

Production and assessment of physicochemical properties of pigments by Solid State and Submerged Fermentation from *Monascus ruber* MTCC 1880.

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ABSTRACT

Research is carried out with optimization of blending carbon sources such as rice grit, rice bran, and groundnut with nitrogen sources such as cottonseed oil cake (CSOC), mustard oil cake (MOC) and niger oil cake in 1: 1 and 2: 1 ratios for maximum production of microbial pigments, optimization of different variables viz. temperature, pH, incubation period, initial moisture and inoculum size in solid state (SSF) and submerged fermentation (SmF) for better recovery of microbial pigments alongwith isolation and partial purification of the extracted pigments. Pigments that are either natural or synthetic play an important role in the food industry as well as in the pharmaceutical industry, mostly for coloring wine, milk products, candy, and pharmaceutical dosage forms. Synthetic red pigments such as azorubin or tartrazin cause allergic effects. Other hand biological pigments are most cautious and useful comparison chemicals, So *Monascus rubar* 1880 using with method of solid state fermentation (SSF), submerged fermentation (SmF) also, highest total yield of pigments was recorded using carbon and nitrogen sources (RG: CSOC) in 2: 1 ratio by maintaining the optimum conditions. The pattern of thin layer chromatography (TLC) indicated that the extracted pigment obtained from both the methods of fermentation.

Keywords: Fermentation methods; pigments; *Monascus ruber*, TLC.

INTRODUCTION

Monascus is an ascomycetous fungus used for the production of food colouring, fermented foods and beverages (Martinkova and Patakova, 1999). Major components of *Monascus* pigments have anti-inflammatory activity and have been reported to suppress skin cancer caused by tumor promoters in experimental animals (Yasukawa *et al.*, 1994 and 1996), in addition to their clinical benefits in treating high blood pressure in humans (Kushiro *et al.*, 1996) It was shown that *M. ruber* is able to produce the mycotoxin citrinin (Blanc *et al.*, 1995). This mycotoxin has antibiotic activities against gram-positive bacteria, but its nephrotoxic properties (Blanc *et al.*, 1995) have limited its use as an antibiotic for therapeutic purposes. Consequently, the production of citrine together with the red pigments rules out the use of *M. ruber* as a

producer of natural colorants for food technology (Blanc *et al.*, 1995, Hajjaj *et al.*, 1997). *Monascus purpureus* belongs to the family Monascaceae and to the class Ascomyceta whose characteristic feature is the ability to produce secondary metabolites with strong yellow, orange or red pigmentation (Juszlova *et al.*, 1996; Pitt and Hocking, 1997). *Monascus* a native organism of China and Thailand can easily grow in several ecosystems and finds several uses, from conferring color to food products to medicinal uses and as meat preservative (Wong and Koehler, 1981; Watanade *et al.*, 1997). *Monascus* helps to lower blood cholesterol, prevent cancer, osteoporosis, stroke, alzheimer's disease and other dementias and muscular degeneration (Cesar *et al.*, 2005).

In the recent years, the number of permitted synthetic colorants has decreased because of undesirable toxic effect including mutagenicity and potential carcinogenicity and efforts to replace them with natural pigments have attracted world wide attention (Vidyalakshmi *et al.* 2009)^a. Though many natural colors are available, microbial colorants play a significant role as food coloring agent because of its flexibility in production and easy down stream processing. Among the various pigment producing microorganigams, *Monascus* is reported to produce non-toxic pigment, which can be used as food colorant. The pigment of *Monascus* improved the coloring appearance of foods and their organoleptic characters. Rice is one of the major agriculture products of economic importance. This primary product could serve as sustainable raw material for secondary value-added products through fermentation of *Monascus* molds.

