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Exploring *Monascus sanguineus* as a Potential Natural Source for Pigment Production

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Abstract

Monascus species are known as producers of bio-pigments, which are used for food coloring. For this study, a Monascus sanguineus strain was isolated from pomegranate. Its molecular identification was done by genome sequencing. The effect of different culture media, temperature, and pH on pigment production and mycelial growth was investigated in submerged culture. Production of the red pigment reached its maximum on the 16th day of incubation (21.9 Color Value Units (CVU)/ml). The optimal temperature for microbial growth and pigment production was 30°C and the maximum pigmentation was observed at pH 6.5 (33.9 CVU/ gram dry substrate (gds). Effect of different solid substrates with varied carbon and nitrogen content on pigment production and optimization was also investigated. Oryza sp. (local polished rice) was found to be the best solid substrate (7.8 CVU/gds) amongst the experimented substrates. The determination of citrinin was carried out by liquid chromatography – mass spectrometry (LC-MS).

Keywords: *Monascus*, pigment, mycelial growth, genome sequencing, citrinin.

Introduction

Monascus sp. belongs to the family *Monascaceae* of the phylum *Ascomycecota.* Based on the cultural characteristics, 9 *Monascus* species are internationally acknowledged. These are *M. pilosus, M. ruber, M. purpureus, M. floridanus, M. eremophilus, M. pallens, M. sanguineus, M. lunisporas,* and *M. argentinensis*. However, over 20 species of *Monascus* have been recorded in the literature since the genus *Monascus* was proposed in 1884[1]. The *Monascus* filamentous fungi have been used in Asia for a long time to color and flavor food and beverages[2,3]. These natural colorants are of practical interest because the red pigments obtained are safe for usage in food industry. Different strains of the genus *Monascus* are used in pigment production[4,5]. *Monascus* sp. has been used primarily in Southern China, Japan, and Southeast Asia for making red rice wine, red soybean cheese and Anka (red rice)[6]. *Monascus* pigments typically comprise six major azaphilone pigments: Yellow pigments: monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$); Orange pigments: monascorubrin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_{22}O_5$); and Red pigments: monascorubramine ($C_{23}H_{27}NO_4$) and rubropuntamine ($C_{21}H_2O_4$)[7]. *Monascus* sp. was also reported to co-produce the mycotoxin citrinin, as well as other potentially toxic metabolites, such as monascopyridines[8].

The goal of this study was to isolate a *Monascus* strain and to investigate the general conditions for growth and pigment production on fungal media. Screening was carried out for different solid substrates and these substrates with varied carbon and nitrogen content were optimized for pigment yield. Qualitative analysis for determination of citrinin was also performed.

Material and Methods

Culture: A wild-type strain of *Monascus* was isolated from pomegranate. 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.

Inoculum preparation: Inoculum preparation for solid-state fermentation was performed as described by Babitha et al.[9] with some modification. One full loop of sporulated (6 days 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.

For morphological investigation, the isolated strain of *Monascus* sp. was grown for 7 days on different growth media such as potato dextrose agar (PDA), sabouraud dextrose agar (SDA), malt glucose peptone agar (MGPA), and malt extract agar (MEA)[10].

For molecular identification, genomic DNA was isolated from the culture. The rDNA fragments of ~500 bp were amplified using the universal primers ITS1f (5'-CTTGGTCATTTAGAGGAAGTA) and NL4 (5'-GGTCCGTGTTTCAAGACGG), the sequencing PCR was set up with ABI-BigDye® Terminatorv 3.1 Cycle Sequencing Kit[1].

Evaluation of the effect of various culture media, temperature, and pH on mycelial growth and pigment production of Monascus sanguineus : Mycelial growth of M.

sanguineus in the listed fungal media was evaluated. In each case, 50 ml of the growth medium in 100 ml flask was used. The medium pH was adjusted to 5.5. After cooling, these media were inoculated with 0.5 ml of the M. sanguineus culture and incubated for 16 days in static condition. Biomass and pigment were assayed on day 4, 8, 12, and 16 after inoculation. The same procedure was adopted to study the effect of temperature and pH on mycelial growth and pigment production. To investigate the effect of temperature, the inoculated media were incubated at 16, 30, 37, and 50°C. The effect of pH was studied at pH values of 4.5, 5.5, 6.5, 7.5 and 8.5 and kept for 15 days in static condition after inoculation[11].

