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COMPARATIVE STUDY OF *MONASCUS SANGUINEUS* AND *MONASCUS PURPUREUS* AS POTENTIAL SOURCES FOR RED PIGMENT PRODUCTION

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ABSTRACT

Monascus spp. namely *Monascus sanguineus* was isolated from pomegranate (*Punica granatum)*. In this study, the isolated *M. sanguineus* was compared with *M. purpureus* MTCC410 procured from MTCC Chandigarh, India for optimising the red pigment yield. It was observed that both strains had produced maximum red pigment on the 16th day of incubation (21.9 CVU/ml for *M. sanguineus* & 16.9 CVU/ml for *M. purpureus*). Both strains had shown 30°C as a favourable temperature for microbial growth and pigment production. The maximum pigmentation was observed at pH 6.5 (33.9 CVU/ml) for *Monascus sanguineus* whereas *M. purpureus* produced maximum pigment at pH 5.5(16.6 CVU/ml). *Oryza* spp. (local unpolished rice) was found as the best solid substrate for both the strains (M. *sanguineus* 6.5CVU/gds and *M. purpureus* 12.5CVU/gds). When substrates were supplemented with glucose, a multi-fold increase in the pigment yield was observed with *M. sanguineus,* whereas no positive impact of glucose was observed with *M. purpureus*. For variable N sources, *M. sanguineus* showed maximum pigment with 1% peptone whereas *M. purpureus* showed similar results with substrate supplemented with 5% yeast extract and MSG. Both strains had shown anti-bacterial activity against gram positive bacteria. Presence of citrinin was confirmed in both the strains by LC-MS.

KEYWORDS: Pigment, Mycelial Growth, Citrinin, *Monascus*

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INTRODUCTION

In recent days, synthetic colours are more commonly used in foods. Previous reports have indicated that synthetic food colours have a higher risk to human health as they may be carcinogenic[1]. Hence attempts have been made to develop natural food colours[2]. The filamentous fungi of the genera *Monascus* have been used for a long time as a meat colourant, meat preservative, and health food and also in the Chinese folk medicine[3]. They comprise of four representative species*: M. pilosus, M. purpureus, M. rubber, and M. anka*. When these moulds are grown on cooked rice, they can produce pigments and some bioactive metabolites. Red mould rice has long been used in East Asia for over 1000 years to colour (as a natural food colorant, such as for red rice wine, red soy bean cheese), aromatize, and conserve meat, fish, and soybean products[4]. Recently, industrial interest in fungi as source of natural colorants has been revived because the carotenoid b-carotene has been produced fermentative from the fungus *Blakeslea trispora* by DSMTM in the Netherlands and approved for food use by EU food legislation[5]. *Monascus* species are known to produce well-known azaphilone pigments like monascorubrin rubropunctatin[6] and more recently monascusones from a yellow *Monascus* mutant have also been identified[7]. Monascorubrin and rubropunctatin have a unique structure responsible for their high-affinity for compounds with primary amino groups. Reactions with amino acids yield the water-soluble red pigments, monascorubramine and rubropunctamine[8]. The aim of present work is to compare isolated strain, which was identified as *Monascus sanguineus* with reference strain *Monascus purpureus* MTCC 410 procured from IMTECH, Chandigarh, India for optimizing pigment yield. Both strains were compared in submerged culture as well as solid state fermentation and optimized with certain physical parameters *viz*. temperature pH in submerged and with variable C & N sources in solid state fermentation. Both strains were also investigated for anti-microbial activity. Citrinin analysis was also done for both the strains.

MATERIALS & METHODS

(1) Culture

Wild strain of *Monascus* was isolated from pomegranate (*Punica granatum)*. The strain was maintained on Potato Dextrose Agar (PDA) medium and incubated at 28-30 °C for 7 days, preserved at 4 °C, and sub-cultured once every 4 weeks.

(2) Source of reference culture of Monascus Spp.

Monascus purpureus MTCC 410 was procured from the Microbial Type Culture Collection, IMTECH, Chandigarh, India. This spp. of *Monascus* was used as reference strain. Both these strains were compared for optimizing pigment yield.

(3) Inoculum preparation

Inoculum preparation of both the strains for solid-state fermentation was performed as described by Babitha et al.[9] with some modification. One full loop of sporulated (6-day old) agar slope culture was diluted in distilled water. The spores were scraped off under aseptic conditions to produce a spore suspension to be used as the inoculum.

