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Influence of Culture Media on Growth and Pigment Production by *Fusarium moniliforme* KUMBF1201 Isolated from Paddy Field Soil

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Abstract: A new strain of *Fusarium moniliforme* KUMBF1201 was isolated from paddy field soil for the production of natural pigment. From an industrial point of view the necessity to obtain a suitable medium for enhanced pigment production by *Fusarium moniliforme* KUMBF1201 was the aim of this work. Out of eight different solid and six liquid media studied, *Fusarium moniliforme* KUMBF1201 grew well on Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media. It required optimum temperature (28±1°C), pH (5.5) for the growth, pigment production and sporulation. From the results, the glucose (2 %) as the carbon source and yeast extract (2 %) as nitrogen source has played a major role in enhanced cell growth and pigment production.

Key words: Bikaverins · Karotenoids · Potato Dextrose Agar · Potato Dextrose Broth · *Fusarium Moniliforme* KUMBF1201

INTRODUCTION

Fungi are more ecological interesting source of pigments, since some fungal species are rich in stable colorants. A wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific culture medium, pH, temperature, light, water availability and surrounding atmospheric gas mixture [1]. *Fusarium moniliforme* is a major fungal parasite occurring worldwide, mainly associated with cereal crops such as rice, oats, wheat, maize, barley and soy beans. This fungus is widespread in both humid temperate zones and extending into subtropical and tropical areas throughout the world [2]. Moreover, this fungus produces further biologically active metabolites, e.g. pigments, mycotoxins, phytotoxins, oestrogens, naphthoquinone and extracellular enzymes. Biosynthesis of secondary metabolites is regulated by medium ingredients such as carbon sources, nitrogen sources and other environmental factors [3]. The production of naphthoquinone pigments was mostly studied in this fungus on nutrient-rich laboratory media for maximal amounts of various pigments and to study their structure. Since naphthoquinones possess a broad-range biological activity and a universal mechanism of action, they can play a significant ecological role as protectors by providing a selective advantage for the producer in its survival in a natural ecosystem. The interest of many investigators in these compounds is due to their broad-range of biological activities: antibacterial, fungicidal, antiparasitic and insecticidal [4-6].

Fusarium moniliforme strains are able to produce two pigment group, bikaverins and the karotenoids, the last of which has existing activity against *Leishmania brasiliensis*. The biosynthesis of some metabolic substances, such as moniliformin, fusaric acid, fusarin C and fusariocin C, is described. From enzymes the milk-clotting rennin, pectinases, phenol-degrading enzymes, cellulolytic and amylolytic enzymes are of economical interest [7-9]. *Fusarium moniliforme* is a source of different bioactive metabolites and the production is dependent on the conditions and strain specificity [10]. Benzoxazolinone, a novel acid was isolated by column chromatograpy from 48 h old cultures of *Fusarium moniliforme* [11]. The culture filtrate was purified by solvent extraction, partition and absorption

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chromatography and yield four new pigments 8-O-methyl derivatives of bostrycoidin, javanicin, solaniol and bikaverin [2]. In another metabolite, kaurene synthetase has been purified approximately 170-fold from cell-free extracts of the fungus *Fusarium moniliforme* Sheld. The two catalytic activities associated with kaurene synthetase, namely the cyclization of trans-geranyl-geranyl-PP to copalyl-PP (activitiy A) and the cyclization of copalyl-PP to (-)-kaurene (activity B) [12]. From an industrial point of view the necessity to obtain a suitable medium for enhanced growth and pigment production by *Fusarium moniliforme* KUMBF1201 was the aim of this work.

MATERIALS AND METHODS

Isolation, Screening and Identification: Eight soil samples were collected from agriculture land in Trichy and Coimbatore district, Tamil Nadu, India. The soil samples were taken from a depth of 1-10 cm and then kept in sterile plastic bags, drying was performed immediately after sampling. Soil samples were sieved with a 0.5 mm sieve to remove larger particle such as stones and plant debris in order to obtain a consistent soil particle size for isolation using the soil dilution technique. Sieved soils and debris were then stored separately in sterile paper bags and kept at 4°C. One gram of the soils were suspended in 100 mL of sterile distilled water and mixed thoroughly. One mL of the soil suspension was used to prepare a dilution series of 10^{-2} and 10^{-3} and one mL of each dilution series was uniformly dispensed onto PDA media, with four replicates. The PDA plates were incubated at room temperature (27±1°C) for 4-7 days or until visible sign of colony growth. This was carried out for eight different soil samples.

