Study of Relationship Between Enzymes Production, Growth Rate and Pigmentogenesis for Five Mutant Strains of Monascus Ruber

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Five mutant strains of Monascus ruber were obtained by gamma and e-beam processing and were characterized in terms of enzyme production, growth rate and pigmentogenesis. These strains were cultivated onto glucose-yeast extract agar slants for maintenance and on rice medium for pigments biosynthesis. It was demonstrated that the parental strain does not produce any amylolytic, lypolytic, proteolytic or cellulolytic enzymes, while mutant strains have enzymatic activity. The biosynthesis of natural pigments was achieved in solid state fermentation on sterilized rice. The biopigments were extracted in ethanol and hexane and the absorption spectra were recorded for the five mutant strains. M5 and M2 mutants produced the highest amount of red and yellow pigments.

Keywords: Monascus ruber, gamma and e-beam irrradiation, enzymes

Application of new technologies in food industry requires untraditional processing of foodstuffs with the aim to improve their quality, durability, storage, nutritious value, and visual attraction.

The use of natural *Monascus ruber* pigment promotes not only fulfillment of all these requirements, but also the positive health aspects from and with *Monascus* prepared food.

Monascus ruber is a red mold species which may be cultivated on starch containing substrates.

The solid state fermentation of rice by *Monascus* has a long tradition in East Asian countries as food dye and for dietary staples. The fermented rice is called ang khak or Hong Zhu in China and beni koji in Japan.

Red yeast rice has also been used as a preservative for meat and fish, for adding color and flavor to food and for brewing wine and liquor. In Japan, these are used for the production of tofuyo, which is a vegetable protein food made from soybean curd by the action of microorganisms [3,4].

Another important characteristic of *Monascus* fungi is their capability for production of proteases and amylases, so they are used for obtaining these enzymes by cultivating them on different natural substrates.

Monascus produces acid proteinase (one of endopeptidases that hydrolyse proteins at internal sites to peptides and small amounts of free amino acids) and carboxypeptidase (an exopeptidase that releases free amino acids from the carboxyl termini of peptides or proteins).

It was found that a large number of free amino acids (e.g. glutamic acid and aspartic acid) were produced during the maturation of tofuyo. This was considered to be the result of proteolytic enzymes produced by *Monascus* hydrolyzed soybean protein. Although a large number of carboxypeptidases have been isolated from various species of fungi, such as *Aspergillus* or *Mucor*.

Monascus fungi are well known as producers of a family of structurally related hexaketide pigments which are red and yellow in colour [5, 6].

Monascus fungi produce at least six major pigments: 2 red colorants named rubropunctamine and monascorubramine, 2 orange colorants, rubropunctatin and monascorubrin and 2 yellowish colorants, monascin and ankaflavin [7, 8].

Monascus ruber is used as a cholesterol-lowering agent in many countries. Other applications of red yeast rice are suggested by recent discoveries that lovastatin and other statin drugs may be useful for treating or preventing cancer, osteoporosis, stroke, Alzheimer's disease and other dementias and macular degeneration.

Red yeast rice has been reported to contain sterols such as beta-sitosterol and campesterol, which are known to interfere with cholesterol absorbtion in the intestines [9]. Red yeast rice also contains fiber, trace elements such as magnesium, unsaturated fatty acids such as oleic, linoleic and linolenic acids and B-complex vitamins such as niacin.

The preservative effect of *Monaşcus* fermentate has also been confirmed; monascidin Á inhibits bacteria of genera *Bacilus, Staphylococcus, Streptococcus* and *Pseudomonas* [10, 11].

In the mouse model, oral administration of monascin inhibited the carcinogenesis of skin cancer initiated by peroxynitrite or ultraviolet light and after the promotion of TPA [12]. Ankaflavin showed selective cytotoxicity to cancer cell lines by an apoptosis-related mechanism and showed relatively low toxicity to normal fibroblasts. The structure analog monascin, however, showed no cytotoxicity to all cell lines tested [13]. The orange pigments, rubropunctatin and monascorubrin, had been found to possess antibiotic activity against bacteria, yeast, and filamentous fungi [14]. Yellow pigments, monascin and ankaflavin, showed immunosuppressive activity on mouse T splenocytes [15].