Monascus sp., a filamentous fungus has been used to make rice wine, soy bean cheese and anka (red rice) in many Asian countries (especially Japan and China) for centuries (Hamano and Kilikian 2006). According to Juzlová *et al.* (1996) this fungus produces at least six types of pigments: two yellow-colored (ankaflavin and monascin), two orange-colored (rubropunctatin and monascorubrin) and two red-colored (rubropunctamine and monascorubramine).

MATERIALS AND METHODS

Monascus ruber MTCC 1880 was employed in the study. The culture was maintained in Malt extract agar (MEA containing malt extract-20g/l, glucose-20g/l, peptone-1g/l, agar-15g/l) at 30-32°C for 10 days. *Monascus* culture was streak inoculated on plates of MEA, PDA and MEA +NH₄NO₃ media and incubated at 300 C for 10 days for comparing the amount of pigment produced. For solid-state fermentation autoclaved rice and boiled autoclaved rice were used and for submerged fermentation MEB and PDB were used to propagate *Monascus ruber* MTCC 1880 culture.

Screening of carbon and nitrogen sources

For microbial pigment production, various low cost waste agro materials were used in order to get maximum production and better quality of end product. In this experiment, substrates such as rice grit (RG), rice bran (RB), groundnut shell (GNS) were taken as carbon sources whereas cotton seed oil cake (CSOC), mustard oil

cake (MOC) and niger oil cake (NOC) were used as nitrogen sources for the cultivation of microbe in the process of fermentation. Different combinations of carbon sources viz. RG, RB, GNS and nitrogen sources viz. CSOC, MOC and NOC were used as substrates for the growth of *Monascus ruber* MTCC 1880 employed for the production of microbial pigments.

| | | |
|-----|---|------|
| RG | : | CSOC |
| RG | : | MOC |
| RG | : | NOC |
| RB | : | CSOC |
| RB | : | MOC |
| RB | : | NOC |
| GNS | : | CSOC |
| GNS | : | MOC |
| GNS | : | NOC |

These 9 combinations of carbon and nitrogen sources were mixed into two proportions i.e. 1: 1 and 2:

1. Such combinations with different proportion were used as substrates in solid state fermentation (SSF) as well as submerged fermentation (SmF).

Cultivation of Fungus

Monascus ruber MTCC 1880 was used for cultivation in the present investigation. The culture was grown and maintained on potato dextrose agar (PDA) media.

Preparation of culture media and culture conditions

The contents of PDA (40 gm) were mixed properly in distilled water and then volume was made upto one litre. On cooling, sterilized slants were inoculated with *Monascus ruber* MTCC 1880 strain with help of sterilized inoculation needle in laminar air flow. Inoculated slants and petridishes were incubated at $28 \pm 2^\circ \text{C}$ for 7 days, and stored at 4°C for further use.

Optimization of blending different combinations of carbon and nitrogen sources for the maximum yield of microbial pigments using different fermentation methods

Based on the findings of earlier workers, it was observed that highest yield was recorded by maintaining a particular set of fermentation variables viz. temperature 30°C , pH 6.0, incubation period of 7 days and initial moisture content 60%. In order to know the best combination of carbon and nitrogen sources resulting into highest yield of microbial pigments, combination of substrates viz RG : CSOC, RG: MOC, RG: NOC, RB: CSOC, RB: MOC, RB: NOC, GNS: CSOC, GNS : MOC, GNS: NOC in the proportion of 1 : 1 and 2 : 1 were used in the process of fermentation.

Solid state fermentation (SSF)

The procedure described by Babitha *et al.* (2006)^a was adopted for conducting the experiments on solid state fermentation using various combinations of carbon and nitrogen sources as already mentioned above. Five grams of each combination of carbon and nitrogen sources were taken into a 250 ml Erlenmeyer flask. Initial moisture was set at 60% by adding the requisite amount of distilled water (Fig. 3.1). The contents of the flask were mixed and autoclaved at 121°C at 15 psi for 20 min. cooled and inoculated with 1.5 ml spore suspension (1×10^6 spors/ml) of *Monascus ruber* MTCC 1880. The process of fermentation was carried out on NBS shaker incubator at 30° C for 12 days. A particular combination of carbon and nitrogen source that resulted into the best pigment yield was selected for further experiments.