(4) Evaluation of physical parameters like temperature and pH on mycelial growth & pigment production for both strains

Mycelial growth of *M. sanguineus* & *Monascus purpureus* MTCC 410 was evaluated in Potato Dextrose Broth (PDB) media. 100 ml flasks were taken and 50 ml media were prepared, autoclaved at 121°C for 20 minutes. Medium pH was adjusted to 5.5. After cooling, media was inoculated with 1 ml of *M. sanguineus* & *M. purpureus* MTCC 410 culture separately and incubated for 16 days in static condition. Biomass and pigment were estimated every 4^{th}, $8^{th,}$ 12^{th} and 16^{th} days after inoculation. Same procedure was adopted to study the effect of temperature and pH on mycelial growth and pigment production. For temperature investigation the inoculated media were subjected to different

temperatures viz. 16°C, 30°C, 37°C and 50°C and for pH study,
the pH of the medium was adjusted in varying range of 4.5, 5.5, 6.5, 7.5 and 8.5 and kept for

15 days in static condition[10].

(5) Dry cell weight

The mycelia separated from broth by filtration (Whatmann No. 1) were weighed on an analytical scale, vacuum filtered through pre-weighed membrane filters, washed with distilled water, dried in an oven at 50°C. The results were expressed in grams per litre[11].

(6) Pigment Estimation

Pigment estimation in submerged culture was carried out using culture filtrate. Filtrate was centrifuged at 10000xg for 15 minutes. Pigment concentration determined by colorimeter at 510 nm. The absorbance values were converted into pigment units using by the following formula:
Colour value = O.D. x dilution x volume of extracts / Amount of sample (ml)[10] .

(7) Substrate Selection and Solid-State Fermentation

Both strains were compared for solid state fermentation. For this, four substrates were chosen *viz*. *Oryza* spp. (local unpolished rice), *Fagopyrum* spp. *(*Buckwheat) flour, *Colocasia* spp. (arbi), and *Manihot* spp. (tapioca). These were purchased from a local market of Bangalore, India. Initially, 20 g of substrate was placed in a 250 ml conical flask to which distilled water was added, pH of the medium was adjusted to 6.0 and autoclaved at 121°C for 20 minutes. After cooling, the substrate-based medium was inoculated with 10% of the seed culture of the *M. sanguineus* & *M. purpureus* MTCC 410 separately and incubated at 28°C- 30°C for 20 days[12]. Moisture content was maintained between 56-60% and was calculated based on the following formula, *Moisture content of substrate (%)=100*(wet weight − dry weight) /wet weight*[13].

(8) Screening of Nitrogen & Carbon source on pigment production

Both strains were optimized for pigment production with variable N & C sources. For both the strains, Nitrogen sources such as peptone, yeast extract, monosodium glutamate at 1%, 5%, 10% concentration & Carbon sources such as xylitol & glucose at 4%, 8% & 12% were used separately for pigment production. 20 g of substrate supplemented with these concentrations of N & C sources was then experimented using the procedure mentioned in above section[12,14].

(9) Pigment extraction and estimation

For solid substrate the culture medium was dried at 50°C for 48 hours. One gram of fermented solid substrate was taken for pigment extraction using 10 ml of 95% ethanol shaking on a rotary shaker at 200 rpm for 24 hrs. The extracts were allowed to settle at room temperature and then filtered through Whatman filter paper. Ethanol extracts of unfermented substrates were kept as blanks. Analysis of pigment concentration was done using colorimeter at 510 nm. The absorbance values were converted into pigment units using the following formula:
Colour value = O.D. x dilution x volume of extracts / Amount of sample (g)[15].

(10) Analysis of Citrinin

Qualitative analysis of citrinin was done for both the strains. The Instrument was HPLC Thermo Finnigan Surveyor and MS Thermo LCQ Deca XP MAX. The software used in this experiment was Xcalibur. The column used was BDS HYPERSIL C18 with a length of 250 mm, I.D. of 4.6 mm and particle size of 5 μm. The detectors used were HPLC PDA / UV detector (254 nm) and the temperature was ambient with 10μL as the volume injected. MS experimental conditions were with Probe/ source voltage of 4.5kV, Sheath gas flow of 40.00 and Auxiliary/Sweep gas flow of 26.00. The Source type was Electro Spray Ionisation (ESI) with Capillary temperature of 275°C and Capillary voltage of 16V. The nebulization gas flow was with Helium at approximately 1 mL/min and the Helium in the mass analyzer cavity was maintained at 0.1Pa (10^{-3})[16].

(11) Antibacterial Activity

Anti-bacterial activity of the *Monascus sanguineus* & *Monascus purpureus* MTCC 410 was studied against some bacterial strain. These strains were *Klebsiella spp., E. coli, Staphylococcus aureus & Proteus spp..* Muller –Hinton medium (250 ml) was seeded with bacterial culture at 3% and then poured to Petri plates, placing the paper discs (6 mm diameter), containing filtrate of both the strains, in the centre of the test plates. After 48 hours inhibition zones were recorded[17].

