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ORIGINAL ARTICLE

Taxonomic Investigation of Commercially Important Pigment Producing Fungi *Monascus purpureus*

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ABSTRACT
Monascus sps are well known as producers of structurally related hexaketide pigments which are yellow and red in colour. The use of Monascus pigments as substitutes for synthetic colorants were promoted by their interesting features like therapeutic properties and relatively high stability with respect to pH and temperature. Natural sources extracting dyes to avoid the environmental pollution and also to avoid toxic and allergic reactions associated with synthetic dyes. These natural dyes have emerged as an important alternative to synthetic dyes. Pigments produced from the Monascus were used as a natural food colorant in Oriental countries and these pigments were most stable and edible when comparing to other pigments. Monascus producing pigments were converted to synthetic dye for commercially availability. So this study becomes important because, taxonomical investigation of commercially important organism is crucial. Taxonomical identification of Monascus purpureus was carried out by Lacto Phenol Cotton Blue (LPCB) staining technique, morpohological observation of growth characters on different media, different temperature and different pH. The shapes, colour, type and their ability to form conidia, aleiroconida, peritheca and ascospore were identified. Susceptibility and Resistance Marker against various antifungal agent were also observed.
Keywords*: Monascus purpureus, pigments, synthetic dyes, hexaketide, peritheca, conidia, ascospore, aleiroconida, aerial mycelium , septated hyphae.*

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INTRODUCTION

Monascus is a filamentous and non pathogenic fungus belonging to the genus *Monascus,* family Monascaceae and class Ascomyceta. This has the power to synthesis bio pigments with connected polyketide structure. *Monascus* sp produces six primary pigments, the colours of which are yellow (anka flavin, monascine), orange (rubropunctatin , monascorubrine) and red (rubropuntantamine, monascorubramine) pigment [1]. The *Monascus* species is a Chinese traditional fermentation fungus used on food for over thousands of years in China, and its special effects and application on food were recorded in ancient Chinese records [3]. Red pigments producing *Monascus* sp has been used in the food industry as food colorants with wide applications on meat, fish, ketch up, liquor, etc. It is a best alternative to synthetic colorants that show carcinogenic and teratogenic effects, e.g. the nitrosamines formed from nitrites and nitrates in cured meats [4]. *Monascus purpureus* is used to treat various diseases: infections, diarrhea and indigestion. This information has occurred since ancient times, from Ming Dynasty (1368 - 1644), and the traditional use of *Monascus* is obtained from Japan, China, Korea, India, USA and Germany medical books In China it is known as "Ang kak" or "Hong Qu" , in Japan "Koji" [5]. *Monascus purpureus* is an effective natural dietary supplement for controlling serum cholesterol. It can convert starchy substrates into several metabolites such as alcohols, antibiotic agents, antihypertensives, enzymes, fatty acids, flavour compounds, flocculants, ketones, organic acids, pigments and vitamins [7]. The aim of this study is to carry out taxonomical investigation of *Monascus purpureus* and its marker identification. This will be certainly helpful in strain improvement of *Monascus purpureus* ,which will in turn be commercially beneficial.

MATERIAL AND METHODS

Microorganism and Culture media

Monascus purpureus was obtained from microbial culture collection, Chandigarh, (MTCC) India. The culture was grown on potato dextrose agar medium (HI media) incubated at 25^0Cfor 14 days. spores were collected by flooding the surface of the plates with ~ 5ml sterile saline solution (NaCl, 8.5g/l water) containing Tween 80 (0.1%v/v). The spores were counted using a haemocytometer, and the spore suspension was standardized to concentration of 10^7 spores/ml by dilution with sterile water before use. The viability of was checked using quantitative colony counts at 10^7 CFU/ml.

Taxonomical Investigation

The isolated strain of Monascus *purpureus* was grown for 7 days on different growth culture media: PDA(potato dextrose agar) containing (g/l):Potato infusion from-200.00g/l,Dextrose-20.00g/l,Agar-30.00g/l, Final pH at(25°C)5.6±0.2.SDA (Sabouraud Dextrose Agar) containing (g/l): Mycological peptone -10.00g/l, Dextrose-40.00 g/l,Agar15.00g/l, Final pH at(25°C)5.6±0.2. BM(Basal Media) containing(g/l): Dextrose-70.00g/l,Peptone-7g/l, potassium nitrate-0.14g/l, ammonium di hydrogen phosphate-0.14g/l, magnesium sulphate-0.35g/l, calcium chloride–0.07g/l.All culture media were sterilised at 121°C. The cultivation was performed in petri dishes at 25°Cfor 7days for all culture media. Additionally cultivation with pH difference, pH4, pH5 and pH6 at 25°C was carried out. During the cultivation, morphological and cultural characteristics of *Monascus purpureus* was observed using Lacto Phenol Cotton Blue (LPCB) staining technique.

