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MODIFICATION OF PIGMENT COMPOSITION IN *EPICOCCUM NIGRUM* BY CHEMICAL MUTAGENESIS♦

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Abstract: *Epicoccum nigrum* MIUG 2.15 produces in high concentration a combination of yellow food-grade pigments (i.e. carotenoids and flavonoids in a ratio of 20:1) by solid-state fermentation on maize and molasses based media. By exposing the parental strain to a chemical mutagen, N-methyl-N'-nitro-N-nitroso-guanidine, a mutant strain was obtained, that produces equal shares of flavonoids and carotenoids (i.e. 1:1) in a red pigment complex. UV-VIS spectrometry and HPLC analysis show a major content of glycosylated flavonoids and free phenolic acids, and also conjugated carotenoids, especially rhodoxanthin. The extracts may be used as red food colorants with functional properties.

Keywords: *Epicoccum nigrum, chemical mutagenesis, solid state fermentation, carotenoids and flavonoids biosynthesis, N-methyl-N'-nitro-N-nitroso-guanidine (NTG),*

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INTRODUCTION

Despite the wide variety of compounds available in nature and their potential for use as food colorants, many of these are found in relatively low concentrations, so that biotechnology may represent an excellent option for large-scale production. Thus, obtaining pigments through plant-cell-tissue culture, microbial fermentations, and genetic manipulation has been investigated. However, given that many of the producer organisms are considered as non-GRAS (generally recognized as safe), the products obtained are subjected to a battery of food-safety testing prior to their release for use as food additives [4]. Microorganisms are known to produce a wide variety of pigments, and therefore are a promising source of food colorants. Their use also has the added advantages of rapid growth and ease of control. Current trends in industrial production aim at the introduction of environmentally safer biotechnological processes. Bacteria (*Flavobacterium multivorum, Brevibacterium linens*), fungi (*Phaffia rhodozyma, Blakeslea trispora, Phycomyces blakesleanus*) and microalgae (*Haematococus pluvialis, Dunaliella salina*) are considered for carotenoid production. Beta-carotene is produced by Gist Brocades (Netherlands) using *Blakeslea trispora* through reactor fermentation. Flavonoids are produces exclusively by extraction from plant material.
Previous research revealed the possibility of using *Epicoccum nigrum* for the production of carotenoids and flavonoids. High yields can be achieved in solid-state fermentation systems by cultivation on agro-food by-products such as molasses and maize milling wastes. The pigments can be easily extracted in alcoholic solutions and other polar organic solvents. The extracts show good stability and high antioxidant activity [2, 3].
Epicoccum nigrum is a dematiaceous mitosporic mould widely distributed and commonly isolated from air, soil, a large variety of plants, human skin, insects, foodstuff, and textiles. The genus *Epicoccum* contains a single species. It is considered saprophytic; in some cases poses as an opportunist, being a secondary invader of plants. *E. nigrum* grows rapidly and produces woolly to cottony colonies on potato dextrose agar, at 25 °C. From the front, the colonies are yellow to orange, orange to red or pink initially, and become greenish brown to black by aging. The reverse is always intense yellow. The fungus produces diffusible pigments (flavonoids) which turn the color of the medium to orange yellow. Occasionally, black dots may be observed macroscopically on the colony surface; these are dense masses of clustered conidiophores called sporodochia. Spore formation may not always occur; it may be induced by stress. There are no documented cases of *Epicoccum nigrum* infections in humans or animals [5].
This work was intended to broaden the color palette of the pigments produced by *Epicoccum nigrum* MIUG 2.15, making use of chemical mutagenesis; thus, the fungus was exposed to various concentrations of N-methyl-N'-nitro-N-nitroso-guanidine.

MATERIALS AND METHODS

Microbial Strain and Fermentation Conditions

For the experiments, a fungal strain from "Dunarea de Jos" Galați University's Collection of Microorganisms (coded MIUG) was used. *Epicoccum nigrum* MIUG 2.15

was isolated from air and screened in past work. The culture was preserved covered with liquid paraffin. For reactivation, inoculum preparation and morphological studies a malt extract agar (MEA) medium was used. Fermentations were conducted in Petri dishes, at 25 °C, for 10 days. The media comprised of a solid substrate made out of maize flour and maize embryos, and a moisturizing liquid composed of molasses, glycerol, urea, and mineral salts.