(12) Statistical analysis

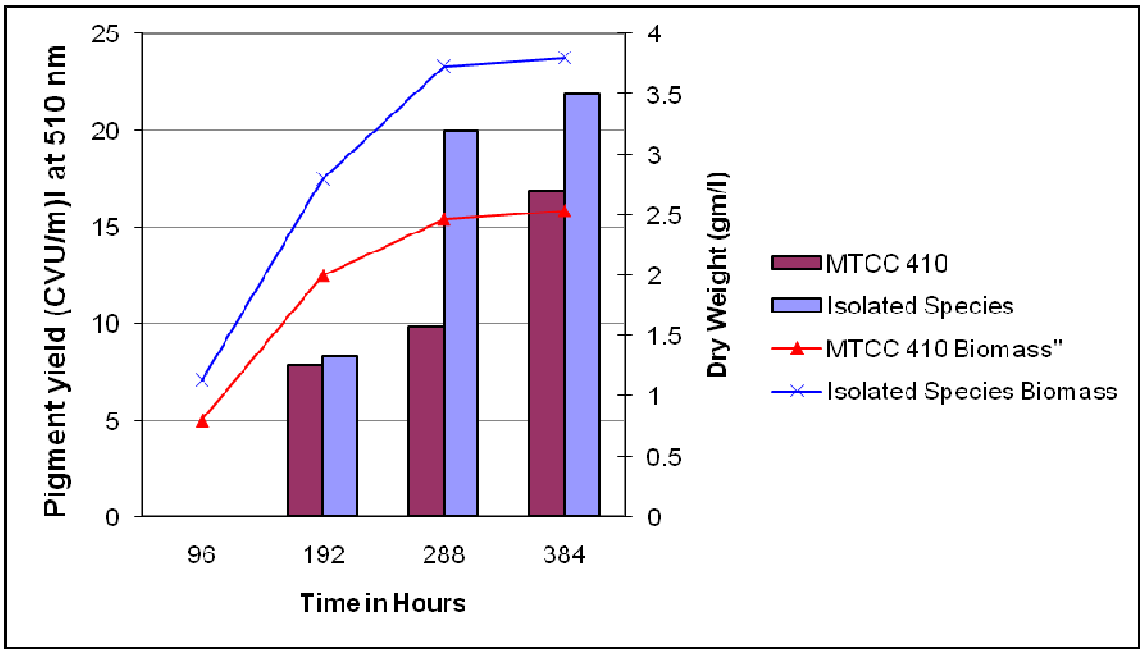
The data was analysed using MS Excel and ANOVA.

RESULTS & DISCUSSION

(1) Growth Pattern of Monascus sanguineus &Monascus purpureus MTCC 410

The growth pattern for both the strains was observed to be more or less linear in nature, with the biomass showing a steady increase against the period of incubation. Since 5th day of incubation, indication of pigment production appeared and it continued to accumulate throughout the fermentation period with the yield reaching its highest level on 16th day. Both the strains had shown maximum biomass on 12th day (*Monascus sanguineus* 3.8 dry wt. (gm/l) & *Monascus purpureus* MTCC 410 dry wt. (gm/l)) and pigment yield on 16th day of incubation (*Monascus sanguineus* 21.8 CVU/ml & *Monascus purpureus* MTCC 410 16.9 CVU/ml) (Fig.1). Chatterjee et al[10] concluded that producer organism turned reddish within 3 days of fermentation and diffusion of pigment started thereafter. In this stage, mycelia became matured thus contained aerial conidia visualized by microscopic observations. Metabolites production by filamentous fungi varies according to the strain, the composition of the growth medium and the cultivation conditions[18].