MARKER IDENTIFICATION

Sensitivity of fungi to fungicide on PDA Agar well cutting method and Disc diffusion method (Anitha et al.,2014). The sensitivity and resistance test was performed with the copper sulphate, Indofil and Hexa Antimyco-01 discs. against the test fungi (*Monascus purpureus*).By using agar well cutting method. 50µl of 50mM, 100mM, 150mM, 200mM concentration Indofil were loaded on to well and were incubated at 25 0 C for 3-5 days.

RESULT AND DISCUSSION

In the present study, an attempt has made to evaluate the taxonomical identification of *Monascus purpureus.* The main morphological and cultural characteristics of the investigated fungus is shown in Table 1.

Growth of *Monascus purpureus* on different media with different temperature

The Figure 1 shows the growth pattern at 25 ^{0}C and 37 ^{0}C on PDA Medium . Red colour Pigment release was observed during the colony formation, short white and yellow colour aerial mycelium was found at 25°C.

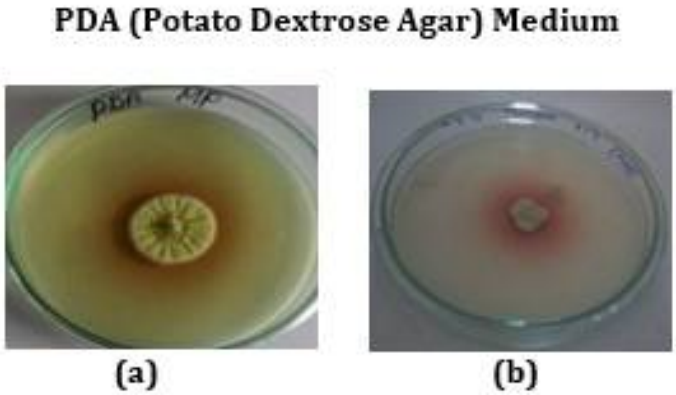

Fig: 1. Growth of *Monascus purpureus* on PDA medium (a) 25 ^{0}C and (b) 37 ^{0}C

The growth rate was higher in 25^{o}C when comparing to 37^{o}C Yellow coloured Pigment production was observed around both the colonies Figure 2.

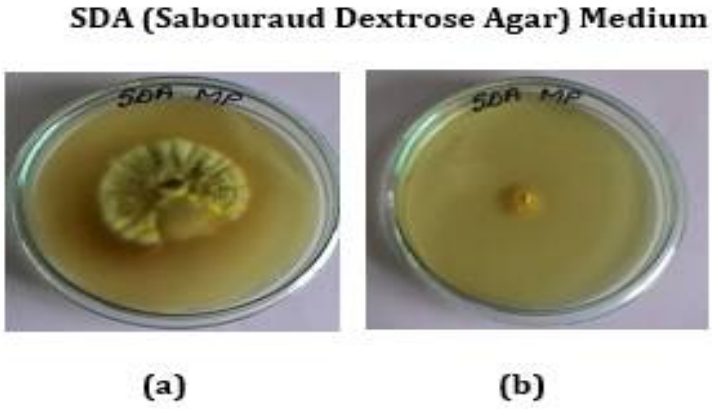

Fig: 2. Growth of *Monascus purpureus* on SDA medium (a) 25⁰ C and (b) 37⁰ C

Table 1 . Morphological and cultural characterisation of *Monascus purpureus* – 369