Microscopic Examination: The selected fungal isolate was microscopically identified [13] and confirmed by Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, G.G. Agharkar Road, Pune, India. For identification, subcultures were made onto PDA, incubated at 28ºC and examined daily.

Measurement of Fungal Growth by Colony Diameter Method: The fungi grow on artificial medium forming colonies of the different shape, size and color. Size of colonies enhances day by day attaining the full growth at definite period. However, fungal growth can be estimated by measuring the size of colonies per hour or per day. Measurement consists of the colony diameter method was taken and recorded by Dubey and Maheswari [14].

Radial Growth and Pigment Production of *Fusarium moniliforme* KUMBF1201 Isolate on Solid Medium: In this study, eight solid media: Potato Dextrose Agar (PDA), Malt extract Agar (MA), Czapex's Dox Agar (CDA), Rose Bengel Agar (RBA), Sabouraud's Dextrose Agar (SDA), Oat Meal Agar (QMA), Yeast Malt Agar (YMA), Nutrient Agar (NA). All the media were prepared according to the manufacturer instructions (HiMedia, India). Each Petri dish were poured with 20 mL sterilized medium and allowed for solidification. With the help of cork borer, 5mm diameter of 7-day old precultured *Fusarium moniliforme* KUMBF1201 grown on PDA were taken out and placed at the centre of each set of Petri plate containing different medium. After inoculation, petri plate was incubated at 28±2°C. The diameter of each tested isolates was recorded in millimeters in two directions at right angles to each other and then average colony diameter in millimeters was calculated and recorded. Measurement of growth was made at the interval of 24 h, till the full expansion of growth and studies of sporulation on different solid media was also undertaken.

Pigment Production and Mycelial Growth of *Fusarium moniliforme* KUMBF1201 Isolate on Liquid Broth Medium: Six different liquid media: Potato Dextrose Broth (PDB) (HiMedia, India), Peptone Glycerol Broth (PGB: 5 g/L peptone; 10 g/L glycerol), Yeast extract Malt extract Broth (YMB: 10 g/L glucose; 5 g/L peptone; 3 g/L yeast extract; 3 g/L malt extract, Malt extract Broth (MB: 20 g/L glucose; 20 g/L malt extract; 1 g/L peptone), Sabouraud Dextrose Broth (SDB: 10 g/L peptone; 40 g/L glucose) and Nutrient Broth (NB: 5 g/L peptone; 3 g/L beef extract; 2 g/L yeast extract; 5 g/L Sodium Chloride) were used in this study. The effect of various liquid media was studied by flask culture method using 250 mL Erlenmeyer flask. The seed culture (*Fusarium moniliforme* KUMBF1201 grown in PDB) was inoculated into various flasks containing different media of 50 mL each. The flasks were kept at 28°C for 8 days at 200 rpm on a rotary shaker. Samples collected at various time intervals from the flasks were centrifuged at 16,000 rpm for 20 min and the final pH of the resulting cell free supernatant was measured. The mycelial biomass yield was estimated by washing with distilled water and drying at 80°C for 48 h. Total fungal pigments in the broth was quantified by double beam spectrophotometer at 500 nm absorbance [15].

RESULTS AND DISCUSSION

Isolation and Screening of Pigment Producing Fungi Isolated from Agriculture Soil: In the present study an attempt was made to evaluate the growth and pigment production by filamentous fungi isolated from agriculture soil. A total of 33 isolates were obtained from the eight soil samples analyzed. Eight morphologically different color producing fungi were isolated. Among the eight isolates, one of the fungi isolated from paddy field soil in Manachanallur, Trichy district, showed the ability to produce dark reddish brown pigment, which was selected in this investigation and was identified as *Fusarium moniliforme* and named as *Fusarium moniliforme* KUMBF1201. The selected isolate were confirmed by Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, G.G. Agharkar Road, Pune, India.

Microscopic Examination: Direct microscopic examination of the colonies performed with lactophenol cotton blue, conidia with a needle-like appearance and hyphal structures suggested *Fusarium moniliforme* [13]. At differential diagnosis, the base color of the reddish-brown colonies having cotton-like appearance, turned from purple color in seventh day. At the end of this period, colony size reached 40-60 mm. Under microscopic examination of the colonies, differential diagnosis was initiated with the observation of oval microconidia with one or two septa and phialide structures exhibiting polyblastic features, with transparent hyphal structures and macroconidia having a needle-like appearance, slightly bent and lightly curved in places, conidiaferous structures and a large number of transparent chlamydospores were present and formed singly or in pairs (Figure 1).