Dry cell weight: The mycelia separated from the broth by filtration (Whatmann No. 1) were weighed on an analytical scale, vacuum filtered through pre-weighed membrane filters, washed with distilled water, and dried in an oven at 50°C. The results were expressed in grams per liter[12].

Pigment content: The pigment content in submerged culture was determined using culture filtrate. The filtrate was centrifuged at 10000 *g* for 15 min. Pigment concentration was determined colorimetrically at 510 nm. The absorbance values were converted into pigment units using by the following formula:
Color value = O.D. × dilution × volume of extracts / amount of sample (ml)[13].

Substrate selection and solid-state fermentation: For solid-state fermentation four substrates were chosen, viz. Oryza sp. (local polished rice), Eleusine sp. (finger millet) flour, *Ipomoea* sp. (sweet potato), and Manihot sp. (tapioca). These were purchased from a local market of Bangalore, India. The physical form of the substrate was as follows:

Eleusine sp. was taken in flour form, *Manihot* was graded into minute threads, *Lpomoea* sp. was cut into small pieces, and *Oryza* sp. was soaked in water overnight.

Initially, 10 g of the substrate was placed in a 250 ml conical flask to which 27.5 ml distilled water was added, pH was adjusted to 6.0, and the medium was autoclaved at 121°C for 20 min. After cooling, the substrate-based medium was inoculated with 10% of the seed culture of *M. sanguineus* and incubated at 28 - 30°C for 20 days[9]. 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[7].

Effect of nitrogen and carbon source on pigment production: Nitrogen sources such as peptone, yeast extract, and monosodium glutamate at 2, 6, and 10% concentration and carbon sources such as xylitol and glycerol at 5, 10, and 15% were used separately for pigment production. The substrate (10 g) was then supplemented with these concentrations of nitrogen and carbon sources used in the experiments according to the procedure described above[14,15].

Pigment extraction and determination: In the case of cultivation on solid substrate, the culture medium was dried at 50°C for 24 hr. One gram of fermented solid substrate was taken for pigment extraction with 10 ml of 95% ethanol on a shaker at 200 rpm for 24 hr. The extracts were allowed to settle at room temperature and then filtered through Whatman filter paper. Ethanol extracts of unfermented substrates were used as blanks. Analysis of pigment concentration was done using a colorimeter at 510 nm. The absorbance values were converted into pigment units using the following formula:
Color value = O.D. × dilution × volume of extracts/amount of sample (g) [13].

Determination of the presence of Citrinin: The instrument used for the determination of citrinin was HPLC Thermo Finnigan Surveyor and MS Thermo LCQ Deca XP MAX. The software 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 10 µL as the volume injected.

MS experimental conditions used were probe/ source voltage of 4.5 kV, sheath gas flow of 40.00 and auxiliary/sweep gas flow of 26.00. The source type was electro spray ionization (ESI) with capillary temperature of 275°C and capillary voltage of 16 V. The mobilization gas flow was helium at approx. 1 ml/min and the helium in the mass analyzer cavity was maintained at 0.1 Pa (10^{-3})[16].

This analysis describes an approach involving the recognition of pattern of mass spectral analysis lines that are produced as result of LC separated analytes. The pattern recognition provides a method for the prediction of chemical structure and can be applied to the sample that has not been examined. The spectral data used were the values of *m/z* and their related intensities.

Statistical analysis: MS Excel and ANOVA were used for data analysis.

Results and Discussion

Isolation and identification of Monascus sp.: The *Monascus* sp. strain was identified by its capacity for pigment production in the medium and pigmented spores. Morphology (the size, color, shape, and aerial hyphae) was studied on colonies. This *Monascus* sp. isolate was able to release the pigment into the media. Microscopic observation of the spores showed that perithecia were born singly on stalk. Coenocytic mycelium was observed and ascospores were round, smooth, and pigmented.

Monascus sp. can be easily distinguished by its ascospores, which may appear to be spherical in shape of 5 µmin diameter or slightly ovoid (6 × 5 µm). The mycelium is white in the early

stage but rapidly changes to a rich pink and subsequently to a distinctive yellow-orange color. Deep crimson color is formed as the culture ages[7].

Molecular identification: Molecular identification was performed on the basis of sequence analysis (548 bases) with NCBI sequence Accession No. AY498586.1 (figure 1). The culture was identified as *Monascus sanguineus*. The isolated strain showed 100% sequence similarity with the genus *Monascus* Tiegh[17].