Figure 1
Growth Pattern

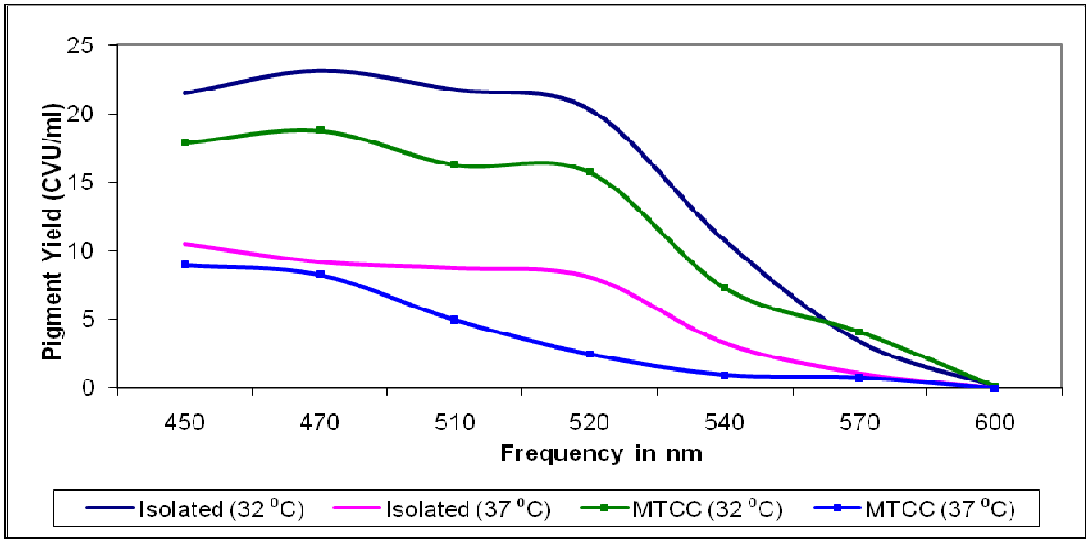


(2) Effect of Temperature on pigment production & biomass

The results showed that *M. sanguineus* grew in all experimented temperature conditions (16°C, 32°C 37°C & 50°C) whereas *M. purpureus* MTCC 410 was not able to grow at 16°C & 50°C. Maximum biomass and pigmentation was observed at room temperature (28°C - 32°C) for both the strains. *M. sanguineus* had shown maximum pigment yield around 23.13 CVU/ml at 470 nm & 21.75 CVU/ml at 510 nm whereas for *M. purpureus* the maximum pigment yield was 18.8 CVU/ml at 470 nm & 16.3 CVU/ml at 510 nm. Both ranges are regarded for maximum absorption towards orange- red colour. After these ranges, a decrease in pigment yield was observed. *M. sanguineus* had shown very less growth and no pigmentation at 16°C and 50°C (Fig 2). The enzymatic activity (multi enzyme complex – polyketide synthase) seems to be optimum for red pigment production at 32°C to 35°C, hence the maximum red pigment was observed at these temperatures. At 37°C, there was a reduction in pigment yield. Thus it is seen that temperature plays a pivotal role in cell metabolism thus influencing pigment yield[10]. It was found that maximum absorbance at 510 nm (red pigment) was obtained around 32°C to 35°C but beyond 40°C, there was drastic reduction in red pigment[19]. Carvatho *et. al*.[15] reported a shift in absorbance maxima of pigment yield with temperature.

Figure 2

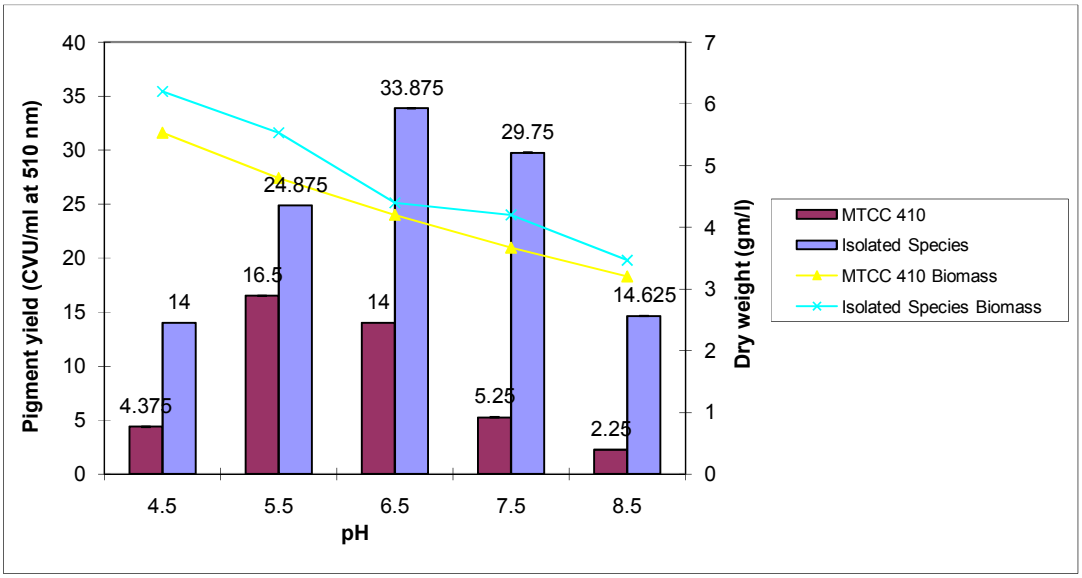
Impact of Temperature on pigment production through spectral analysis