Submerged fermentation (SmF)

The procedure described by Lin *et al.* (1992) was adopted for conducting the experiment in submerged fermentation using various combinations of carbon and nitrogen sources. The chemical composition of growth medium for cultivation of *Monascus ruber* MTCC 1880 in submerged fermentation.

Physicochemical analysis of pigment solution

Optimization of different fermentation variables viz. initial moisture content, incubation temperature, pH, incubation period, inoculum size etc. in solid state and submerged fermentation for better recovery of microbial pigments

Based on the finding of previous experiments on different substrate combination of carbon and nitrogen sources with a set of fermentable variables using two different method of fermentation (SSF and SmF), the best combination of carbon and nitrogen source showing the higher yield of pigments was selected for further studies on optimization of fermentation variables for achieving the higher yield of microbial pigments. For the purpose of optimization of fermentation variables, different sets of conditions were used for two different methods of fermentation process.

Solid state fermentation (SSF)

The method of solid state fermentation was used for carrying out two different experiments for optimization of process parameters for attaining the maximum yield of microbial pigments. In the initial stage, first experiment was conducted at five levels of moisture contents viz. 50, 55, 60, 65 and 70% and second with four ranges of temperature viz. 25, 30, 35 and 40° C. Based on the result of earlier studies in this investigation, best combination of substrate consisting of rice grit (RG) and cotton seed oil cake (CSOC) in the ratio of 2: 1 was used in the medium as carbon and nitrogen sources for the growth of *Monascus ruber* MTCC 1880 with an incubation period of 12 days, as fixed in the earlier studies in the laboratory.



Plate 1: Culture plate of strain *Monascus ruber* MTCC 1880



2
3
4
Plate 2: Fermented biomass after fermentation period of 12 days
Plate 3: Incubator shaker used in investigation (New Brunswick Scientific Co.)
Plate 4: Fermented broth after fermentation period of 7 days

Fig 1:- Microbial culture of *Monascus ruber* on Petridis and fermentor unit

After optimizing these two process parameters (moisture content of medium and incubation temperature), other parameter i.e. incubation period was optimized using the same combination of carbon and nitrogen source (RG: CSOC, 2: 1). Keeping in view the findings of first experiment, the optimum conditions of process parameters (moisture content of 60% in medium and incubation temperature of 30°C) were maintained for optimizing the other process parameters. By maintaining the optimum condition of moisture in medium and temperature of incubation, cultivation of *Monascus ruber* MTCC 1880 was carried out for different incubation periods viz. 4, 6, 8, 10, 12 and 14 days with varied inoculum size viz. 0.5×10^6 spores/ml, 1×10^6 spores/ml, 1.5×10^6 spores/ml, 2.0×10^6 spores/ml and 2.5×10^6 spores/ml, so as to know the optimum incubation period and inoculum size for attaining the maximum yield of microbial pigments. The method employed for cultivation of fungal strain *Monascus ruber* MTCC 1880 was the same as reported earlier in this investigation.

Submerged fermentation (SmF)

Similar to solid state fermentation method, submerged fermentation process was also used for carrying out optimization of different fermentation variables in order to get maximum yield of microbial pigment. First experiment was conducted at 4 levels of pH viz. 5.0, 5.5, 6.0 and 6.5 and second with four ranges of temperature viz. 25, 30, 35 and 40° C. In the process of optimization of these fermentation variables in

submerged fermentation, substrate combination of rice grit (RG): cotton seed oil Cake (CSOC) in 2: 1 ratio, selected from earlier experiments in this study, was used as carbon and nitrogen source for the growth of fungus with an incubation period of 7 days as fixed in earlier studies in the laboratory.