Growth pattern of Monascus sanguineus on different media: It was seen that *Monascus sanguineus* grew rapidly on all media, while the color and texture of the mycelium produced depended on media type. The pattern was observed to be more or less linear in nature for all the media with biomass showing a steady increase with time. Maximum biomass was observed on 12th day of incubation in all the growth media. The maximal biomass was observed in MEB (dry weight of 6 g/l), followed by MGPB (dry weight of 5.8 g/l) and others. Mycelial growth occurred as a thick mat in all the above media except for the sabouraud dextrose broth, where the growth of mycelium was observed as pellets.

The pigmentation in malt extract medium was observed from 96 hours (4th day) (5.7 CVU/gds) onwards, whereas in other media it was observed from 144 hours (6th day) onwards. Maximum pigmentation was observed on the 16th day in PDB (21.9 CVU/gds) followed by MGPB (19.1 CVU/gds) (figure 2).

Monascus sanguineus growing on MGPB and PDB has shown maximum pigment yield because the media had a balanced C-N ratio along with starch content. Growth in sabouraud dextrose broth resulted in very poor pigmentation but satisfactory biomass. Biomass was observed in pellet form, which could be due to more glucose stress that inhibited pigmentation. It was found that glucose at 18 g/l was optimum for red pigment production. Reduction in pigment production was observed at higher glucose concentrations, perhaps due to respire fermentative metabolism[12]. Nimnoi and Lumyong,[15] concluded that high amount of yeast extract and glucose found in mixture of tryptone glucose yeast extract (TGY) and yeast malt (YM) promoted faster growth compared to other media.

Effect of temperature on pigment production and biomass of Monascus sanguineus: Maximum biomass and pigmentation was observed at room temperature (28 - 30°C) and very slow growth with no pigmentation was seen at 16 and 50°C. On MGPB, the biomass showed a dry weight of 5.8 g/l and pigment yield of 19.5 CVU/gds whereas on PDB the biomass dry weight was 5.46 g/l and the pigment yield was 21.8 CVU/gds (Figure 3). In the case of malt extract, the pigmentation was nearly uniform at all temperatures, whereas on sabouraud medium maximum pigmentation developed at 37°C. Temperature plays an important role in metabolic activities and microbial growth. The result obtained above clearly indicates the mesophilic nature of the fungus.

It was found that maximum absorbance at 510 nm (red pigment) was obtained around 32 to 35°C, while beyond 40°C, there was a drastic reduction in the amount of red pigment[9]. Carvalho et al.[18] reported a shift in absorbance maxima of the pigment extract at different incubation temperatures. Lin[19] reported an incubation period of 3 days, temperature of 32°C, and pH 6.0 as the optimum cultural conditions for *Monascus* sp..

Effect of pH on the pigment production and biomass of Monascus sanguineus: The growth of *Monascus sanguineus* was observed within the entire tested range of pH (4.5 to 8.5), though it showed a downward trend with increasing pH. Maximum biomass was observed at pH 4.5 (dry weight of 7, 6.93 and 6.2 g/l for MGPB, malt extract, and PDB, respectively) and decreased biomass production was observed at pH 8.5. Pigment yield was maximum at pH 6.5 (33, 31.4, and 33.9 CVU/gds for MGPB, malt extract and PDB, respectively) and decreased pigment yields were observed at acidic pH of 4.5 and basic pH of 8.5 (figure 4). Different pH levels influenced the physiology of fungi, conidial development and pigment synthesis. At acidic pH 4.5, conidiation was found to be increasing whereas the red pigment synthesis showed reduction[12]. The red pigment was more pronounced at pH 6, but acidic pH supported yellow pigment production[11]. It has been reported that there was predominance of yellow pigments at lower pH and red pigments at higher pH[20].

Screening of the substrates for pigment production by Monascus sanguineus: It was found that the maximum pigment production occurred with *Oryza* sp. (7.8 CVU/gds) followed by *Lpomoea* sp. (6.5 CVU/gds) and *Manihot* sp. (3.6 CVU/gds). *Eleusine* sp. was found to be the weakest substrate with pigment yield of 2.2 CVU/gds. Pigment production can be accomplished with *Monascus* sp. by fermentation technique using agricultural products other than rice. Corn meal has been reported to be the best substrate for the pigment production followed by peanut meal, coconut residue, and soybean meal[15].