Measurement of Fungal Growth by Colony Diameter Method: Take average value of three readings and calculate radial growth of *Fusarium moniliforme* KUMBF1201, day by dividing this value by total number of days incubated. At the end of this period, colony diameter of *Fusarium moniliforme* KUMBF1201 attained 54mm diameter in 5 day of incubation and then its mean growth rate was 10.8 mm/day (Figure 2). (i.e. 16 (16-0) + 12 (28-16) + 6 (34-28) + 10 (44-34) + 10 (54-44) = 54/5 = 10.8 mm/day).

Radial Growth and Pigment Production of *Fusarium moniliforme* KUMBF1201 Isolate in Solid Medium: Fungal mycelium can adopt flat or cottony type of mycelial growth, on the basis of availability of nutrition, although it differs from species to species and strain to strain [16]. The findings of this study that different culture media influenced the growth and pigmentation of *Fusarium moniliforme* KUMBF1201. Out of Eight solid media (PDA, MA, RBA, OMA, YMA, CZA, SDA and NA) were used for the cultural studies (Figure 3).

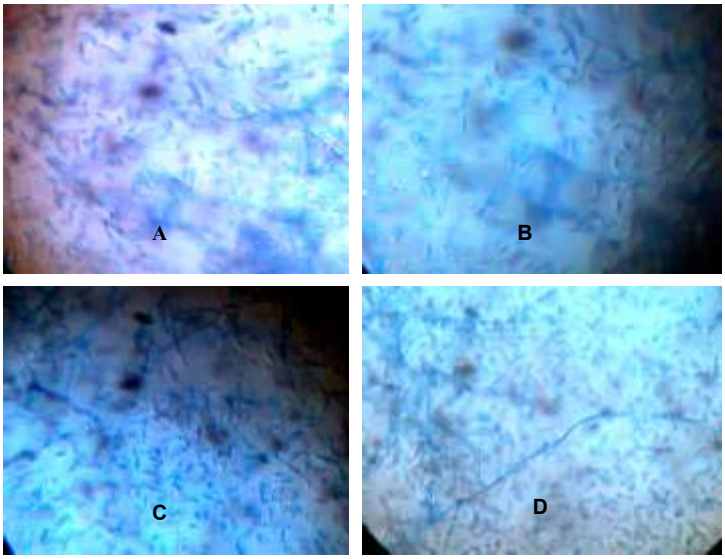

Fig. 1: Direct Microscopy (Lactophenol Cotton Blue Stain): Morphological characteristics of assorted *Fusarium moniliforme* KUMBF1201 (A) Chlamydospores formed singly or in pairs. (B) Macroconidia are slightly sickle-shaped, thin-walled and delicate, with an attenuated apical cell and a foot-shaped basal cell; (C) Oval to kidney-shaped microconidia; (D) Microconidia produced in false heads on short monophialides.

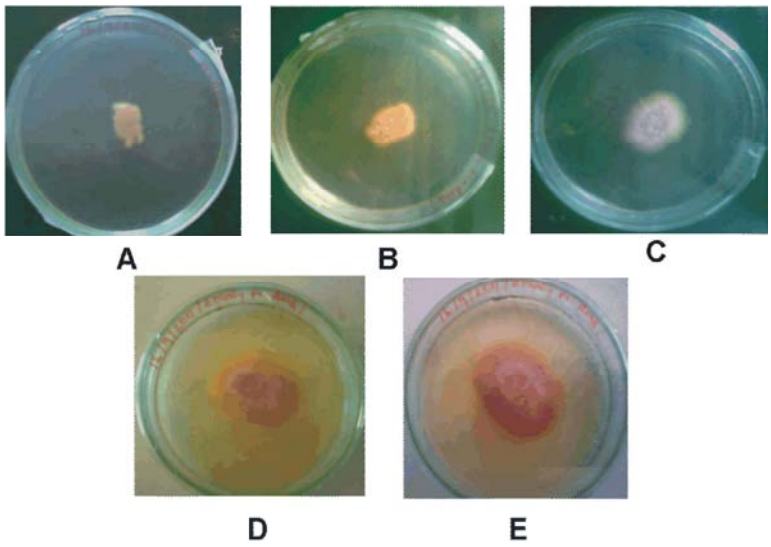

Fig. 2: Measurement of *Fusarium moniliforme* KUMB1201 by colony diameter method (A-Day-1, B-Day-2, C-Day-3, D-Day-4, E- Day-5).