After optimizing these two fermentation variables (pH of medium and incubation temperature), other variables like incubation period and inoculum size were optimized using the same combination of carbon and nitrogen source. Based on the finding of the first experiment, the optimum conditions of fermentation variables (pH of 6.0 in medium and incubation temperature of 30° C) were used for the growth of fungal strain in order to optimize the other variable. Keeping these two variables (pH and temperature) at optimum condition, cultivation of *Monascus ruber* MTCC 1880 was done for different incubation periods viz. 4, 5, 6, 7 and 8 days. Likewise, to find out the effect of varied inoculum size of pigments yield in submerged fermentation, the blend of substrate was inoculated with different inoculum size viz. 0.5×10^6 spores/ml, 1.0×10^6 spores/ml, 1.5×10^6 spores/ml, 2.0×10^6 spores/ml and 2.5×10^6 spores/ml. The process for cultivation of fungal strain was the same as mentioned earlier.

Extraction of pigments

Extraction of total crude pigment produced by SSF, 5 gm of the fermented biomass was suspended in 25 ml of acetone, incubated on rotary shaker at 30° C with 200 rpm for an hour. After this, the pigment extracted in acetone was decanted. This step was repeated till all the pigment was extracted completely from the fermented biomass. The total crude pigment extract in acetone was pooled together and flash evaporated in a rotary vacuum evaporator at 40° C with 100-120 rpm under 556 mbar vacuum to dryness to remove the acetone traces with the pigment. The dried extract was taken in 10 ml of distilled ethanol for spectrophotometric analysis.

Extraction of pigment produced by SmF

Intracellular pigment

The washed mycelia was suspended in 25 ml of 80% alcohol, incubated on a rotary shaker (150 rpm) at 30° C for 30 min. and filtered. Extraction was repeated 2-3 times. Finally the filtered extracts were pooled together and made upto 50 ml.

Estimation of dry weight from fermented biomass obtained from SSF and SmF

After extraction of total pigment, the fermented biomass was taken in a preweighed aluminium dish to which about 5 ml of ethanol was added. The dish was kept in an oven maintained at 105° C. After about 3-4 hour. the dish was transferred to a desiccator with the help of forceps. When the temperature of the dish reached ambient temperature, the dish with the dry fermented biomass was weighed. Drying was continued till the constant weight was obtained. The difference in weight gave the moisture content of fermented mass.

Estimation of pigments (SSF and SmF)

After suitable dilution of dry biomass with respective organic solvent, 2ml of each pigment extract was scanned in UV spectrophotometer with same solvent as blank. Optical density (O.D.) was measured at 500, 475, and 375 nm wavelength corresponding to red, orange and yellow pigment respectively. The pigment yield (OD units/g. dry biomass) of individual pigments was calculated using the following formula :

$$\text{Pigment yield (O.D. units/ gm dry biomass substrate)} = \frac{\text{O.D. (Abs)} \times \text{Dilution factor} \times \text{Total vol. of pigment}}{\text{Dry weight fermented mass}}$$

Isolation and partial purification of the extracted pigments

The dilutions of the extracted pigments were concentrated at low temperature under vacuum in a rotary vacuum evaporator. The concentrated extract was further separated for the isolation of compounds using the method described by Babitha *et al.* (2006)^a.



Partial purification of the crude filtered extract obtained from solid-state fermentation using column chromatography



Partial purification of the crude filtered extract obtained from submerged fermentation using column chromatography

Fig 2:- Partial purification of the crude filtered extract obtained from solid state and submerged fermentation using chromatography.

Thin layer chromatography (TLC)

For preparation of TLC chamber the developing solvent (30 ml of Benzene, 10 ml. of methanol, 9 ml of chloroform), was placed into a TLC chamber. The solvent was covered the bottom of the chamber to a depth of approximately 0.5 cm. The chamber was closed, shaken and then kept covered so that evaporation doesn't change the composition of the developing solvent mixture. After 15 minutes, the chamber was saturated with the solvent vapour.