Effect of carbon source on pigment production by Monascus sanguineus: Maximum pigment yield was observed when the substrates were supplemented with 5% glycerol (wt/wt) (37.5 and 30.9 CVU/gds for *Oryza* sp. and *Ipomoea* sp., respectively), whereas at 10 and 15% glycerol, a decrease in the pigment yield was observed (figure 5). *Monascus sanguineus* exhibited very slow growth and no red pigment yield with the substrates supplemented with xylitol since it is a sugar alcohol (data is not presented). Our results agreed with the findings of Babitha et al.[9] who concluded that addition of glycerol enhances pigment production. Although glycerol, which is able to induce osmotic stress in the microorganism, it may be served as carbon source due to this having great importance as medium constituents in pigment biosynthesis.

Effect of nitrogen source on pigment production by Monascus sanguineus: The results were highly dependent on the percentage of the nitrogen source as well as on the substrate type. *Oryza* sp. showed a mammoth increase in the pigment production with 2% peptone (35.4 CVU/gds). This may be due to *Oryza* sp. being a rich source of carbohydrates and the ratio being the optimum for this combination. Nitrogen sources concentrations of 2 and 6% were found to be suitable for pigment production, whereas 10% nitrogen content in all the substrates was found to be inhibitory (figure 6). Shepherd and Carels[21] reported that nitrogen source affected the growth and pigment production. This also depends on the cultural conditions and C-N ratio[12]. Chairote et al.[22] demonstrated that most intense red color was observed when RD6 (rice) as a substrate was supplemented with soybean milk. The nitrogen sources monosodium glutamate and yeast extract favored the growth of the *Monascus ruber* strain[8]. Miyake et al.[23] reported enhanced yellow pigment production upon addition of 0.5% MSG.

Determination of Citrinin by LC-MS: For the determination of Citrinin the qualitative analysis was done by the means of LC-MS. *Monascus sanguineus* extract (spectrum A of figure 7) was detected at 10.80 minutes.

Spectrum B of figure 7 shows LC separated analytes detected by ESI mode (*m/z* of 104.79, 114.86, 145.82 and 279.98). Therefore the peak detected at 10.98 minutes for *Monascus sanguineus* extract was identified as citrinin. *Monascus* is a genus that produces this toxic metabolite, just as many other fungi in the order *Eurotiale*[24].

The toxicity of most *Monascus* species appears to be minimal since there has seldom been reports of adverse medical effects reported in the populations that consume *Monascus*-fermented food. All of the species produced citrinin regardless of pigment production, but the quantity varied with (386, 120, and 78 mg/l for *Monascus purpureus, Monascus ruber*, and *Monascus sanguineus,* respectively[25].