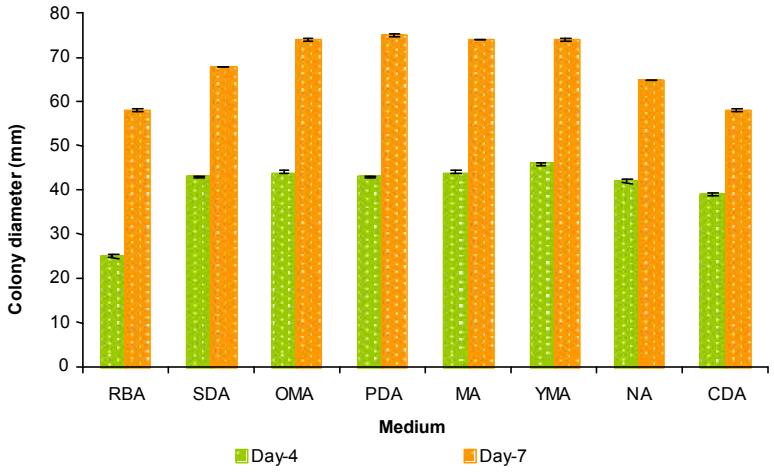

Fig. 3: Growth of *Fusarium moniliforme* KUMBF1201 on eight solid media, after fourth and seventh day of incubation. Results are mean of independent experiments ± SD and are expressed as colony diameter (mm).

The data revealed that maximum mycelial growth and pigment production was best on PDA followed by MA and OMA with a mean maximum growth of 74.96±0.35, 74.03±0.25 and 74.03±0.15 mm respectively (Table 1). Mycelium were whitish color in RBA, NB, CZA medium and color mycelial growth appear in PDA (Reddish brown), OMA (Dark pinkish), MA (violet), YMA (yellow) medium (Figure 4). The most remarking change in pigmentation was observed on CDA, YMA and NA, which produced no pigmentation or only yellowish white pigmentation after one week onwards along with the formation of pale yellow margins.

Peptone, a commercially available digest of a particular plant or animal protein, made available to organisms as peptides and amino acids to help satisfy requirements for carbon, nitrogen, sulfur and energy. Yeast and malt extracts are frequently used as a source of amino acids, vitamins and coenzymes, growth factors by fastidious organisms. Media without peptone or yeast extract or other organic nitrogen sources produced lighter pigmentations than the media containing them [17]. The specific role of yeast extract on pigmentation of *Fusarium moniliforme* KUMBF1201 was evaluated by observing the difference in pigmentation between NA and YMA. Based on the observed growth characteristics from the present study, it is suggested that starch based media, such as PDA, MA and OMA were good substrates for the species of *Fusarium moniliforme* KUMBF1201.

Table 1: Cultural characteristics of *Fusarium moniliforme* KUMBF1201 on eight solid media

S.No	Medium	Colony diameter (mm) Day-4	Colony diameter (mm) Day-7	Growth and pigment production	Sporulation (Microscopic field (100X)
1.	Potato Dextrose Agar	43.10±0.26	74.96±0.35	Pink cottony growth with irregular growth was observed.	++++
2.	Oat Meal Agar	43.96±0.35	74.03±0.15	Purple violet colony growth was observed.	+++
3.	Rose Bengal Agar	24.96±0.45	57.9±0.36	White cottony growth with irregular growth was observed.	+++
4.	Nutrient Agar	42.06±0.35	64.80±0.43	White cottony growth was observed	++
5.	Czapex-Dox Agar	39.03±0.20	58.03±0.15	White cottony growth was observed.	+++
6.	Malt extract Agar	43.93±0.47	74.03±0.25	Purple violet colony growth was observed.	++
7.	Sabouraud Dextrose Agar	43.03±0.25	67.96±0.15	Pink cottony growth, irregular margin was observed.	++
8.	Yeast Malt Agar	43.06±0.20	73.93±0.20	White cottony growth was observed	++++

++ Poor sporulation: 1-20 spores; +++ Medium sporulation: 11-60 spores; ++++ Good sporulation: More than 100 spores. Results are mean of independent experiments ± SD and are expressed as colony diameter (mm).