(3) Effect of pH on pigment production & biomass

Maximum biomass for both the strains was observed at pH 4.5 with linear decrease. Maximum red pigment was observed at pH 6.5 for *M. sanguineus* (33.8 CVU/ml) and pH 5.5 for MTCC 410 (16.5 CVU/ml) (Fig.3). The present study divulges the influence of pH on pigment production & biomass. The pigment yield varied with change in pH. At higher pH, consolidation of red pigment production & decrease in biomass was observed. Quality of red pigment production by *Monascus* spp. was found to be reducing in acidic pH[10] and it appeared that the product reacted with ammonium ion or free amino group to transform itself to red amine derivatives towards neutrality and beyond[20]. Different pH levels influenced the physiology of fungi, conidial development and pigment synthesis. At acidic range pH (4.5) conidiation was found to be increasing whereas the red pigment synthesis showed reduction[21]. Pigment production by *Monascus* spp. was entirely dependent on the pH of the media, reversible in acidic and alkaline pH[22].

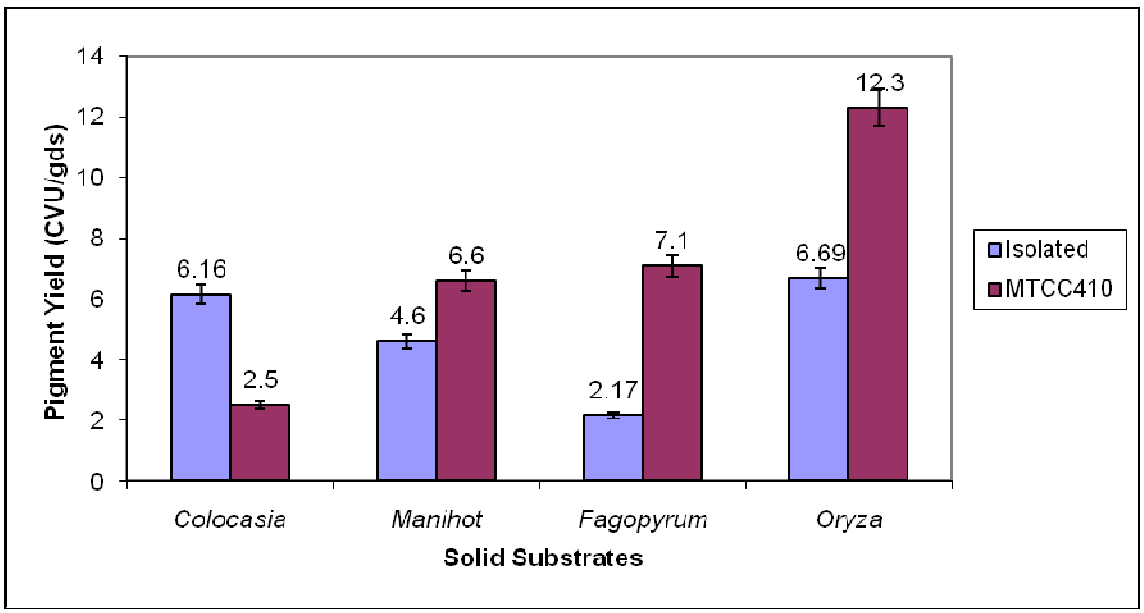
Figure 3
Effect of pH on pigment production & biomass



(4) Screening of substrates for Monascus sanguineus & Monascus purpureus MTCC 410

It was found that both the strains grew on all experimented substrates though pigment yield was different. Both strains had shown maximum pigment yield with *Oryza* spp. (rice) (Isolated spp.6.69 CVU/gds & MTCC 410 12.5 CVU/gds) (Fig.4). Pigment production can be achieved with *Monascus* spp. by fermentation technique using agricultural product other than rice. Dioscorea (cushcush) is concluded to be the best substrate for *Monascus* spp. to produce the cholesterol- lowering agent –monocolin K and anti- inflammation agent – monascin, which is also a cheap agricultural product[23].