Figure-1
Sequence Analysis of *Monascus sanguineus* for molecular identification

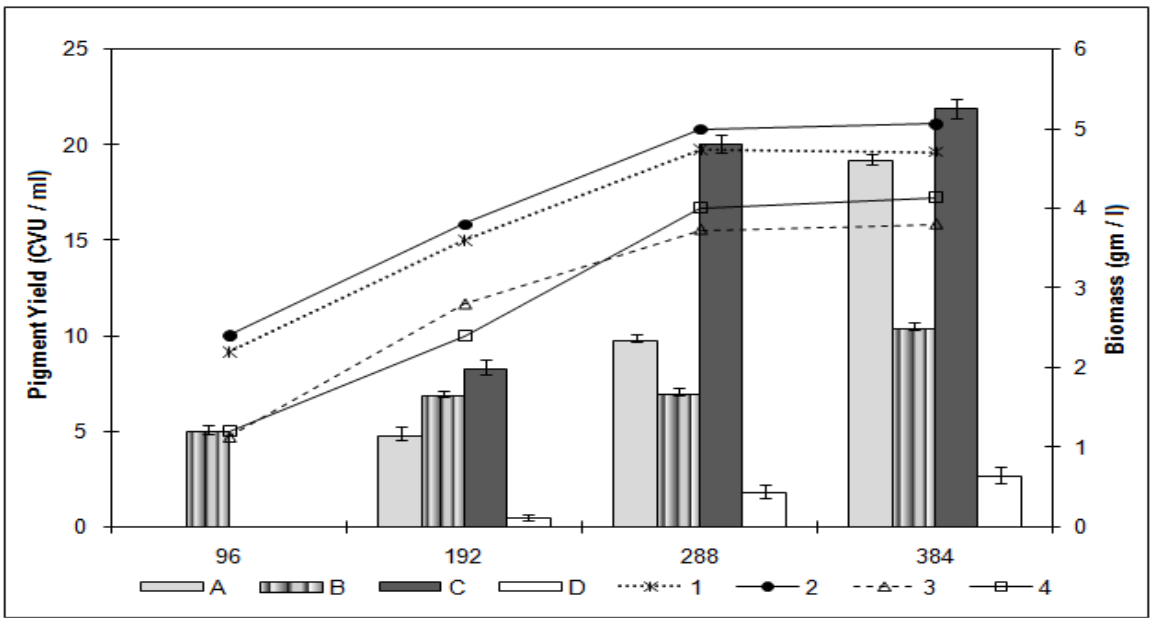

Figure-2

Growth Pattern of *Monascus sanguineus* on different media (- Malt Glucose Peptone Broth; - Malt Extract Broth; - Potato Dextrose Broth; - Sabouraud Dextrose Broth; - Malt Glucose Peptone Broth; - Malt Extract Broth; - Potato Dextrose Broth; - Sabouraud Dextrose Broth; left y-axis depicting scale for pigment yield in Color Value Units/ml; right y-axis depicting scale for biomass in gram/liter and x-axis indicating scale for time in hours) at 32°C and pH 5.5. The experiments were carried out in triplicate and the data were expressed as mean value with error bars as their standard error

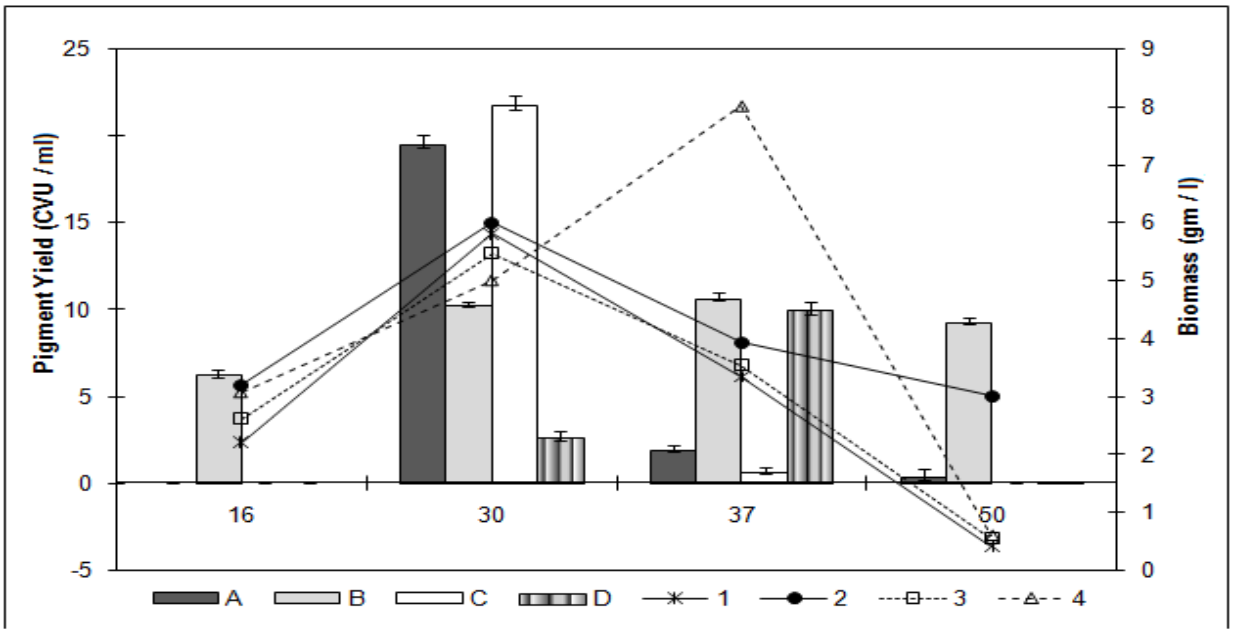

Figure-3

Effect of temperature on pigment production and biomass of *Monascus sanguineus* (- Malt Glucose Peptone Broth; - Malt Extract Broth; - Potato Dextrose Broth; - Sabouraud Dextrose Broth; - Malt Glucose Peptone Broth; - Malt Extract Broth; - Potato Dextrose Broth; - Sabouraud Dextrose Broth; left y-axis depicting scale for pigment yield in Color Value Units/ml; right y-axis depicting scale for biomass in gram/liter and x-axis indicating scale for temperature in °C). The experiments were carried out in triplicate and the data were expressed as mean value with error bars as their standard error