The aim of this investigation is to determine morphological, biochemical and some kinetic characteristics of some mutant strains selected for fermented red rice production.

Experimental part

Strains

Five *Monascus ruber* mutant strains were used for comparative study of the enzyme production, the growth rate and the pigments synthesis. The mutant strains were obtained by electron beam and ^{60}Co gamma irradiation

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[16] at 1-10 kGy doses applied on a spore suspension of parental strain *Monascus ruber* MUCL 28962. The fungal mutant strains are called M1, M2, M3, M4 and M5.

Culture media

These strains were cultivated onto glucose-yeast extract agar slants for maintenance, in tubes, at 30 °C, for 7 days.

Enzymes production is accomplished on specific media. Amylases production was performed on starch containing medium (yeast extract 4 g/L, soluble starch 10 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, agar-agar 15g/L). Cellulase activities were tested after cultivation of *Monascus* strains on a medium with cellulose powder 2.5 g/L, peptone 0.5 g/L, K_2HPO_4 0.2 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, K_2CO_3 0.4 g/L, $CaCl_2$ 0.02 g/L, $FeSO_4 \cdot 7H_2O$ 0.02 g/L, NaCl 0.02 g/L, agar-agar 15 g/L.

Proteases biosynthesis was estimated using a casein containing medium (casein 2.5 g/L, $Ca(OH)_2$ 0.15 g/L, $CaCl_2$ 0.05 g/L, agar-agar 15 g/L). For the lipases production it was used nutrient agar supplemented with 1% $CaCl_2$ solution and 1% Tween 80 to form a Ca-oleate insoluble precipitate.

The growth rate was assigned by measurement of diameters of *Monascus* colonies in Petri dishes, on potato-dextrose agar, at 30°C.

Pigments biosynthesis is carried out on sterile ground rice, in Erlenmeyer flasks.

50 g of rice was inoculated with 10 mL of spores suspension and incubated 7 days at 30°C.

Measurement of enzyme activity

To evaluate the enzyme biosynthesis, all the *Monascus* strains were inoculated in the middle of Petri plate on adequate media and the surrounding haloes dues to the enzyme gel diffusion were measured. The hydrolytic index was calculated as I_{enz} = halo diameter/colony diameter. Using a semi-quantitative screening method, enzymatic indices were determined for each mutant strain after 9 days of growth.

Growth rate

In order to estimate the growth rate of *Monascus* strains the maximum diameter of colonies was measured after 216 h and the ratio diameter/time was calculated.

Pigments production and extraction

The production of red and yellow pigments was tested by growing *Monascus* strains on sterilized rice for 10 days, at 30 °C.

Pigments were extracted in ethanol and hexane from the red yeast rice and the visible absorption spectra were drawn on a 6715 UV/VIS Jenway Spectrophometer.