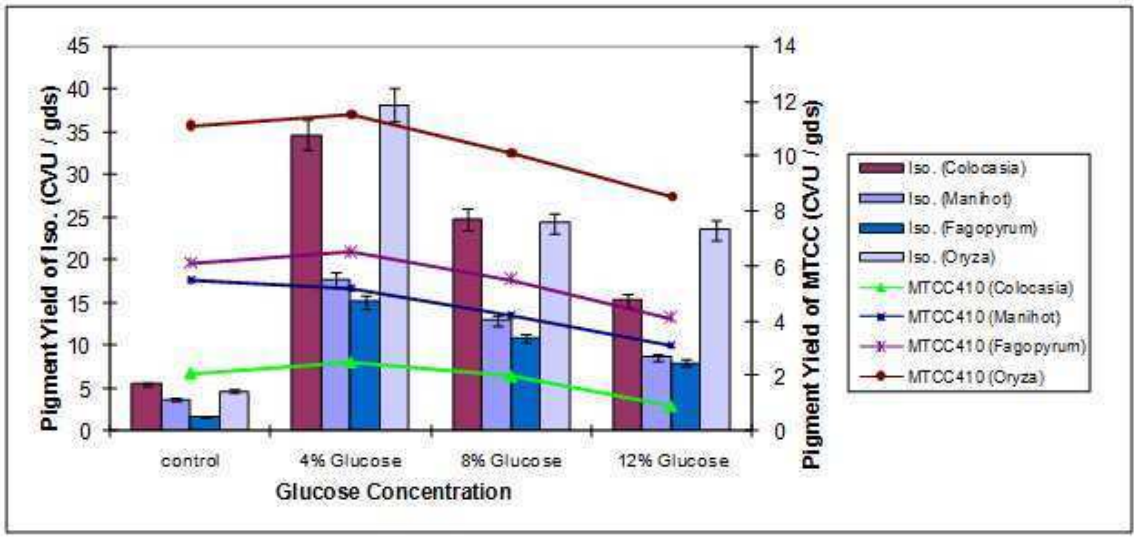
Figure 4
Screening of Substrates



(5) Effect of Carbon Source on pigment production by Monascus sanguineus & Monascus purpureus MTCC 410

The results showed multi-fold increase in the pigment production by *M. sanguineus* and negligible pigment yield for *Monascus purpureus* MTCC 410. Maximum pigment was observed when substrates were supplemented with 4% & 8% glucose (38.8 CVU/gds). *Monascus spp.* produces protease and amylase enzymes that break down proteins and amylase respectively (Fig.5). Strains of Monascus spp. have the capability to produce enzyme amylase. The more amylase the strain produces, the more it can be hydrolysed into glucose to produce more pigment. Glucose is needed as the source of energy during the development of secondary metabolite. Amylase in white rice is higher than red rice; therefore, white rice will produce more red and yellow pigments[24]. Solid-state fermentation done with Jackfruit seed powder with a particle size between 0.4 and 0.6 mm by *Monascus purpureus* without any additional carbon source was found to be the best for pigment production[9].

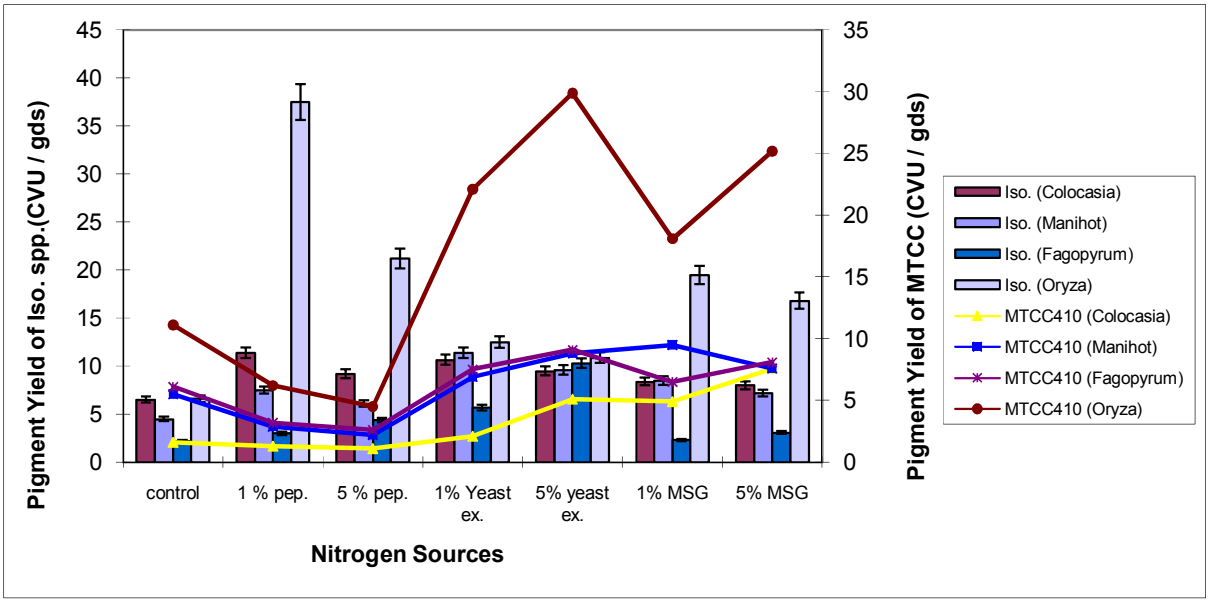
Figure 5
Effect of C Source on pigment production