Cultural and Morphological Characters and pH	Culture Media,7 days of cultivation								
	PDA			SDA			Basal Medium		
	25°C	37°C	4°C	25°C	37°C	4°C	25°C	37°C	4°C
	Monascus purpureus – 369								
CONIDA									
Shape	Pyriform	Pyriform	No Growth	Pyriform	Pyriform	No Growth	Pyriform	Pyriform	No Growth
Colour	No	No		No	No		No	No	
Type of conidia	Straight	Straight		Straight	Straight		Straight	Straight	
Ability of conidia	+	+		+	+		+	+	
PERITHECIA									
Shape	Globose	Globose	No Growth	Globose	Globose	No Growth	Globose	Globose	No Growth
Colour	No	No		No	No		No	No	
ASCOSPORE									
Shape	Oval	Oval	No Growth	Oval	Oval	No Growth	Oval	Oval	No Growth
Colour	No	No		No	No		No	No	
Formation	……	……		……	……		……	……	
COLONY									
pH4, pH5, pH6	Well growth (acidic range)	……	……	Well growth (acidic range)	……	……	Well growth (acidic range)	……	……
Colour	Yellow, White edge	……	……	Yellow, White Edge	……	……	Yellow, White edge	……	……
Shape	Flat	Flat	No Growth	Flat	flat	No Growth	flat	Flat	No Growth
Aerial Mycelium	Long, yellow, white, edge	……	……	Long, Yellow with gray edge	……	……	Short white, rare	……	……

Good growth and slight colour change was observed on both 37⁰C and 25 ⁰C temperature Figure 3,Yellow and white colour was found at centre of the culture plate and long white aerial mycelium was present in both temperatures (25°C and 37°C).

BA (BASAL AGAR MEDIA)

Fig: 3. Growth of *Monascus purpureus* on Basal Agar medium (a) 25⁰ C and (b) 37⁰ C

Growth of *Monascus purpureus* on different media with different pH 4, 5 and 6 in different media
Good growth and colour change was observed on both 25⁰ C with different pH 4, 5 and 6 on Potato Dextrose Agar Medium. In pH4 the *Monascus purpureus* was brown in colour and the mycelia colour was pale yellow colour in both pH5 and pH6 it had same yellow colour with white edge colour .

PDA (Potato Dextrose Agar) Medium

Fig: 4. Growth of *Monascus purpureus* growth on PDA at 25⁰ C with different pH (a) pH4 (b) pH5 (c) pH6 at 25⁰ C.

Good growth and colour change was observed on both 25⁰ C with different pH 4, 5 and 6 with Sabouraud Dextrose Agar Medium too, In pH4 the *Monascus purpureus* was brown in colour and the mycelia colour was pale yellow colour and both pH5 and pH6 has same colour like yellow gray colour both are in acidic range.

SDA (Sabouraud Dextrose Agar) Medium

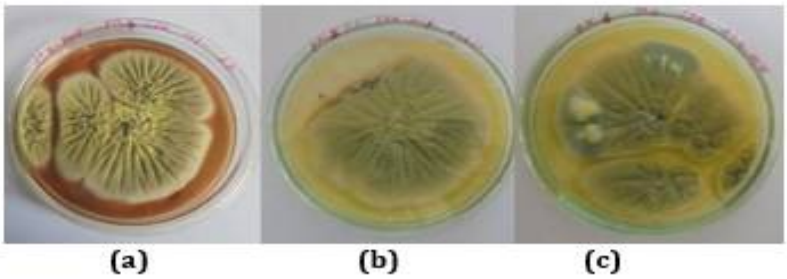

Fig: 5. Growth of *Monascus purpureus* on SDA 25⁰ C with different pH (a) pH4 (b) pH5 (c) pH6 at 25⁰ C.

Slightly growth and good colour change was observed on both 25⁰ C with different pH 4, 5 and 6 with Basal Agar Medium. In pH4 the *Monascus purpureus* was dark red in colour and the mycelia colour was

light white colour and both pH5 and pH6 has same colour like yellow with white colour both are in acidic range.

When comparing the growth of *Monascus purpureus* on different media with different pH 4, 5 and 6 all plates had more pigments as well as grow.

BAM (Basal Agar Medium)

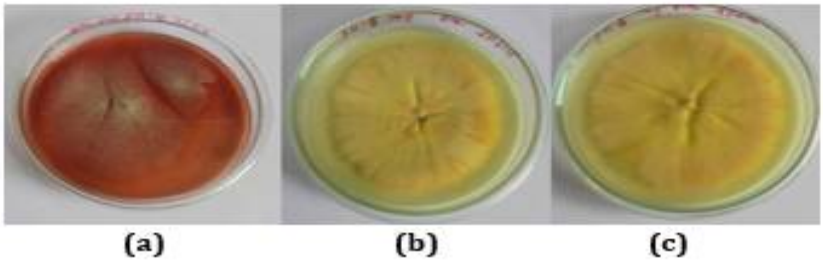

Fig: 6. Growth of *Monascus purpureus* on BAM at 25⁰ C with different pH (a) pH4 (b) pH5 (c) pH6 at 25⁰ C.