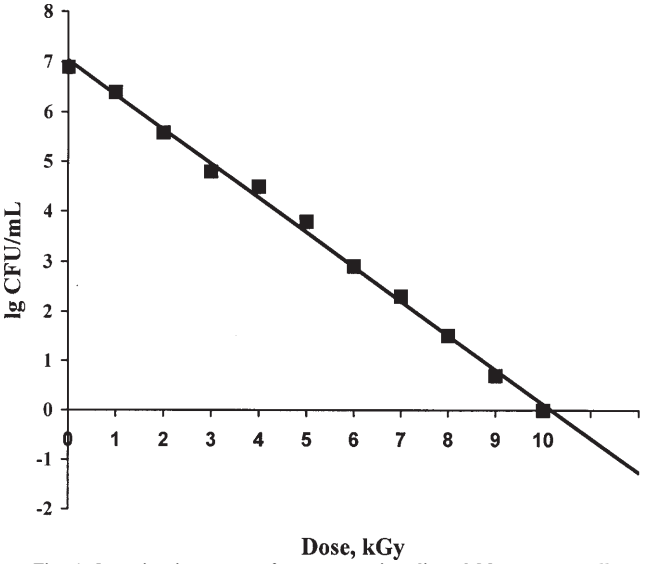

Fig. 1. Inactivation curve for gamma irradiated Monascus cells

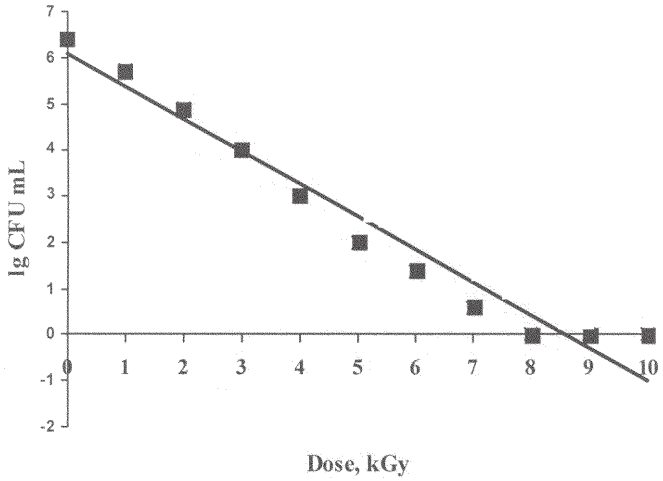

Fig. 2. Inactivation curve for electron beam processed Monascus cells

Results and discussion

The aim of this investigation was to determine morphological, biochemical and some kinetic characteristics of 5 mutant strains selected for fermented red rice production.

Five mutant strains of *Monascus ruber* were obtained by gamma and electron beam irradiation and were characterized in terms of enzyme production, growth rate and pigmentogenesis [17].

Irradiation treatment was carried out to select mutant colonies with higher pigment production potential than parental strain.

Table 1
ENZYMATIC INDICES OF MONASCUS RUBER WILD-TYPE AND MUTANT STRAINS

Strain	Amylolytic index	Proteolytic index	Lipolytic index	Cellulolytic index
Wild-type	0	0	0	0
M1	1.1	1.11	1.71	0
M2	1.53	1.17	2.33	0
M3	0	1.11	1.2	0
M4	1.66	1.66	3	0
M5	1.1	1.2	1.5	0

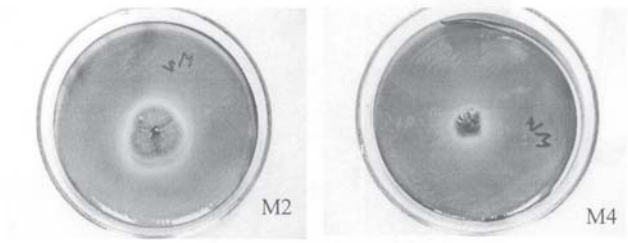

Fig.3. Amylolitic enzymes produced by M2 and M4 mutant strains (remained unhydrolyzed starch colored in brown with Lugol reagent)

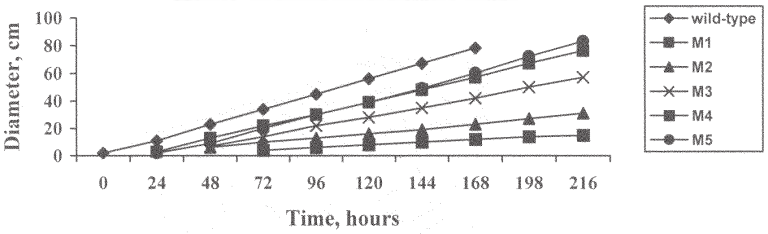

Fig.4. The variation in colony diameters for the Monascus ruber parental and mutant strains

Gamma irradiation was achieved at a ^{60}Co source at doses among 0-10 kGy. D_{10} (absorbed dose that inactivates 90% cells from initial population) was 1.4 kGy and the inactivation dose was 10 kGy.