(6) Effect of N Source on pigment production by Monascus sanguineus & Monascus purpureus MTCC 410

The results were highly dependent on the percentage of the Nitrogen source as well as the substrate type for both the strains. *M. sanguineus* had shown mammoth increase in the pigment production with 1% peptone (37.5 CVUU/gds) whereas *Monascus purpureus* had shown maximum pigment with rice supplemented with 5% yeast extract (29.9 CVU/gds). This may be due to local red rice being rich source of carbohydrate and the ratio being the optimum for this combination. 1% and 5% nitrogen sources were found to be suitable for pigment production (Fig.6) where as 10% Nitrogen content in all the substrates was found to be inhibitory in nature for both the strains. Pigment production could notably vary with N supplementation in *Monascus spp.*.Vidyalakshmi *et. al.*[14] determined the pigment yield of *Monascus ruber* by the solid state fermentation of rice supplemented with different N sources and maximum pigment yield was observed when rice was supplemented with monosodium glutamate. The addition of external nitrogenous compounds showed a positive impact on water-soluble pigment production[9]. Wong and Koehler[25] indicated that the growth of *M. purpureus* and pigment production are influenced by the amount of Carbon and Nitrogen in the media, and that the amount of Carbon and Nitrogen will determine the amount and the type of the pigments.

Figure 6
Effect of N Source on pigment production



(7) Antibacterial activity of Monascus pigments

Both strains had shown antibacterial activity against gram-positive *Staphylococcus aureus* (*Monascus purpureus* MTCC 410 with inhibition zone of 85.40 sq.mm & *Monascus sanguineus* inhibition zone of 112.95sq.mm) (Table.1). Therapeutic properties of culture filtrate of *Monascus* spp. could be due to monascidin A, which is secondary metabolite produced by *Monascus* spp. Camelia Ungureanu[17] reported that culture filtrate of *Monascus* strain had an antifungal action against some spp. of *Aspergillus, Mucor, Penicillium* & *Fusarium* and anti bacterial action against *bacillus spp.*. The two strains of Gram (+) bacteria belonging to *Bacillus* genus has been more sensitive to the action of the extract containing *Monascus* pigments whereas the *Pseudomonas* Gram (-) strains has been less influenced by the extract presence. Antibiotic action of *Monascus purpureus* against bacteria might be attributed to the presence of orange pigment[26]. Nameirakpam & Wahab[27] reported that the crude fungal extract from isolated *Penicillium* spp. showed inhibitory activity against all bacterial tested pathogens except *Proteus* spp. and no activity against fungal pathogen also the highest zone of inhibition was observed against *Pseudomonas aeruginosa*., *Serratia marcescens* and *Staphylococcus aureus*. Further, in NMR analysis, it was identified that aromatic compounds were present in the extract which were responsible for antimicrobial activity.