Mutagenesis Procedure

A homogenous suspension of hyphae was prepared by mixing in stomacher sterile distilled water with the mycelium from a submerged culture of Epicoccum nigrum MIUG 2.15. The suspension was standardized nephelometrically, using an empirical method that correlates turbidity with the number of colony forming units (cfu) determined by the pour plate technique. For inducing mutations, a chemical agent was used, i.e. N-methyl-N'-nitro-N-nitroso-guanidine (NTG). The concentrations of active ingredient ranged from 0 to 200 μg/mL. A value of 10^2 cfu/mL was constant for all samples. A phosphate buffer (pH = 7.4, 0.2M) was used to complete the volume of the samples to a fixed value. The exposure time was set to 30 min. A temperature of 30 °C was applied for incubation. Upon completion of treatment time, the hyphae were separated by centrifugation (9000 rpm, 8 min), washed with sterile distilled water, re-centrifuged, re-suspended and inoculated in Petri dishes. After an incubation period of 72 h at a temperature of 25 °C, the resulted colonies were analyzed as to assess survival rates, morphological and color variations, and genetic stability of the mutant strains.

Pigment Extraction and Characterization

Fermented media were dried at a maximum temperature of 40 °C and finely ground; obtaining a reddish brown powder that was used for extracting the pigments. For pigment characterization, the following procedures were used:

- *UV-VIS absorbance spectrum scan* – extracts in various polar and non-polar organic solvents (i.e. petroleum ether, ethanol, and methanol with 1% HCl) were analysed by determining the absorbance for wavelengths of 190-700 nm.
- *HPLC* – gradient separation with solvent A, nitrile-acetate:methanol, 9:1, TEA buffer; and solvent B, ethyl acetate 100 %, with TEA buffer; according to the following elution program : min. 0-16min, solvent B from 0 to 60 %, min. 16-40 min , solvent B = 60%, min. 40-42 min, solvent B from 60 to 0 %;
- *Total carotenoids* – the UV-VIS spectrometric method;
- *Total poly-phenols* – the Folin-Ciocâlteu method.

RESULTS AND DISCUSSION

Effect of the Mutagen Agent upon Morpho-Physiological Properties

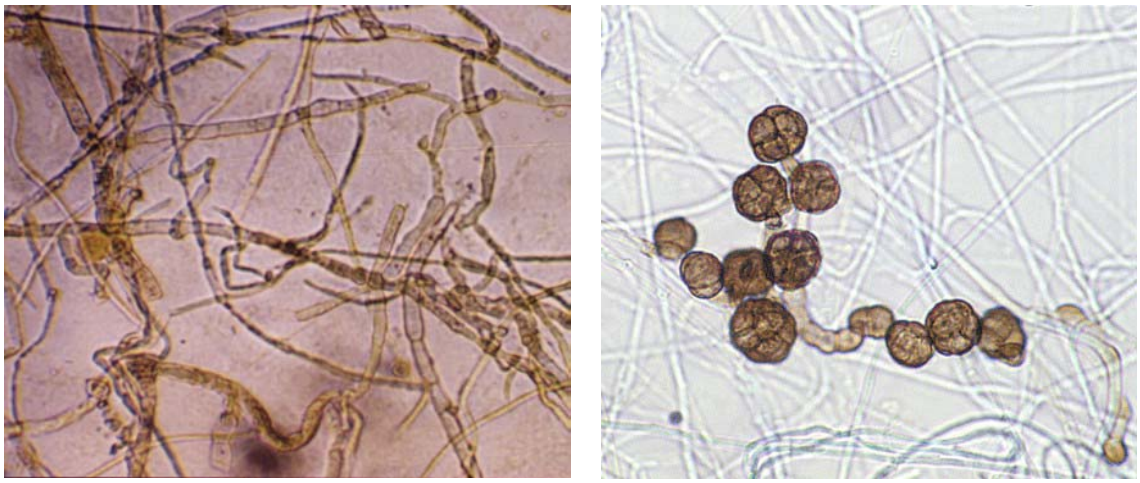
NTG is an alkylant agent which functions by nucleotide substitution (G≡C→A=T), preferably at the DNA replication fork. Hence, mutation rate is quite high and death rate relatively low. Concentrations of NTG above 120 μg/mL have proven to be lethal for

Epicoccum nigrum MIUG 2.15, resulting in null survival rates. Mutations affecting the mycelium color were noticed for NTG levels above 20 μg/mL. Mutant strains were isolated and screened for genetic stability and pigmentation properties. Out of these, *Epicoccum nigrum* MIUG 2.15^{m} was selected, mutant strain resulted from the exposure to a concentration of 60 μg/mL that translated into a survival rate of 40%. A comparative analysis of morphological and pigmentation properties between the parental strain and the mutant is presented in table 1.

