

Study of relationship concerning the pigment production and growth rate for five mutant strains of *Monascus purpureus*

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The purpose of this study was to obtain a hyperproductive strain of *Monascus purpureus* used in the biosynthesis of a natural red food dye in solid state fermentation, on rice. Five mutant strains of *Monascus purpureus* were isolated after gamma and electron beam irradiation of conidiospores, at doses lower than 10 kGy. The mutant fungi show different morphocolonial aspects, varied red nuances and distinct microscopic reproductive structures and have lower growth rates on petri dishes culture media than the parental strain. All the radioinduced mutants were cultivated in solid state fermentation system to obtain the red fermented rice and four of them produced more pigment than the parental strain.

1. Introduction

Monascus purpureus, known as "red yeast" are used by almost two millennia in the countries of East Asia both for the preparation of food and drink, to obtain food coloring and for the synthesis of dietary products and pharmaceuticals. Rice fermented with red *Monascus purpureus*, Ang-Khak, Hong Qu or Koji in China, Ang-Khak, Beni Koji, Red Koji in Japan, is mentioned in ancient Chinese pharmacopoeia, which is described as a treatment for improving digestion and blood regeneration, and later in a book of Chinese medicine published in 1590, Li Shih-Chun mentions the use of this preparation as a coloring agent and as a medical cure for various diseases (Bakosova et al., 2001).

The fungus *Monascus purpureus* produces at least 6 pigments, of which 2 yellow - monascidin and ankaflavin, 2 orange - rubropunctatin and monascorubrin and 2 red - rubropunctamin and monascorubramin. Red fermented rice contains compounds with activity of inhibitors of 3-hydroxy-3-metilglutaril-coenzime A reductase (CoA reductase inhibitors), which are responsible for inhibiting cholesterol synthesis in the liver. Coloring raw *Monascus* also includes unsaturated fatty acids that help reduce serum lipids, extracts of *Monascus* decrease the level of total cholesterol and cholesterol-LDH and of serum triglycerides (Heber et al., 1999; Erdogru et al. 2004).

The antibacterial activity of *Monascus purpureus* was first reported in 1977. The wild type isolated from red-mold rice and some induced mutants were shown to produce antibiotic(s) active to *Bacillus*, *Streptococcus*, *Pseudomonas*, *Listeria*, *Staphylococcus* and some molds due to a substance called monascidin A (Wong and Bau, 1977).

2. Biological material and growing media

In experiments conducted was used a strain of *Monascus purpureus*, which has been treated with gamma radiation and electron beam accelerated order to isolate and selection of the mutant productive potential for increased production of red pigments food. Maintenance and cultivation of fungal species was achieved on glucose-potato-extract agar, malt extract-agar and glucose-yeast extract-agar (4% glucose, yeast extract 1%, KH_2PO_4 0.3%, agar-agar 1.5). The average hourly growth rate was calculated as the ratio of the diameter of colony grown on average above in Petri dishes at a time and the number of hours of development. The additive color biosynthesis of *Monascus* has been conducted on milled rice and sterilized.

3. Irradiation methods

Study of the possibility of obtaining some hyperproductive mutant strains was achieved by using physical mutagen factors, such as gamma and electron beam irradiation. The aim of irradiation was to select hyperproductive mutant strains useful in the production of food pigments from *Monascus purpureus*. For both types of irradiation it has been used a suspension of conidiospores in sterile water, obtained on the culture medium potato-dextrose-agar.

3.1 Gamma irradiation

Gamma irradiation was carried out using a source of ^{60}Co standard type "pencil", in the Institute of Physics and Nuclear Engineering "Horia Hulubei" Magurele-Bucharest. Source presents an activity of about 105 TBq and items arranged so that allow a plane geometry of radiation. Irradiation have been carried out on 1-10 kGy dose at a range of fixed dose of about 3 kGy/h, by varying the time of radiation and ensure a uniform dose of less than 10%.

3.2 Electron beam irradiation

Electron beam irradiation was accomplished in the Electronic Accelerator Laboratory of the National Institute of Physics Laser, Plasma and Radiation Magurele-Bucharest, using a linear electron accelerator "ALIN" which presents the following characteristics: electrons mean energy 6 MeV, mean current in beam 5 μA , pulse time length 3.5 μs , frequency 100 Hz and mean power of about 60 W. Irradiation doses were in the range of 1-10 kGy and the dose rate was 100-2000 Gy/min.

4. Biosynthesis and analysis of pigments

The color additive of *Monascus* was obtained by cultivating the mutant strain on ground rice, in solid state fermentation system in 1500 mL Erlenmeyer flasks, at a temperature of 30 °C for 14 days.