Table 1
Antibacterial activity of Monascus pigments

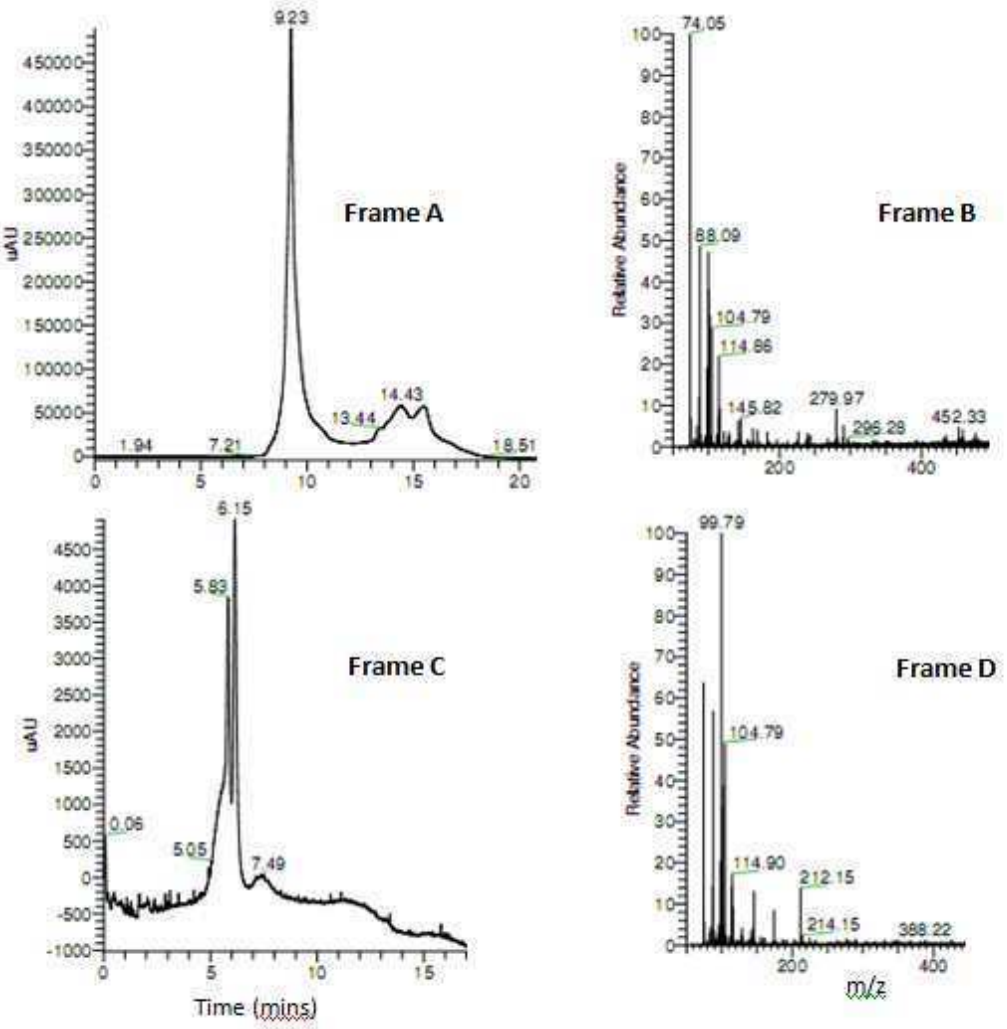
Strains	*Monascus purpureus* Inhibition zone (dia in mm)	*Monascus sanguineus* Inhibition zone (dia in mm)
Staphylococcus aureus	85.40	112.05
Escherichia coli	0.00	0.00
Klebsiella spp.	0.00	0.00
Proteus spp.	0.00	0.00

(8) Citrinin analysis of Monascus sanguineus & Monascus purpureus MTCC 410 by LC-MS

Citrinin presence in both the spp. is identified by means of LC-MS. LC-MS analysis describes an approach involving the recognition of pattern of mass spectral analysis lines that are produced as result of LC separated analytes. The spectral data used where the values of *m/z* and their related intensities. *Monascus sanguineus* extract (Frame A) (Fig.7) was detected at 10.80 minutes whereas *Monascus purpureus* MTCC 410 extract was detected at 5.95 minutes (Frame C). Frame B shows (*Monascus sanguineus)* LC separated analytes detected by ESI mode (*m/z* of 104.78, 114.90, 212.14 and 214.14). LC separated analytes in frame D (MTCC 410) was detected by ESI mode (*m/z* of 104.79, 114.86, 145.82 and 279.98).

Figure 7

Mass fragmentation spectrum of ESI chromatogram of Monascus spp.



Monascus is a genus that produces a toxic metabolite, just as many other fungi in the order Eurotiale[27]. The toxicity of most *Monascus* sp. appears to be minimal since there has been seldom reports of adverse medical effects reported in the populations that consume *Monascus* fermented food. All of the spp. produced citrinin regardless of pigment production, but quantity varied with *Monascus purpureus* being 386 mg/l, *Monascus ruber 120* mg/l & *Monascus sanguineus* as 78 mg/l[28].

CONCLUSIONS

Both strains can survive a wide range of pH (4.5-8.5) but maximum biomass was observed at acidic pH (4.5) and maximum red pigment yield was noticed around pH 5.5-6.5. *Oryza* spp. was found best substrate for pigment production. Both strains had shown positive impact on N sources. *Monascus sanguineus* had shown multi-fold increase in pigment yield with addition of glucose whereas there was no positive impact observed with *Monascus purpureus.* Both the strains had shown anti-microbial activity against gram positive bacterial strain. Citrinin was also found to be present in both the strains. Though there are many reports of work done on *Monascus purpureus*, it is clear from the study that *Monascus sanguineus* strain is better than *Monascus purpureus* when compared for the production of the pigment. There is not much information available on pigment production by *Monascus sanguineus*. Hence there is a need to explore *Monascus sanguineus* to make it more adaptable and acceptable to the food industry for its industrial usage and production.

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