Microbial pigment production in solid state fermentation (SSF)

Yield of microbial pigments in SSF showed that the initial moisture level of 60%, incubation temperature of 30° C, inoculum size (1.5×10^6 spores/ml.) and incubation period of 12 days were found to be optimum and gave highest total pigment yield of 165.4 OD unit/g dry biomass consisting of 59.1 for yellow, 56.2 for orange and 50.1 OD units/g dry biomass for red pigment using RG: CSOC combination in 2: 1 ratio. The observations record that the gradual increase in the incubation period, there was gradual increase in the yield of pigment. The incubation period of 12 days was found to be optimum for obtaining maximum yield of different fractions of pigment. After the incubation period of 12 days, yield of pigment was slightly decreased to total pigment yield of 153.9 OD units/g biomass consisting of 55.3 for yellow, 50.4 for orange and 48.2 OD units/g dry biomass for red.

In the present investigation, it was observed that formation of polyketide pigment was highly sensitive to the initial moisture content of different blends. Following fermentation, blends of mixed substrate with initial moisture content of 55% and below appeared dry with poor growth of *Monascus* whereas blend with 60% of initial moisture showed better growth and pigment production and with high moisture at 65% and above, substrate blends became too stickly hindering the growth of mold and thereby pigment synthesis and its secretion into the substrate.

Likewise, the fermentation temperature was a very important and sensitive factor that controls the synthesis of pigments. Maximum total pigment yield of 165.4 OD units/g dry biomass was recorded at 30° C followed by 25° C (148.0 OD units/g dry biomass), 35° C (134.0 OD units/g dry biomass) and 40° C (89.2 OD/units/g dry biomass). Higher incubation temperature might have resulted in severe moisture loss thereby altering the water activity of substrate blend.

A large number of reports have been published in the literature on the yield of microbial pigments using different substrates and fermentation variables (Mukherjee *et al.* 2003, Babitha *et al.* 2006 and Ochaikul *et al.* 2006). In a study conducted by Babitha *et al.* (2006), it was reported that under optimization conditions such as initial moisture 60%, temperature 30° C, inoculum size 1.5×10^6 Spore/ml and incubation period 12 days, the maximum pigment production was obtained. It was also reported that pigment production decreased above or below the optimum conditions.

Microbial pigment production in submerged fermentation (SmF)

Microbial pigments in submerged fermentation showed that the initial pH of 6.0, incubation temperature 30° C, inoculum size of 1.5×10^6 (spores/ml) and incubation period of 7 days were found to be optimum for obtaining maximum total yield of pigment (724.7 O.D. units/g dry biomass) which comprised of 200.0 for yellow, 190.1 for orange and 182.3 O.D. units/g dry biomass for red pigment as extracellular pigment whereas intracellular consisted of 55.1 for yellow, 50.1 for orange and 47.1 O.D. units/g by biomass for red pigment using RG: CSOC substrate combination in 2 : 1 ratio. These observations showed that the blending of RG: CSOC in 2 : 1 ratio might have played some role by meeting the requirements of essential nutrients for the better growth of strain used for bioconversion of carbon and nitrogen sources into the microbial pigments. These findings also revealed that with the relative increase in carbon level in different blends, there was a gradual decrease in the yield of microbial pigments.