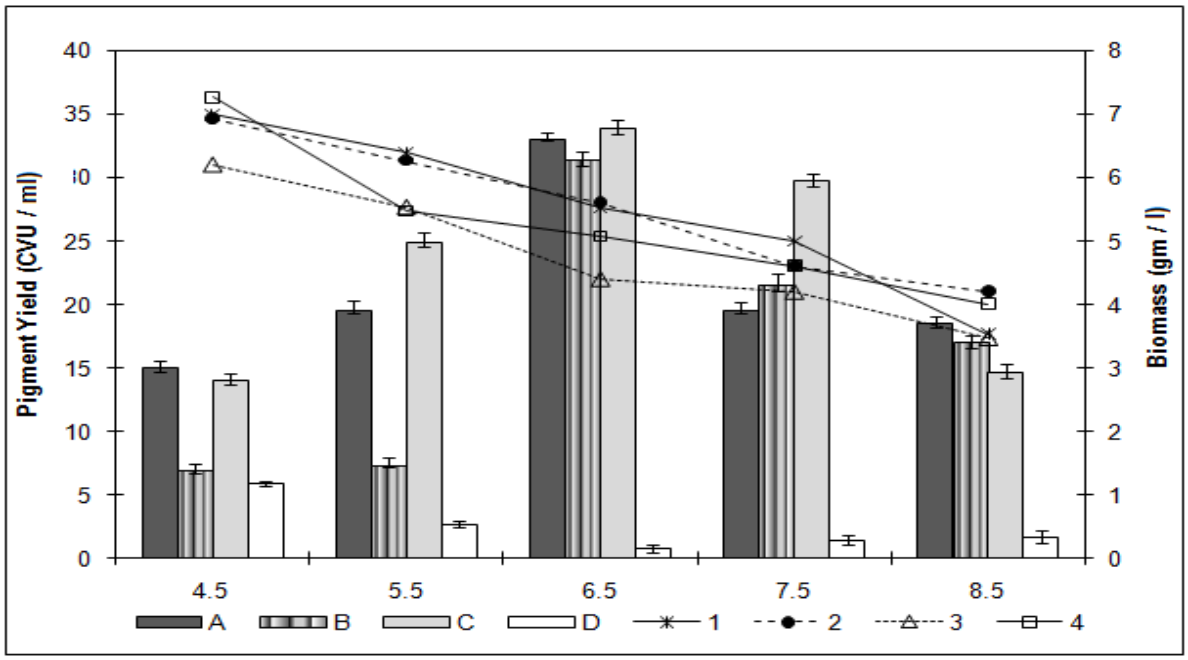

Figure-4

Effect of pH on pigment production and biomass of *Monascus sanguineus* (- Malt Glucose Peptone Broth; - Malt Extract Broth; - Potato Dextrose Broth; - Sabouraud Dextrose Broth; - Malt Glucose Peptone Broth; - Malt Extract Broth; - Potato Dextrose Broth; - Sabouraud Dextrose Broth; left y-axis depicting scale for pigment yield in Color Value Units/ml; right y-axis depicting scale for biomass in g/l and x-axis indicating scale for pH variation). The experiments were carried out in triplicate and the data were expressed as mean value with error bars as their standard error