From the gamma irradiated suspensions of spores had been selected two colonies, the most pigmented ones, M1 from the 6kGy irradiated culture and M2 from 4 kGy irradiated suspension of spores.

Electron beam treatment was achieved at 0-10 kGy, in a linear irradiator.

The D_{10} was 1.4 kGy and the inactivation dose was 8.6 kGy.

It had been isolated 3 hyperpigmented colonies, M3, M4 and M5.

Mutations can alter the phenotype of a microorganism in several different ways. Morphological mutations change the microorganism colonial or cellular morphology. It is the case of the 5 mutant strains obtained from the wild-type *Monascus* MUCL 28962 that differ in growth rate, colony and microscopic morphology, enzyme and pigment production.

Gamma rays, indirectly generating of ionizing particles because of high energy photons that act on irradiated matter, give rise to fast electrons with important damages in DNA molecules. In the same manner, electron beams directly producing ionizing particles have enough energy to alter the DNA structure from fungal chromosome.

Enzymatic activity

Fungal colonies produce enzymes to convert the substrat molecules such as starch, proteins, lipides into a smaller ones which can come through the cellular enveloppe particularly the plasma membrane. In the first stage of catabolism, larger nutrient molecules (proteins, polysaccharides, and lipids) are hydrolyzed or otherwise broken down into their constituent parts. The chemical reactions occurring during this stage do not release much energy. Amino acids, monosaccharides, fatty acids, glycerol, and other products of the first stage are degraded to a few simpler molecules in the second stage of catabolism.

Many fungi degrade external polysaccharides by secreting hydrolytic enzymes that cleave polysaccharides into smaller molecules, which can then be assimilated. Starch and glycogen are hydrolyzed by amylases to glucose, maltose and other products. Cellulose is more difficult to digest; many fungi produce cellulases that hydrolyze cellulose to cellobiose and glucose.

Microorganisms frequently use lipids as energy sources. Triglycerides or triacylglycerols, esters of glycerol and fatty acids can be hydrolyzed to glycerol and fatty acids by microbial lipases.

Some fungi, particularly pathogenic, food spoilage, and soil microorganisms, can use proteins as their source of carbon and energy. They secrete protease enzymes that hydrolyze proteins and polypeptides to amino acids, which are transported into the cell and catabolized.

It was demonstrated that the parental strain does not produce any amylolytic, lypolytic, proteolytic or cellulolytic enzymes, while mutant strains have enzymatic activity. However, the wild-type strain can growth on solid starched substrates like rice to produce red, orange and yellow pigments but slower and less than mutant strains. This can be explain by the low amounts of amilases, cellulases and proteases produced by the parental strain. It seems

Table 2
THE RADIAL GROWTH RATES VALUES FOR WILD-TYPE AND MUTANT MONASCUS STRAIN

Strain	Time, hours	Colony diameter, mm	Radial growth rate, mm/hours
Wild-type	168	78	0.46
M1	216	76	0.35
M2	216	31	0.14
M3	216	57	0.26
M4	216	15	0.07
M5	216	83	0.38

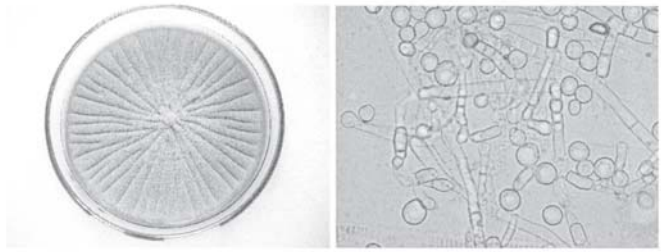

Fig.5. Morphological characteristics and microscopic view for wild-type Monascus strain