Table 1. *Phenotypic modifications induced by NTG chemical mutagenesis in the parental strain Epicoccum nigrum MIUG 2.15*

Characteristics	**Parental strain**	**Mutant strain**
Mycelium colour	Yellow orange	Pink red
Colony morphology	Woolly-cottony mycelium, expanded	Cottony-velvety mycelium, dense; black dots – clustered conidiophores (sporodochia)
Microscopic aspect	Medium length thin hyphae, no spores	Shorter thin hyphae, aleuriospores

Data in table 1 show an obvious modification of morphological properties and pigmentation characteristics, as a result of the mutations induced by NTG treatment. Mycelium color has changed from yellow orange to pink red; hyphae have become shorter and more compact resulting in a denser mycelium. In addition, because of the exposure to NTG, the fungus has developed aleuriospores, displayed in dense clusters called sporodochia. Figure 1 includes microscopic images of the two strains.



(a) (b)

Figure 1. *Microscopic images of the parental strain (a) and mutant (b)*

It should be mentioned that the spore-forming ability was noticed only at the first isolate, disappearing after replication. This is not unusual for *Epicoccum nigrum*, given the fact that the fungus prefers hyphal growth as a form of dissemination. Spores appear only in certain conditions, in reaction to environmental stress, such as low levels of water activity (method for producing suspensions of conidia used as bio-control agents in agriculture for combating fungal plant parasites) or exposure to toxic chemicals. In

this case, sporulation must have been caused by the NTG treatment. *Epicoccum nigrum* MIUG 2.15^{m} is characterised by morphologic and genetic stability.

Modification of Pigment Profile in the Mutant Strain

The mutation determined a drastic decrease (i.e. 83.2 %) in carotenoid content and a 230.7 % increase in poly-phenols. A comparison of pigment compositions, expressed as total poly-phenols and total carotenoids, between the parental strain and the mutant is given in figure 2.