MICROSCOPICAL OBSERVATION

Figure 7 shows that the mycelium has oval pyriform (a) Ascogonium (b) Conidiophores with conidia (c) Conidia, single or in a chain up to 6 equivalents in young *Monascus purpureus* culture.

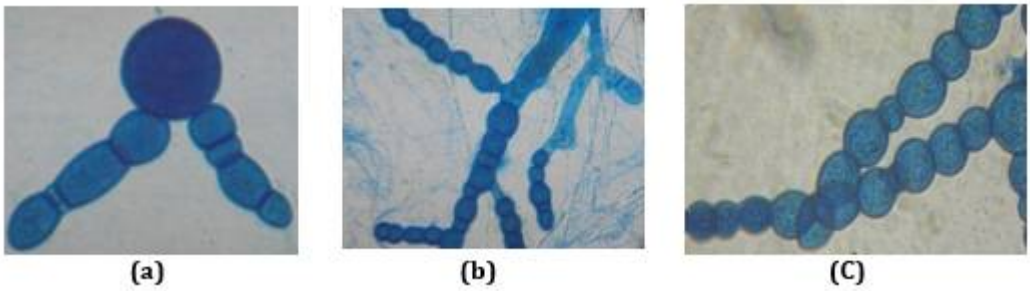

Fig: 7. Morphological identification of *Monascus purpureus* on PDA medium

Figure 8 shows that the formation of Aleiroconidia, aerial mycelium, septated hyphae was identified and filled up with lipid droplet.

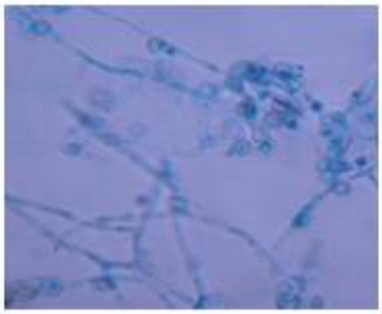

Fig: 8. Morphological identification of *Monascus purpureus* on SDA Medium

Figure 9 shows that the mycelium is filled with lipid droplets on Basal Ager Medium

Fig: 9. Morphological identification of *Monascus purpureus* on Basal Medium

The table: 1 shows that, the morphological characters of Monascus purpureus on PDA medium was observed under the light microscope. The flat and straight type conidia, globose shaped Perithecia and oval shaped ascospore were observed in both temperature (25°C,37°C). According to SDA medium the Conidia were observed in both temperature (25°C, 37°C) and it was straight and slightly raised on the centre. Globose shaped Perithecia were observed in both temperature (25°C, 37°C) and oval shaped ascospore were identified. When comparing to Basal medium also same as the flat like structure and straight shape conidia, globose shaped perithecia and oval shaped ascospore was observed in both temperatures under the light microscope at 45X. Good growth and colour change was observed on both 250C & 370 C with different pH 4, 5 and 6 on Potato Dextrose Agar Medium, Sabouraud Dextrose Agar & Basel Media. Both are in acidic range .The colonies are colour changed and it visualized in yellow with white edge, flat shapes the Aerial Mycelium was observed in long, yellow, white edge in 250 C, in 370 C the Aerial Mycelium was observed with long, yellow with grey edge but compare to basal media short white mycelia was observed. There was no growth found in 4°C.

MARKER IDENTIFICATION

Spore suspension was prepared and the concentration of spore was determined using a Haemocytomer. The spore suspension containing 10^6 spores/ mL was used as the inoculum of *Monascus purpureus*.

According to Marker Identification By using disc diffusion method were dipped in to the agar plate and were incubated at 25 ^{0} C for 3-5 days. Both sensitivity and resistance were showing seen inhibitory action of HexaAntimyco-01 disc. Clotrimazole and Nystatin Were sensitivity and both Amphotericin-B, Fluconazole, Itraconazole- and Ketoconazole were resistance showed in (table - 2).