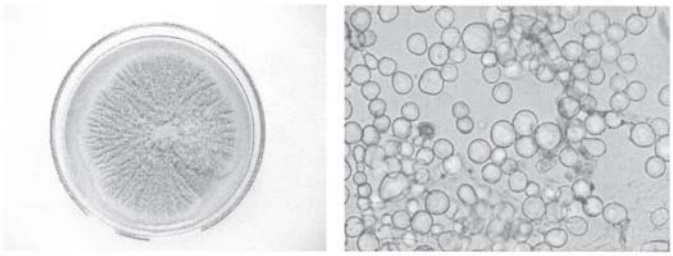

Fig.6. Morphological characteristics and microscopic view for M1 strain

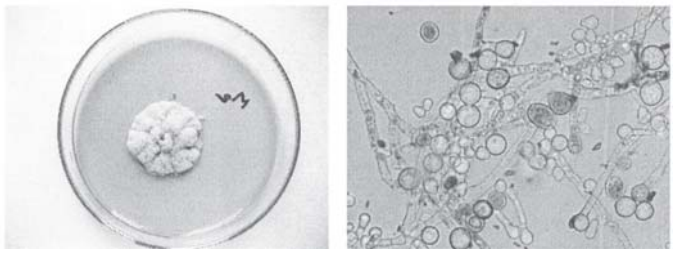

Fig.7. Morphological characteristics and microscopic view for M2 strain

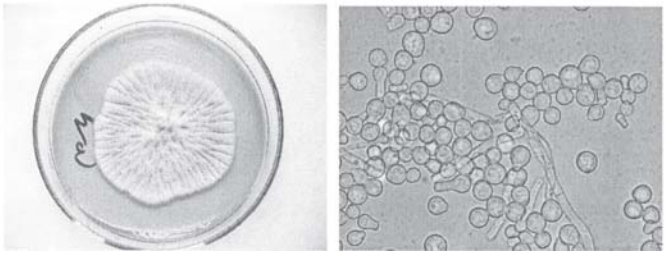

Fig.8. Morphological characteristics and microscopic view for M3 strain

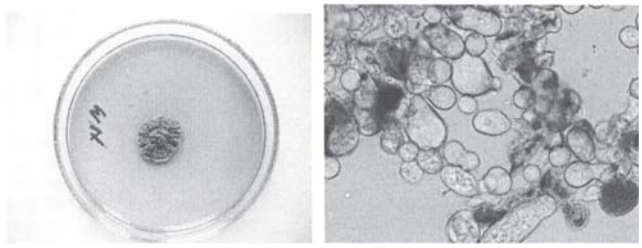

Fig.9. Morphological characteristics and microscopic view for M4 strain

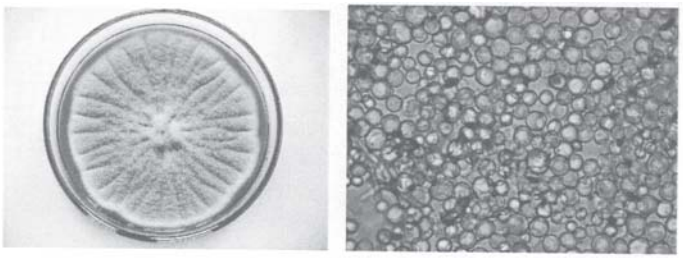

Fig.10. Morphological characteristics and microscopic view image for M5 strain

that these quantities of enzymes are undetectable in the conditions of method or the *Monascus* wild-type strain synthesises enzymes after a longer time than the mutant strains. The most productive are M2 and M4 strains that form high pigmented colonies. No strain produces cellulolitic enzymes.

Growth rate

Radial growth rate was estimate on glucose-yeast extract-agar medium, at the time before fungal colony reachs the edge of plate, 168 hours for wild-type strain and 216 hours for mutants. The growth rate shows highest value for wild-type strain against all other strains; M4 strain colony was the smallest one.