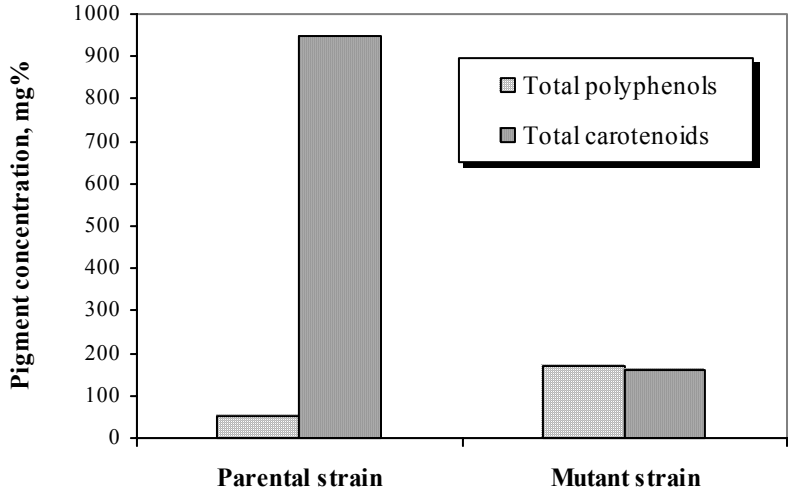


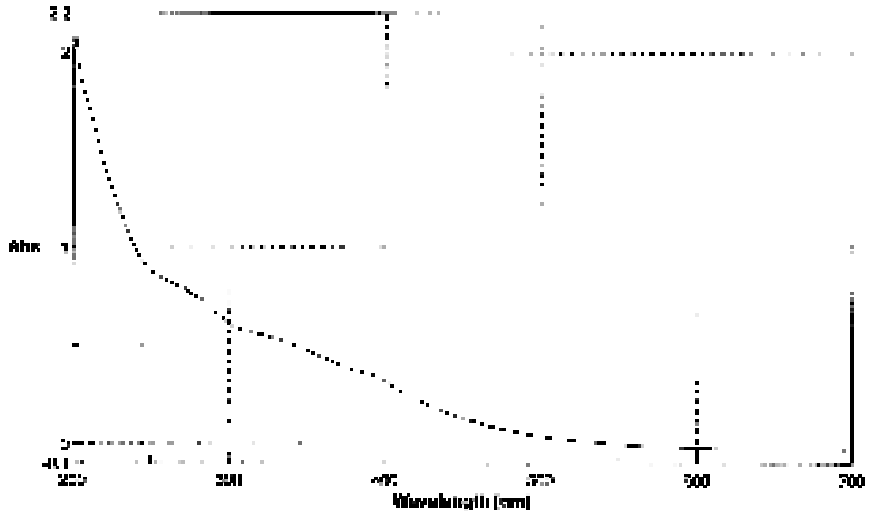
Figure 2. *Changes in pigment content as a result of the chemically induced mutation in Epicoccum nigrum MIUG 2.15*

Starting with a ratio of 20:1 between carotenoids and poly-phenols in the parental strain, a 1:1 ratio has been reached in the mutant strain by NTG treatment. This characteristic is constant for the descendents of the mutant.
The HPLC analysis reveals more details relative to the composition of glycosylated pigments. Two types of signals are visible: specific to polar groups of glycosylated flavonoids and rhodoxanthin, and specific to free forms of phenolic acids (i.e. RT = 13-15 min). Data are given in table 2.

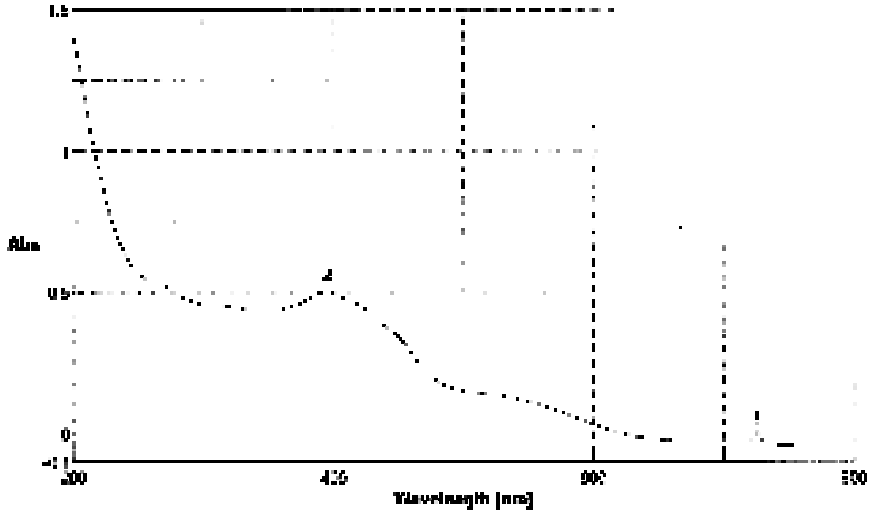
Table 2. *HPPLC signals for pigments produced by Epicoccum nigrum MIUG 2.15^{m}*

RT, min	λ_{max}, nm	Comments
2,24	240, 340, 420, 550	Combination of flavonoids, carotenoids
3,54	240, 340, 500	Combination of flavonoids, carotenoids
13,4	240 ,340	Flavonoids
14,7	280	Phenolic acids and quinones

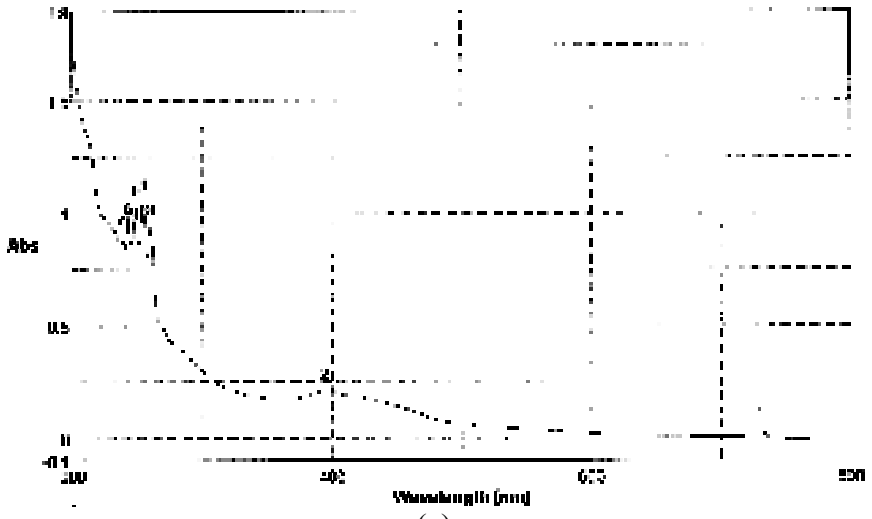
An UV-VIS (190-700 nm) absorbance spectrum scan was conducted for extracts in petroleum ether, ethanol, and methanol with 1% HCl. The spectra are shown in figure 3. Spectrometry data show that the pigments are found as glycosides as the absorbance peaks are moved towards the UV domain. In petroleum ether there are no visible signals denoting the lack of free carotenoids.



