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Optimization of Fermentation Conditions and Effect of Nutritional Supplementation in Production of Food Grade Pigments by *Monascus ruber*

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Monascus ruber culture was cultured in submerged and Solid-state fermentation using rice as a substrate for the production of pigments using a fungal culture of _M. ruber_. It was used to study the regulation of pigment production by nitrogen sources. We found that the formation of red pigments by yeast extract as the sole nitrogen source. In this study, yeast extract was found to yield maximum pigment 0.524 (370nm), 1.084 (400nm) and 0.616 (500nm) Units/g of red yeast rice for orange, yellow and red pigments respectively. The highest red yeast rice yield of 21% was recorded in yeast extract treatment. The inorganic nitrogen sources were found to produce better results. It was used to study the regulation of pigment production by carbon sources. We found that the formation of red pigments by starch as the sole carbon source. In this study, starch was found to yield maximum pigment 0.599 (370 nm), 1.058 (400 nm) and 0.639 (500 nm) U/g of red yeast rice for orange, yellow and red pigments respectively. The highest red yeast rice yield of 19 % was recorded in starch treatment. Therefore it is concluded from the study the best nitrogen source is yeast extract and the carbon source is starch for culturing of _Monascus ruber_ in solid state fermentation.

Key words: *Monascus ruber*, Red Yeast Rice, Natural Pigments, Monascorubin, food coloring agent, Spectrophotometer.

There is a worldwide interest in process development for the production of pigments from natural sources due to a serious safety problem with many artificial synthetic colorants which have widely been used in foodstuff, cosmetic and pharmaceutical manufacturing processes (Kim *et al*., 1995).

There are number of microbes which have the ability to produce pigments in high yield, including species of *Monascus, Paecilomyces, Serratia, Cordyceps, Streptomyces* and yellow red and blue components produced by *Penicillium.*

Monascus produce at least six major related pigments which can be categorized into three groups based on colour as follows. Two yellow-colored (ankaflavin and monascin), two orange-colored (rubropunctatin and monascorubrin) and two red-colored (rubropunctamine and monascorubramine). (Chen and Johns *et al*., 1993). Generally, *Monascus* pigments are intracellular and they are insoluble in water. But appropriate cultivation conditions (mainly the pH and the use of monosodium glutamate as nitrogen source) can result in extracellular and water-soluble pigments (Lin and Demain, 1991; Jung *et al*., 2003).

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The common names of this fungal product are red yeast rice, red rice, angkak, red leaven, *beni- koji* (Japanese), *hung-chu, hong qu, zhitai* (Chinese), *rotschimmelreis* (Europe), red mould (USA). Angkak or red yeast rice is a product resulting from rice fermentation using *Monascus spp*. It has been used extensively in Asia as a natural food colorant in fish, Chinese cheese, red wine and sausages .

M. ruber red yeast rice is an effective natural dietary supplement for controlling serum cholesterol. Secondary metabolites, Monacolin (J, L, X, M) from *M. ruber* fermented rice acts as the potent hypocholesterolemic agent. The implementation of *Monascus* pigment as a coloring agent in food provided an additional advantage of specific flavor in the products. Product is supported by long-standing traditional use by millions of people. The applications of some synthetic colorants in food such as azorubin or tartrazin have been limited due to their possible allergeric effects (Multon, 1992). It is possible to use Angkak as food colorant in order to avoid allergic problem.

MATERIAL AND METHODS

Culture collection and maintenance

Pure culture of the M- 410 - *Monascus ruber* was maintained at Indian Institute of crop processing technology, Tanjore.

Pigment production

Yeast phosphate soluble starch agar medium was used for pigment production. (Component -gm/L ,Yeast extracts - 4gm, Soluble starch - 15gm, Di potassium hydrogen phosphate - 1gm, Potassium di hydrogen phosphate - 1gm, Agar - 20gm, pH- 6.5. The above components of the medium were weighed and dissolved in 1000 ml of distilled water). After that the final pH of the medium was set to 6.5 with the help of pH meter. Then the medium was autoclaved at 121°C at 15lbs for 15 minutes. After sterilization the medium was poured into the sterile petridishes inside the laminar air flow chamber.

Pigment Production Using Submerged Fermentation

Yeast phosphate soluble starch broth was prepared and sterilized at 121°C at 15lbs for 15 minutes. After sterilization, the medium was inoculated with the *Monascus ruber* and incubated for 7-15 days at 25-30°C.

Effect of incubation period on pigment production

Yeast phosphate soluble starch agar media was prepared were autoclaved at 121°C at 15 lbs pressure for 15 mins. After sterilization, each conical flasks containing 10ml of medium was inoculated with 1ml of the broth culture of *Monascus ruber* and shake well for even spreading of culture. After inoculation, inoculated media were incubated at room temperature. After every two days interval, pigments were extracted and estimated using UV spectrophotometer.

Isolation and extraction of pigments

Extracellular pigments

After incubation period the culture was sterilized at 121⁰C at 15lbs pressure for 15mins. After sterilization, the broth was filtered through Whatman No. 1 filter paper. Then the fungal mat was washed twice with 10ml of distilled water. Pigments were measured at 370nm(Orange), 400nm (Yellow) and 500 nm using UV Spectrophotometer.