Also, for all *Monascus* mutants it was shown a slower growth rate correlated with a few modifications of microscopic appearance. The parental strain has the highest growth rate on petri dishes in relation with a high production of conidiospores and a undetectable enzymatic activity in terms of method. The results are displayed in figure 4.

The decline of mutants growth rate comparative with parental strain is correlated with a raised production of intra or extracellular pigments, which can be observed by aspect and color of colonies study. This should be explained by an inhibition of growth process because of pigments synthesis or by the competition for nutrients between growth and pigmentogenesis.

Table 3
ABSORBANCE VALUES FOR ETHANOL AND N-HEXANE EXTRACTS OF MONASCUS PIGMENTS

Ethanol extract	Abs_{400} nm	Abs_{510} nm	Abs_{510}/Abs_{400} (nuance)	% of Abs_{510} against wild-type strain	Hexan extract	Abs_{400} nm
wild-type	0.632	0.504	0.79	100	wild-type	0,064
M1	1.331	0.692	0.52	137	M1	0.223
M2	1.532	1.67	1.09	331	M2	1.717
M3	0.06	0.056	0.93	-	M3	0.124
M4	0.152	0.116	0.76	-	M4	0.027
M5	2.314	1.355	0.58	268	M5	0.891

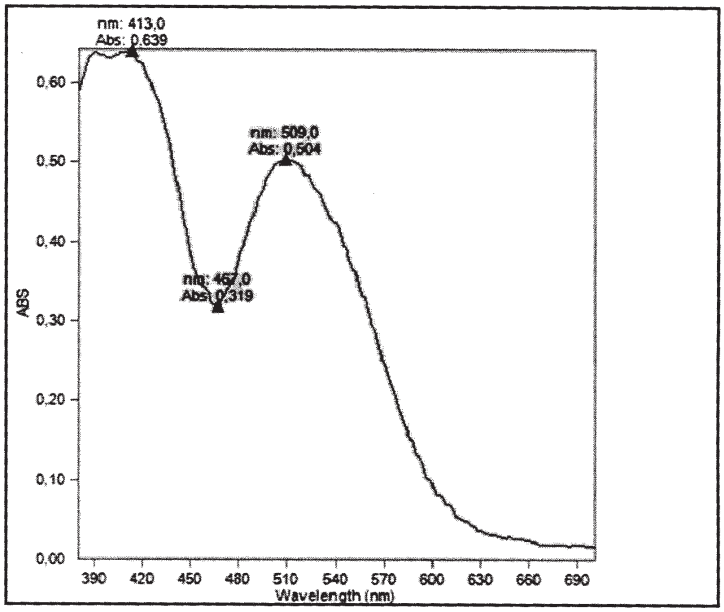

Fig.11. Absorption spectrum of ethanol extract from wild-type strain of Monascus ruber