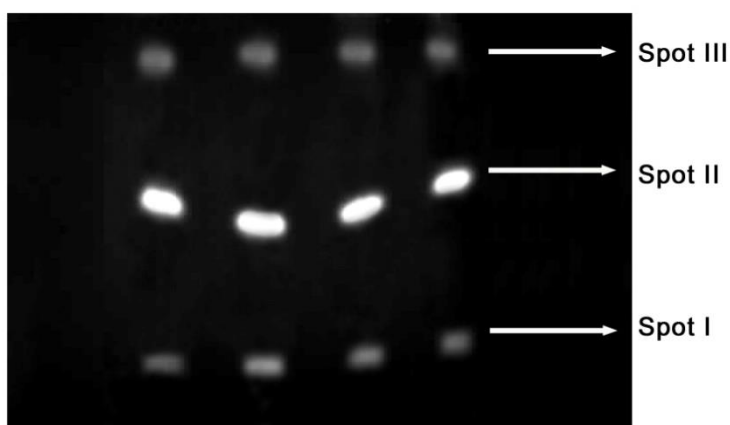


Fig 3:-Thin layer chromatography of pigment extracted from solid state fermentation

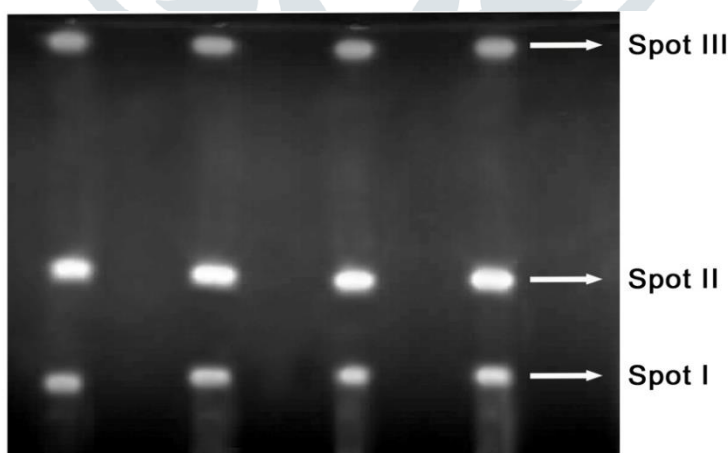


Fig 4:-Thin layer chromatography of pigment extracted from submerged fermentation (SmF)

Results

The observations recorded in the present investigation revealed that maximum production of pigments (724.7 OD units/g dry biomass) was obtained at initial pH of 6.0 and it got decreased with the increase or decrease in the pH value of 6.0. Likewise, incubation temperature also played a very important role in attaining the maximum yield of microbial pigment. The investigation showed that an incubation temperature of 30° C was found to be optimum for achieving the maximum yield of pigment. The yield of microbial pigment got decreased with the increase or decrease in the incubation temperature from the optimum level of 30° C. Another fermentation variable, inoculum size also played a key role in obtaining maximum pigment yield. The inoculum size of 1.5×10^6 (spores/ml) was found to be optimum for attaining the maximum yield of pigment. This pigment yield got decreased with the decrease on increase in the inoculum size from the optimum level of 1.5×10^6 (spores/ml.)

The incubation period of 7 days was found to be optimum for obtaining the maximum total yield (724.7 OD units/g dry biomass) of pigments consisting of 200.0 for yellow, 190.1 for orange and 182.3 OD units/g dry biomass for red pigment as extra cellular pigments whereas intracellular pigment yield comprised of 55.1 for yellow, 50.1 for orange and 47.1 OD unit/g dry biomass for red pigment fraction under a particular set of fermentation conditions. After the incubation period of 7 days, yield of pigment got slightly decreased.

A large number of reports have been published in the literature on the yield of microbial pigments using different substrates and fermentation variables (Lee *et al.* 2001, Mukherjee *et al.* 2003 and Silvana *et al.* 2008). In a study conducted by Mukherjee *et al.* (2003) it was reported that the maximum production was observed at the end of 5 day of fermentation in shake culture at 35° C and at pH 6.0. Thus results of present investigations at large were also in accordance to the findings of earlier workers. The slight variations in the findings of present investigation might be due to the type of substrates and methodology used along with the varied fermentation conditions. Based on the results of this study it could be concluded that yield of microbial pigments obtained in SmF was found to be better than yield of microbial pigments in SSF under optimum conditions of different fermentation variables.

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