(a)

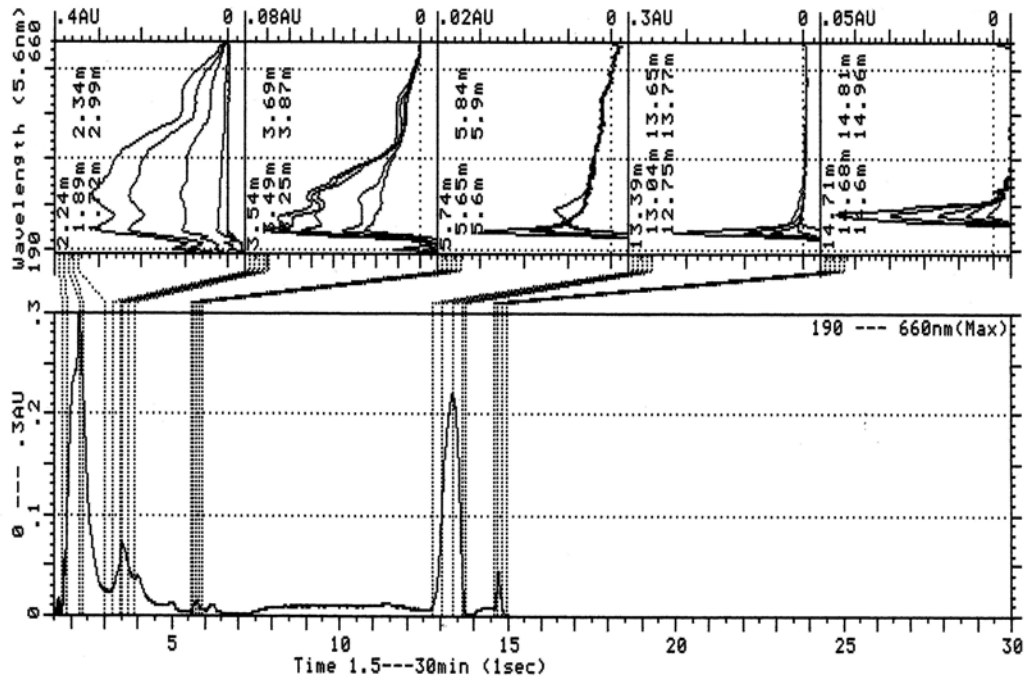


(b)