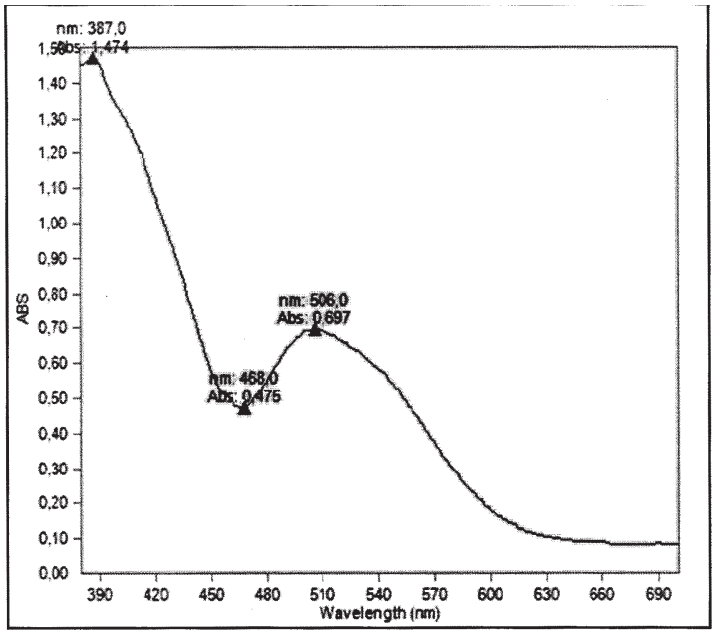

Fig.12. Absorption spectrum of ethanol extract from M1 strain of Monascus ruber

Morphological changes of mutants compared to wild-type strain

Microscopic slides (Olympus U-CMAD 3) showed enough important alterations of mutants' morphology. Concerning the hypha aspect, these are shorter and sometimes thinner, that, in correlation with the raised pigment production, should be explained by the inhibition of cell wall constituents' synthesis because of monascorubrin and rubropunctatin yield. Otherwise for all mutant strains there have been observed many hyphal areas or conidiospores strongly colored in red or orange. In this manner it should be explained the decrease of growth rate that seems to be in correlation with the pigment production. The slowdown of glucans, cellulose, chitin or other components of cell wall determines the decrease of growth and the increase of the hypha brittleness. This aspect is the most obvious for M4 mutant strain, with the lowest growth rate but strongly pigmented colonies.

Pigment biosynthesis

The biosynthesis of natural pigments was achieved in solid state fermentation on sterilized rice, at 30°C, for 14 days.

The biopigments were extracted in ethanol and n-hexan (1:100 ratio) and the absorbtion spectra were recorded for the parental and mutant strains and the OD values at 400 nm (yellow components) and 510 nm (red components)