The dark-red product was dried, ground and subjected to a heat treatment for reducing microbial load, resulting the final powdered product with tinctorial properties. Pigments extraction was done in the Et-OH 96%, for an extraction ratio depending on the type of mutant strain.

The absorbance of the mixture of yellow, orange and red pigments was measured at a Spectrophotometer Cary 3E, at a wavelength of 400 nm (corresponding to maximum absorption of the yellow components) and 510 nm (corresponding to maximum absorption of the red components) [Ferdes, 1998].

5. Results and discussion

The improvement of biosynthetic potential of microorganisms can be achieved through a series of methods, of which one of the most inexpensive is to use mutagen agents, even if this method requires a laborious task not always successfully completed, because of the unpredictable emergence of colonies with useful mutant character.

Gamma irradiation was carried out to a source of ^{60}Co , with the characteristics outlined above.

For each irradiated sample it was determined the $\text{CFU}\cdot\text{cm}^{-3}$ value and the viability of spores suspension. The results are presented in Table 1.

Table 1. The viability of *Monascus* cells in the gamma irradiated samples

	DOSE (kGy)										
	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
CFU·cm ⁻³	7.9·10 ⁶	2.5·10 ⁶	3.9·10 ⁵	6.3·10 ⁴	3.1·10 ⁴	6·10 ³	6·10 ²	199	32	5	0
Viability (%)	100	31.6	4.9	7.9·10 ⁻¹	3.9·10 ⁻¹	7.6·10 ⁻²	1·10 ⁻²	2.5·10 ⁻³	4.1·10 ⁻⁴	6·10 ⁻⁵	0

Selection of hiperproductive strains has been carried out using a culture medium with glucose and potato extract, in petri dishes, inoculated with a diluted suspension of irradiated conidiospores and then stored at 30 °C for 5 days.

After the examination of color differences between the colonies grown in the plates, it have been selected a number of 25 possible mutants, which were isolated and cultivated in the same condition to check their characters and to choose the best producers.

Finally there were selected only two colonies, the most intensely colored. They were named M1, isolated from the spores suspension irradiated at 6 kGy dose and M2 strain, isolated from the spores suspension irradiated at 4 kGy.

Electron beam irradiation was performed in a linear irradiation installation ALIN 7, in INFLPR Magurele-Bucharest institute.

Radiation doses were between 1 and 10 kGy. On the basis of the performed investigations there was determined the viability percentage versus the unirradiated (control) sample, separately for every irradiation dose, as it follows (Table 2).

Table 2. The viability of *Monascus* cells in the electron beam irradiated samples

	DOSE (kGy)										
	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
CFU \cdot cm $^{-3}$	24.7· 10 5	5.1· 10 5	7.5· 10 4	10·10 3	1·10 3	10 2	25	4	0	0	0
Viability (%)	100	20.6	0.03	4.04· 10 $^{-3}$	4.04· 10 $^{-4}$	4.04· 10 $^{-5}$	1.01· 10 $^{-5}$	1.62· 10 $^{-6}$	0	0	0

To select a mutant strain with high productive potential concerning the synthesis of red pigments, from a number of 11 colonies there was isolated 3 colonies from the spores suspension irradiated at 2 kGy. The 3 strains were named M3, M4 and M5.

5.1 Cultural characters of the mutant strains

Mutant strains isolated after irradiation have been grown on mentioned culture media, studying their appearance and color and determining the growth rates at different temperatures, compared with the parental strain.

For all culture media used, it notes large differences between the size of parental and mutant colonies. The most rapid growth characterized the parental strain and the M5 strain cultivated on yeast extract- glucose-agar medium. The lowest values of colony diameter were recorded for M4 strain for all the media used.

The growth hourly rates for mutant strains at 20°C, 30 °C and 37 °C comparatively with parental strain are shown in Figure 1.

All the five mutant strains shown lower growth rates than the parental strain for each temperature.

For example, at 30 °C, parental strain has grown with a rate of 0.393 mm/hour, while M1 and M5 had growth rates of approximately 0.25 mm/hour. The lower value of this size, only 0.06 mm/hour was recorded for the M4 strain.

The lower growth rates of mutant's strains comparatively with the parental strain are related with higher pigment production, a phenomenon observed even in the study of appearance and color of the colonies. This can be explained either by an inhibition of growth process due to the synthesis of pigments or by competition for nutrients between growth and pigmentogenesis (Ferdes, 1998).