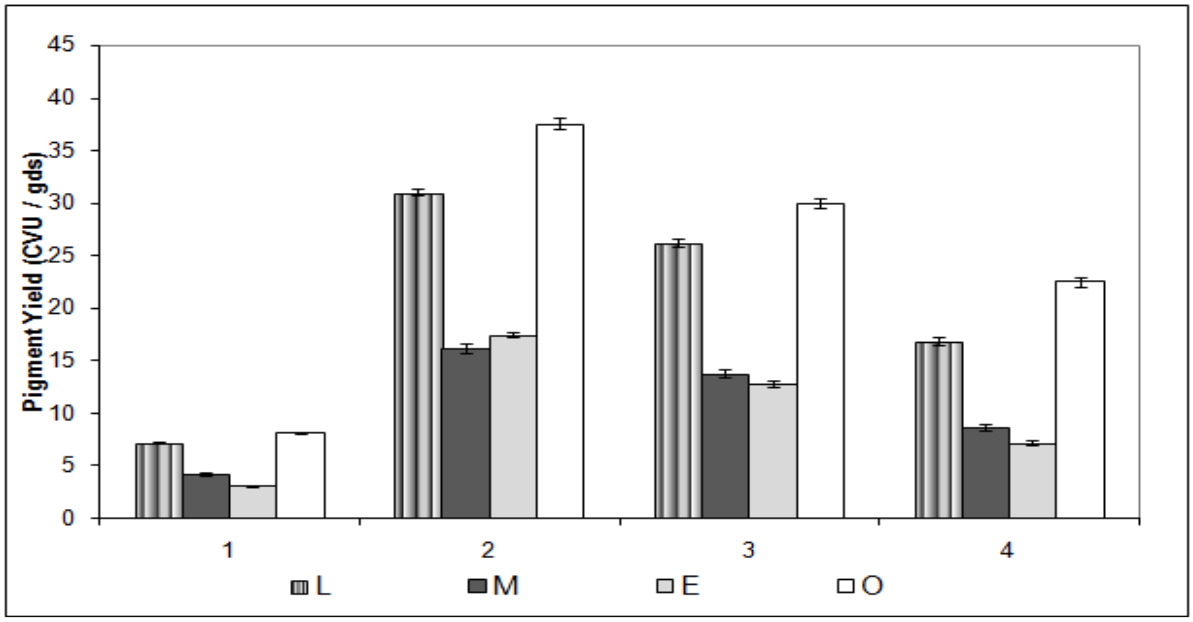

Figure-5

Effect of carbon source on pigment production by *Monascus sanguineus* (-*Ipomoea* sp., - *Manihot* sp., - *Eleucine* sp., - *Oryza* spp.; y-axis showing the pigment yield in Color Value Units/gram dry substrate and x-axis substrate with varied carbon sources viz. 1-control, 2 – 5% glycerol (w/w), 3 – 10% glycerol (w/w), 4 – 15% glycerol (w/w),). The experiments were carried out in triplicate and the data were expressed as mean value with error bars as their standard error