Intracellular pigments

After filtering the extracellular pigments, the fungal mat was collected. The moisture of the fungal mat was dried through filter paper and the wet weight of the mat was measured. The fungal mat was homogenized with 75% of ethanol and then it was made up to 50ml with 75% of ethanol. This was taken for pigment estimation (Fig. 1).

Optimization of Pigment production using Solid State Fermentation

Effect of Carbon sources

Rice broken was used as substrate for production of pigments given in figure 2.10g of broken rice was added with 25 ml of distilled water. Rice media supplemented with the carbon sources like Starch, Mannitol, Lactose, and Fructose at the concentration of 3% (Fig. 3).

Treatments

T1- Rice + Starch; T2- Rice + Mannitol
T3- Rice + Lactose; T4- Rice + Fructose
T5-Rice (Control)

After the media was autoclaved at 15lbs for 15 mins. After sterilization, the medium was inoculated with the Monascus ruber and incubated for 7-14 days at 25-30°C.

Estimation of pigment in red yeast rice powder

0.1 g of red rice powder was taken and added with 10 ml of 75% ethanol. It was sonicated at 120 w for 60min. Then it was centrifuged at 3000rpm at 4^0C for 10 min. The supernatant was collected. The above extraction procedure was repeated three times. The obtained 30ml of supernatant was made up to 50ml with 75%of ethanol. After standing for 30 min, the solution was filtered through, 0.2 micrometer membrane The yellow, orange and red pigments of *Monascus spp* was detected by a spectro- photometer at 400, 470, 500 nm, respectively (Chen and Johns *et al*., 1993).

RESULTS

Monascus ruber culture was cultured on yeast phosphate soluble starch broth (Table 1 and Fig. 1) and agar for pigment production.

Effect of Incubation period on pigment production

The growth of *Monascus ruber* was periodically monitored the intervals of every 2 days up to 14days. The pigment production was increased as the increase in the incubation period of the *Monascus ruber* (Fig. 4 & 5).

In order to study the influence of nitrogen and carbon source on the pigment production by *M.ruber*, the strain has been cultivated on rice supplemented with different nitrogen and carbon sources. The results are presented in this chapter.

Effect of carbon sources on pigment production using *M.ruber*

The growth was periodically monitored at intervals of 2, 4, 6, 7 and 14 days. On the second day a slight growth of *M.ruber* was observed in all treatments. After14 days of incubation, improved growth and pigment production was observed in carbon supplemented samples. The treatment containing starch favoured more growth of *M.ruber* when compared to other treatments. The starch treatment had an appealing red appearance, while other carbon sources produced faint or foggy red coloration. The results are presented in the Table 1.

Pigment yield

From the spectral analysis, slight shift in absorbance maxima was observed for different carbon sources. In this study, starch was found to yield maximum pigment 0.599 (370nm), 1.058 (400nm) and 0.639 (500nm) U/g of red yeast rice for orange, yellow and red pigments respectively. Lactose yielded 0.451, 0.713 and 0.511 U/g of

Table 1. Carbon sources

Treatments	After 2 days	After 7 days	After 15 days
Rice + starch	+	+++	+++
Rice + mannitol	+	++	++
Rice + fructose	+	++	++
Rice + lactose	+	++	+++
Control	+	++	++

+ Slight growth; ++ Dense growth; +++ Dense growth with dark pigmentation

Table 2. Influence of different carbon sources on Red yeast rice yield

Weight of raw rice(g)	Weight of steamed rice(g)	Carbon sources	Carbon sources(g)	Dried weight of red rice(g)	%yield
10	30	Starch	0.3	5.7	19.0
10	30	Mannitol	0.3	5.1	17.0
10	30	Lactose	0.3	5.37	17.9
10	30	Fructose	0.3	5.25	17.5
10	30	control	-	5.04	16.8

Fig. 1. Extraction of Intra and Extra cellular pigments

Fig. 2. Monascus pigment production using Solid state fermentation

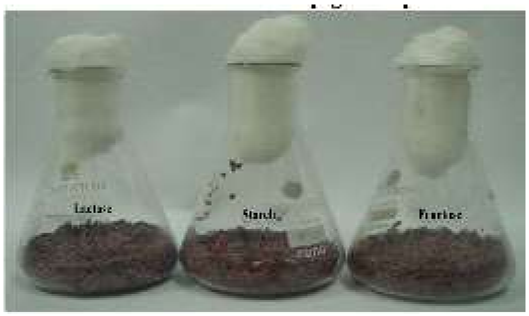

Fig. 3. Effect of Carbon sources for pigment production using Solid state fermentation

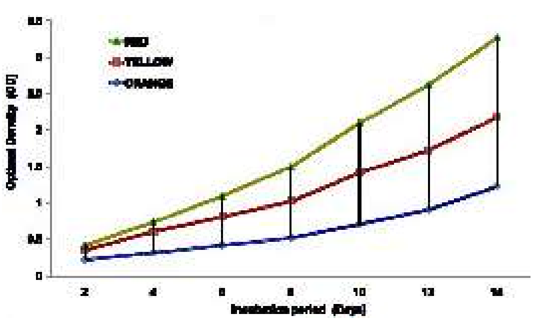

Fig. 4. Effect of Incubation period on pigment production (Intracellular pigments)