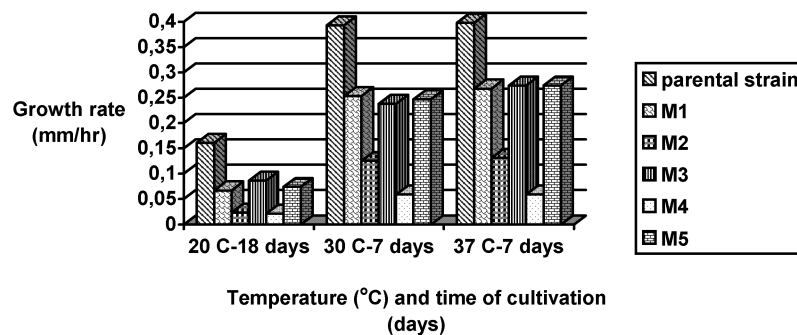


Figure 1. Growth rate at temperatures of 20 °C, 30 °C and 37 °C on potato-dextrose-agar plates

5.2 Microscopic appearance of the mutant strains

Microscopically characters of *Monascus* mutant strains were carried out on potato-dextrose-agar in petri dishes after 6 days at 30 °C.

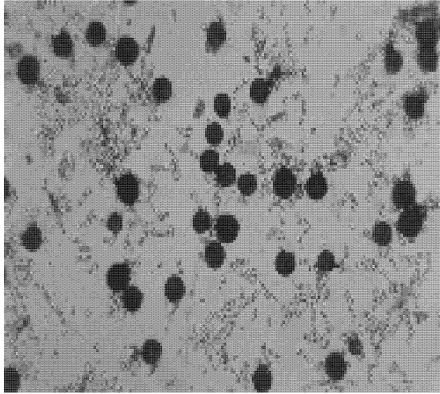


Figure 2. The microscopic appearance of parental strain

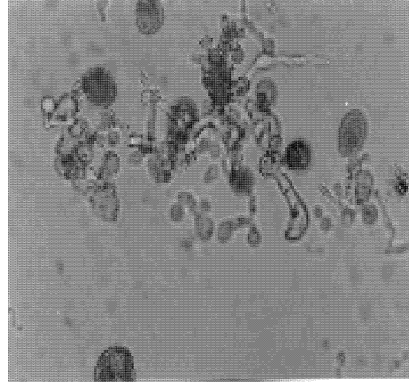


Figure 3. The microscopic appearance of M4 strain

Microscopic slides indicate quite important morphological changes of the mutant fungal mycelium. In regard to the appearance of hypha, these are shorter and sometimes thinner than the parental hypha, a phenomenon correlated with an increased production of pigments, and could be explained by the inhibition of cell wall constituents because of the monascorubrin and rubropunctatin high level of synthesis. This observation can explain the decrease of growth rate which appears to be related to the production of pigments. The diminution of glucan, cellulose and chitin or other wall constituents, synthesis produces the decrease of growth rate and higher fragility of hypha.

This aspect was better observed for M4 mutant strain, characterized by the lowest growth rate and a strong pigmentation of colonies. Therefore, it seems that the pigments production is a process in competition with the synthesis of materials of the cell wall. Mutant strains have a reduced number of asce, asexual spores being the main modality of reproduction.

Biosynthesis of red pigments using mutant strains grown on solid medium

The production of red pigments was analyzed after the growth of *Monascus* mutant strains in solid state fermentation on rice, comparatively with the parental strain, to select the most productive strain.

Reports of increased production of red and yellow pigments, expressed as units mutant OD/OD stem parental units, are shown in Figure 4.

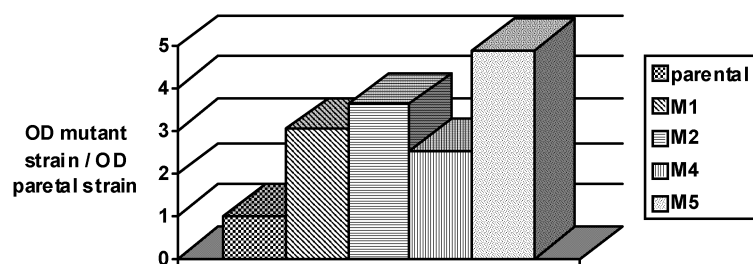


Figure 4. Values of OD_{510} mutant/ OD_{510} parental in alcoholic extract

6. Conclusions

Gamma and electron beam irradiation was an effective procedure for mutant induction in the pigment production with *Monascus purpureus* species. It was obtained 5 mutant strains which can be used for the biosynthesis of red fermented rice, a natural food dye. Hyperproductive M1, M2, M3, M4 and M5 mutant strains selected differ from the parental strain by pigmentation, growth rate, macro-and microscopic morphology and biosynthetic potential of red pigments on solid media.

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