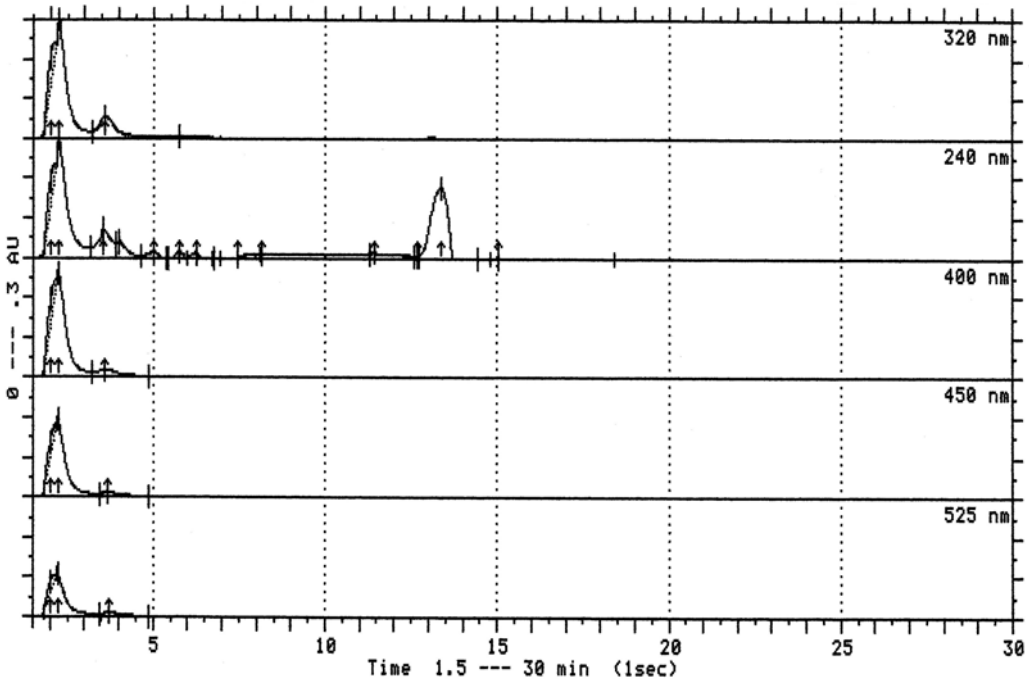


(c)

Figure 3. *Spectra scans for pigment extracts of Epicoccum nigrum MIUG 2.15[m] in methanol with 1% HCl (a), ethanol (b), and petroleum ether (c)*



(a)



(b)

Figure 4. *HPLC chromatograms for pigment profiles in Epicoccum nigrum MIUG 2.15*[m]

The chromatograms of the HPLC analysis are included in figures 4 and 5. Figure 4 points out signals between 190 and 660 nm for retention times (RT) of 0-30 min. In the chromatogram from figure 5 (450 nm) signals are visible only at 2.24 and 3.54 min.

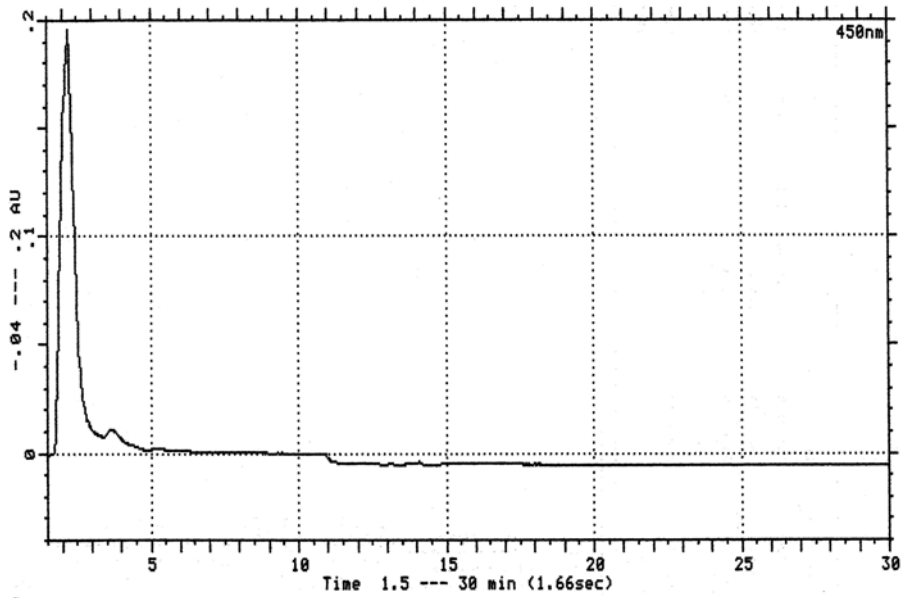


Figure 5. *HPLC signals for readings at 450 nm*

CONCLUSIONS

By exposing the parental strain to N-methyl-N'-nitro-N-nitroso-guanidine, a mutant strain was obtained, that produces equal shares of flavonoids and carotenoids (i.e. 1:1) in a red pigment complex.

Data show that the pigments are found as glycosylated forms of flavonoids and carotenoids (rhodoxanthin), as well as free phenolic acids and quinones.

The mutant strain could be used as a source of red food-grade pigments with improved functional properties, as flavonoids and carotenoids are synthesized in equimolecular shares, hence more potent in fighting the cytotoxic activity of free radicals.

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