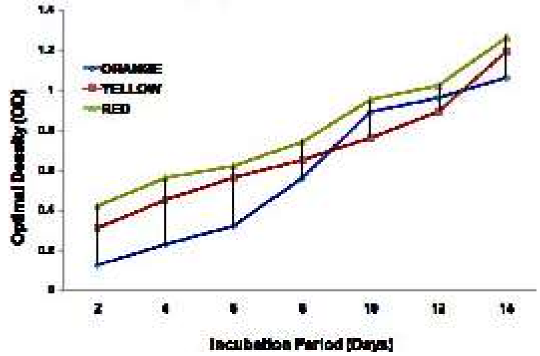

Fig. 5. Effect of Incubation period on pigment production (Extracellular pigments)

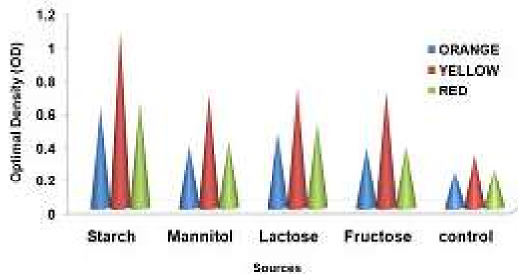

Fig. 6. Effect of Carbon source for Pigment Yield

Fig. 7. Red yeast rice yield

red yeast rice production. Compared to the control addition of carbon increased the yield of pigment. The carbon source, starch increased pigment production by *M.ruber*. The results are presented in the Fig: 6.

Red yeast rice yield

The red yeast rice yield was collected and the results were shown in Fig: 7 & Table 2. The yield content varied from 19 % to17%. All the treatments were found to be significantly different. The highest yield of was recorded in yeast extract treatment. This was followed by Lactose 17.9 % and fructose 17.5%. Lesser yield was registered in control of 16.8%.

DISCUSSION

The results obtained in the study of the influence of incubation period, nitrogen sources and carbon sources on pigment production by *M.ruber* are discussed in this chapter.

Growth of *M.Ruber* in rice

The growth observation was monitored at 2, 4, 7 and 14 days. It is well known that utilization of carbon sources affect the growth and pigment production (Wong *et al.,* 1981, Shepherd *et al*., 1983). During growth *Monascus spp* breaks down starch substrate into several metabolites. The structures of pigments as secondary metabolites depend on substrate types and mother specific factors during cultivation such as pH, temperature, and moisture content. Carbon (glucose, maltose) may be used to induce pigment production in *M.ruber* (Lin *et al*., 1991).

Carbon source, nitrogen source and pH have been shown to influence pigment production by *Monascus ruber* (Su, 1978; Wong *et al*., 1981; Lin and Demain 1991; Chen and Johns 1993). Pigments produced by *M. ruber* offer a possible alternative to certified food dyes or natural pigments used currently (Dweek, 2002). The solid-state culture technique is preferred to the submerged culture technique because the solid state technique is simple, requires less capital investment lower levels of catabolite repression and end product inhibition, produces lower amount of waste water output. (Lee, Piao, 2002). In the above study pigment estimation is carried out in the solid state fermentation in which rice used as the substrate supplemented with carbon sources.

Pigment yield

From the results obtained yeast extract was found to be better for both red (0.616 U/g of red yeast rice) and yellow pigment (1.084 U/g of red yeast rice) production. Regarding nitrogen source utilization, yeast extract and ammonium sulphate were preferred by *Monascus sp*., strains for pigment production (blanc *et al*., 1995). There exists some controversy in the literatures as the best nitrogen source for red pigment production with organic nitrogen (Yoshimura *et al*., 1975, Su and Huang 1980). The effect of organic nitrogen is complicated, because it may serve as carbon source and can promote the protein bound dissolution of red pigments into the culture broth. Pigment secretion into the medium was favoured at pH 6.5, particularly for red pigments and by the use of peptone.

The *Monascus* sp. appeared to utilize maltose better, while sucrose and lactose were utilized well by *M. ruber. M. ruber* utilized galactose well,but starch, mannitol and glycerol were poorly utilized. *M. ruber* utilized raffinose and starch verywell. The Monascus sp. utilized starch well, but not when compared with *M. ruber* (Omamor *et al*., 2008). This confirms the result obtained in which starch is best carbon source for culturing of *Monascus ruber.*

Red yeast rice yield

Yeast extracts stimulated condition, repressed the sexual cycle and increased biomass production. Due to the formation of copious amount of conidia, pigment production remained at a relatively low level. Sodium nitrate supported sporulation, limited the growth and gave intermediate pigment yields; the use of ammonium chloride resulted in a repression of condition and the sexual cycle and led to the best pigment interactions giving origin to red pigments. In addition to ammonium chloride, peptone also yielded superior growth and pigment amounts when compared with sodium nitrate.

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