
- 46-Vázquez, M.; Santos, V. and Parajó, J.C. (1997). Effect of the carbon source on the carotenoid profiles of *Phaffia rhodozyma* strains. Journal of industrial Microbiology and Biotechnology, 19, 263-268.
- 47-Chen, M.Y.; Wu, S.H.; Lin, G.H.; Lu, C.P.; Lim, Y.T.; Chang, W. C. and Tsay, S.S. (2004). *Rubrobacter taiwanensis* sp. nov., a novel thermophilic resistant species. International Journal of Systematic Evolution Microbiology, 54, 1849-1855.
- 48-Belli, M.; Cherchini, G.; Vecchia, M.D.; Dini, V.; Esposito, G.; Moschini, G.; Saporà, O.; Signoretti, C. and Simone, G. (2001). DNA fragmentation in mammalian cells exposed to various light ions. Advances in Space Research, 27(2): 393-399.
- 49-Ziuzikóv, N.A.; Korogodin, V.I. and Korogodina, V.L. (1999). Features of action of low doses of gamma-radiation on yeast cells. Radiats. Biol. Radioecol., 39(6):619-22.
- 50-Carlos, E.E. and Johnson, E.A. (2004). Stimulation of astaxanthin formation in the yeast *Xanthophyllomyces dendrorhous* by the fungus *Epicoccum nigrum*. FEMS Yeast Research , 4(4-5): 511-519.
- 51-Wang, W.; Yu, L. and Zhou, P. (2006). Effect of different fungal elicitors on growth, total carotenoids and astaxanthin formation by *Xanthophyllomyces dendrorhous*. Bioresource Technology, 97(1): 26-31.
- 52-Junghanns, K.T.; Kneusel, R.E.; Gröger, D. and Matern, U. (1998). Differential regulation and distribution of Acridone Synthase in *Ruta graveolens*. Phytochemistry, 49(2): 403-411.
- 53-Ebel, J. and Scheel, D. (1992). Gene involved in the plant defense. In: plant gene research., pp: 183-205. Boller, T. and Meins, F., Editors, Springer-Verlag, Wien, Vienna, Austria.
- 54-Andrews, A.G.; Phaff, H.J. and Starr, M.P. (1976). Carotenoids of *Phaffia rhodozyma*, a red pigmented fermenting yeast. Phytochemistry, 15, 1003-1007.
- 55-Kamat, A.; Pingulkar, K.; Bhushan, B.; Gholap, A. and Thomas, P. (2003). Potential application of low dose gamma radiation to improve the microbiological safety of fresh coriander leaves. Food Control, 14(8): 529-537.
- 56-Davoli, P. and Weber, R.W.S. (2002). Identification and quantification of carotenoid pigments in aeciospores of the daisy rust fungus, *Puccinia distincta*. Phytochemistry, 60(3): 309-313.