Table 2. Culture Growth with diameter in different media

Number Of Days	Culture media								
	SDA(mm)			PDA(mm)			Basel Media(mm)		
	25^0 C	37 ^{0}C	4 ^{0}C	25^0 C	37^0 C	4^0 C	25^0 C	37 ^{0}C	4^0 C
1	---	---	---	----	-----	-------	-----	----	-----
2	1	2	----	---	-----	-------	------	----	-----
3	3	3	----	2	5	-----	----	----	----
4	6	6	----	8	9	------	2	3	----
5	9	9	----	11	15	----	5	6	----
6	10	13	----	15	17	----	7	9	----
7	13	14	----	19	19	---	9	10	---
8	13	19	---	21	23	----	10	11	-----

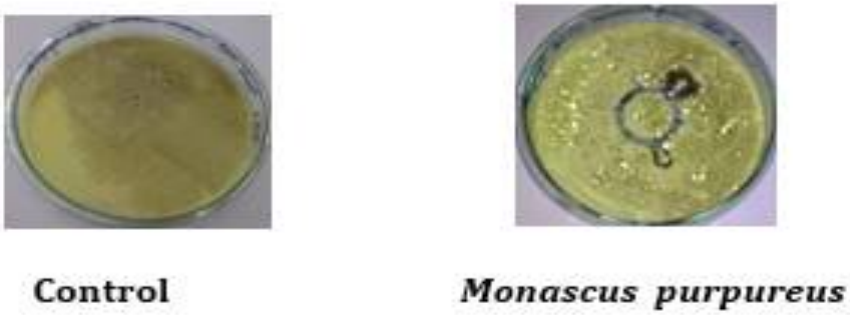

Fig: 10. Selective marker identification using different fungicides in SDA Medium

The table 2 shows the grown on *Monascus purpureus* at three different temperatures. On the first day, no growth was observed in all media and in the second day, slight growth was measured on (1mm, 2mm) in SDA media. In third day, normal growth was observed in both media (SDA, PDA) and not in basal medium, the growth on basal medium started on forth day. There was no growth observed at 4°C.

Table 3. Shows diameter of the clear zones showing inhibitory action of fungicide and pesticide tolerance

FUNGUS	FUNGICIDE	CONCENTRATION			
Monascus Purpureus		50mM	100mM	150mM	200mM
	Indofil	14.33±4.50	14.66±6.80	11.66±7.23	25±7
	Copper sulphate	25.55±115	28. ±4.35	35.66±1.52	38.66±2.08

The table-3 exhibit that, the Antifungal agents Indofil and Copper sulphate was used in different concentration (50mM,100mM,150mM,200mM) in the plates showed clear zones around the 50 µl of each concentration in the plates showed clear zones around the culture which revealed that *Monascus purpureus* was sensitive to both Indofil and Copper sulphate.

Table 4. Shows diameter of clear zones showing inhibitory action of HexaAntimyco-01

Antibiotics of Hexa Antimyco-01	Growth of fungi	Zone formation
Amphotericin-B	Growth	-
Clotrimazole	No Growth	10mm
Fluconazole	Growth	-
Itraconazole-	Growth	-
Ketoconazole	Growth	-
Nystatin	No Growth	5mm

The table 4 exhibit that, the Antibiotic disc Hexa Antimyco-01 rings with Amphotericin-B, Fluconazole, Itraconazole, Ketoconazole, Clotrimazole and Nystatin was used. Measurement of clear zone around the culture exhibited the sensitivity and resistant pattern of *Monascus purpureus* against the antifungal agent. *Monascus purpureus* was sensitivity to Clotrimazole and Nystatin and it was resistant to Amphotericin-B, Fluconazole, Itraconazole, and Ketoconazole.

CONCLUSION

In the present study, Taxonomical investigation of *Monascus purpureus* on different media, incubation temperature and pH was carried out. Best growth was observed in Basal media at 25^0C than other media and temperature studies. But *Monascus purpureus* showed effective growth in all the different pH variation studied ie pH4,pH5 and pH6.But in the pH4 pigment production was faster than other pH provided. *Monascus purpureus* was resistant to Amphotericin-B, Fluconazole, Itraconazole, and Ketoconazole. These marker identified will be very beneficial in further studies. And over all the study carried out will be certainly useful in *Monascus purpureus* researchers.

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