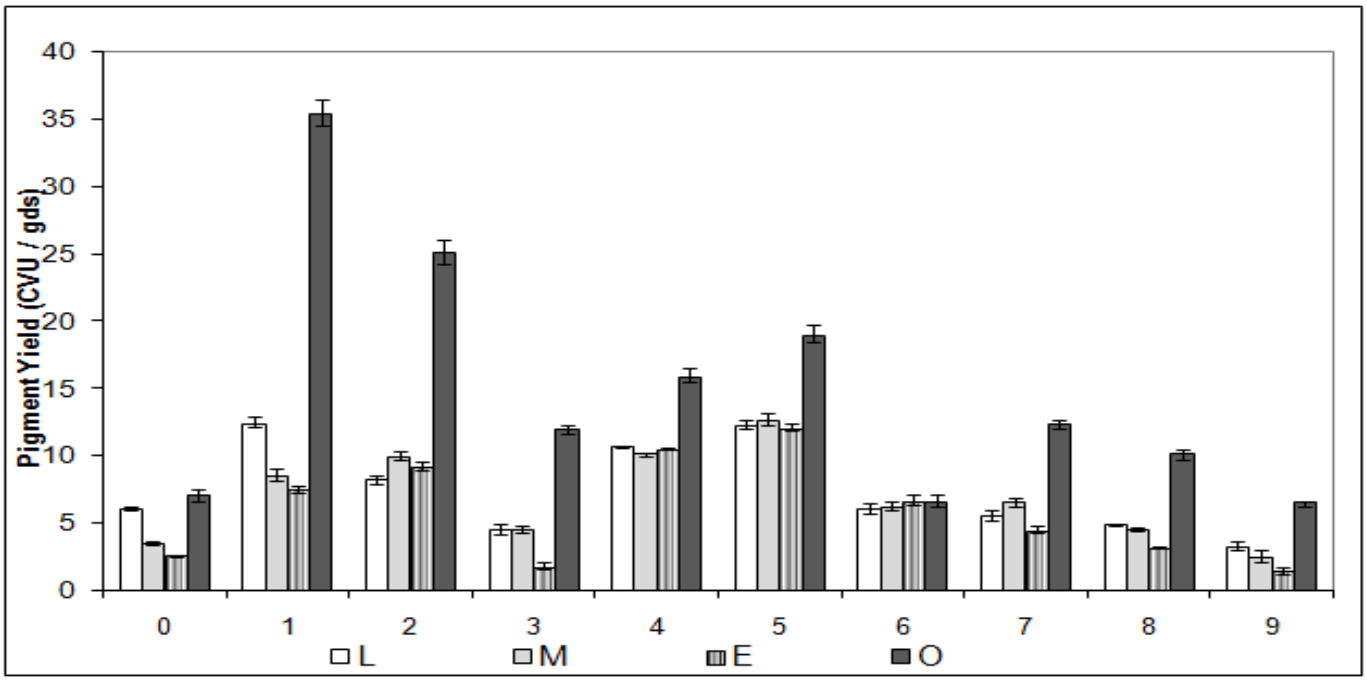

Figure-6

Effect of nitrogen source on pigment production by *Monascus sanguineus* (-*Ipomoea* sp., -*Manihot* sp., -*Eleucine* sp., - *Oryza* sp.; y-axis showing the pigment yield in Color Value Units/gram dry substrate and x-axis substrate with varied nitrogen sources viz. 0-control, 1 – 2% peptone (w/w), 2 – 6% peptone (w/w), 3 – 10% peptone (w/w), 4 – 2% yeast extract (w/w), 5 – 6% yeast extract (w/w), 6 – 10% yeast extract (w/w), 7 – 2% mono sodium glutamate (w/w), 8 – 6% mono sodium glutamate (w/w), 9 – 10% mono sodium glutamate (w/w)). The experiments were carried out in triplicate and the data were expressed as mean value with error bars as their standard error.

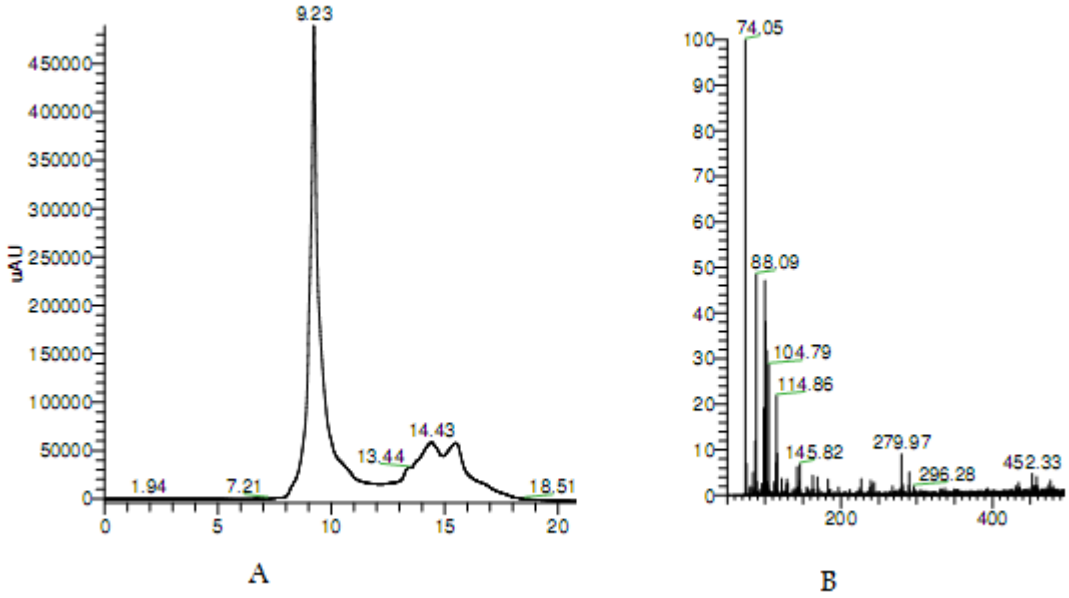

Figure-7

Mass fragmentation spectrum of ESI chromatogram of *Monascus sanguineus* (A – detection of *Monascus sanguineus* extract, B - LC separated analytes) with x-axis showing the wavelength and y-axis the relative abundance

Conclusion

The present study revealed that while *M. sanguineus* can tolerate temperatures within the range of 16-50°C, increase in biomass and pigment yield was observed at 30-32°C. This species 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 6.5. *Oryza* and *Ipomoea* sp. were found to be the best substrates for pigment production. Maximum red pigment yield by SSF was observed when the substrates were supplemented with 5% glycerol and *Oryza* sp. with 2% peptone.

Though there have been many reports on the usability of *M. purpureus*, the isolated *M. sanguineus* strain also needs attention and exploration from researchers to establish itself as potential natural source for pigment production.

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