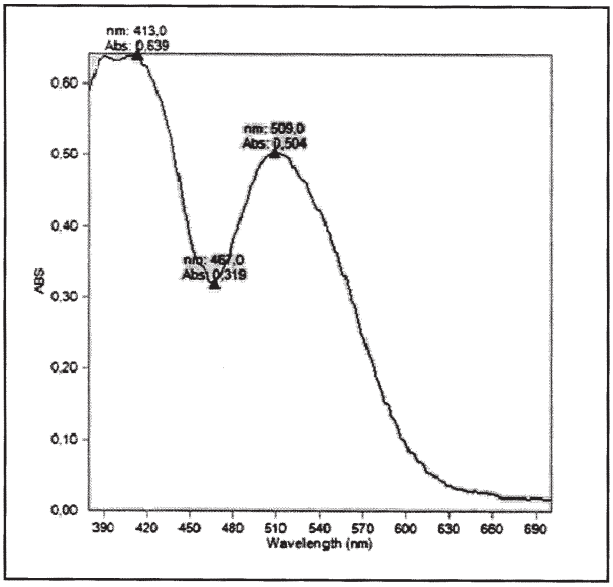

Fig.13. Absorption spectrum of ethanol extract from M2 strain of Monascus ruber

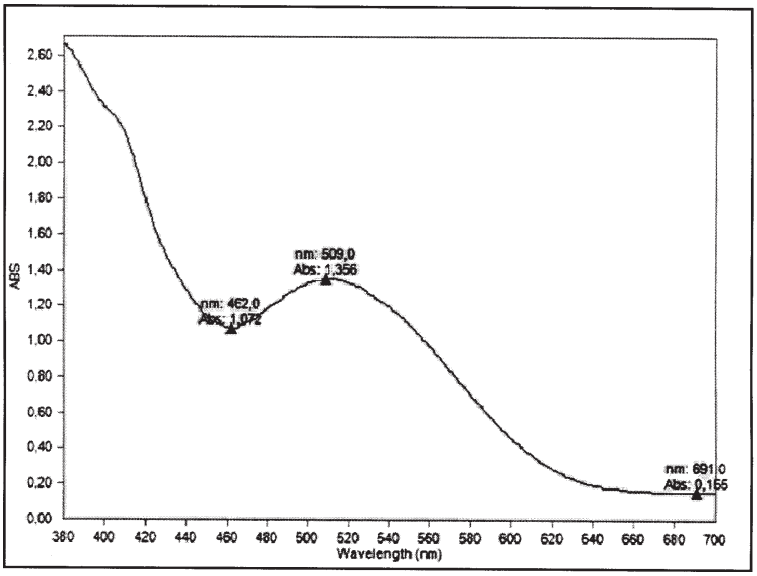

Fig.14. Absorption spectrum of ethanol extract from M5 strain of Monascus ruber

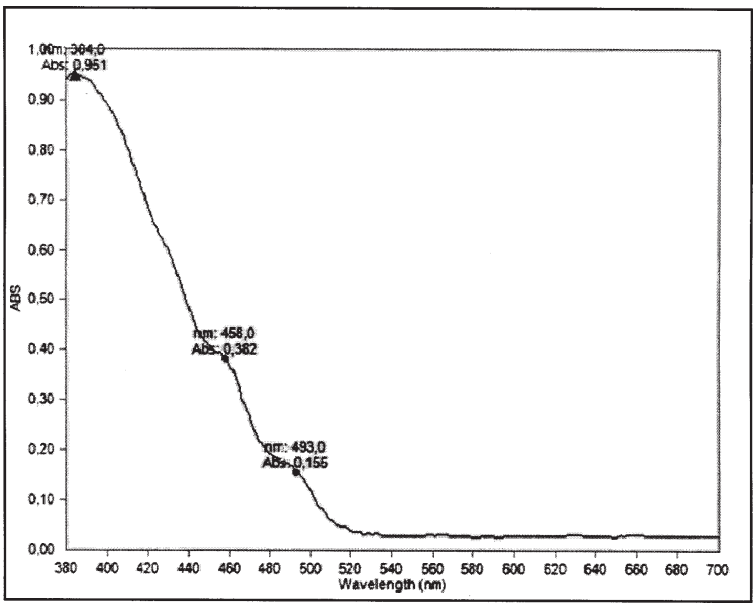

Fig.15. Absorption spectrum of hexan extract from M5 strain of Monascus ruber

were compared. The nuance calculated as Abs_{510}/Abs_{400} ratio (commercial use) was estimated too.

In all cultures on wet rice the *Monascus* strains produced yellow and red pigments as seen in the visible spectra.

The fermentation of rice shows that the M5 and M2 *Monascus* mutant strains produced the largest amount of red and yellow pigments, almost three times more than the parental strain. The M3 and M4 mutant strains produce a small amount of pigments in the solid state fermentation system. M4 strain produces less pigment probably because of the very low growth rate compared to other strains. The calculated nuances as $Abs_{510}nm/Abs_{400}$ nm ratio make us to choose the M2 strain producing more red and less yellow color, interesting for food industry.

Conclusions

Five mutant strains of *Monascus ruber* were obtained after gamma and electron beam irradiation at doses of 1-10 kGy and were characterized in terms of enzyme production, growth rate and pigmentogenesis. The wild-type strain does not produce any amylolytic, lypolytic, proteolytic or cellulolytic enzymes, while mutant strains have enzymatic activity. The enzymatic activity facilitates the absorption of extracellular nutrient components which are transformed into smaller molecules necessary for microbial growth.

For all *Monascus* mutants it were observed lower values of radial growth rate correlated with a few modifications of microscopic appearance. The parental strain has the highest growth rate on petri dishes and a high production of conidiospores.

The biosynthesis of natural pigments was carried out in solid state fermentation on sterilized wet rice and the biopigments were extracted in ethanol and hexane to measure the absorbancy values at 400 nm (yellow pigments) and at 510 nm (red pigments). M5 and M2 mutants produced the highest amount of red and yellow pigments.

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