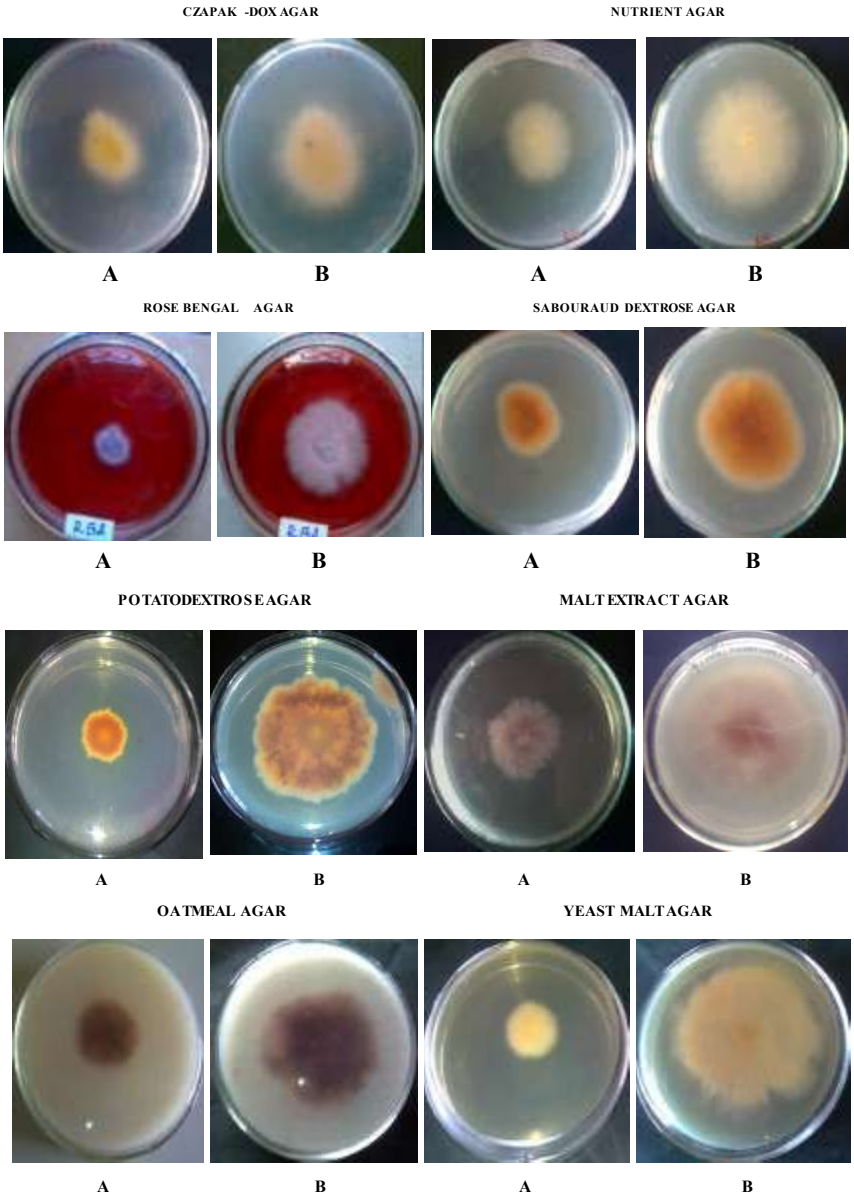

Fig. 4: Effect of growth and pigment production from eight solid media. A- Fourth day mycelia growth and pigment production, B - Seventh day mycelia growth and pigment production.

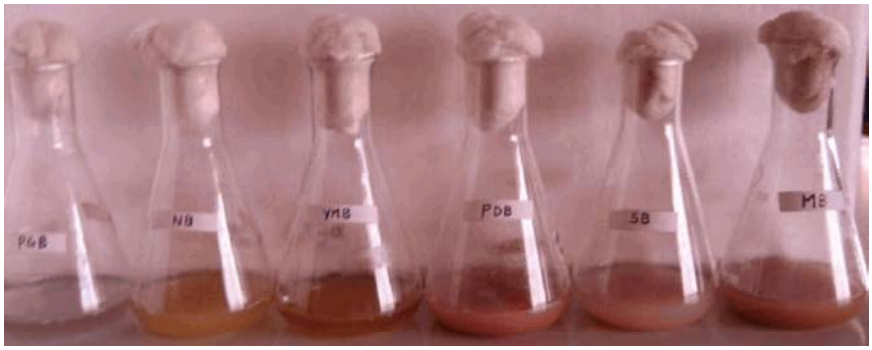

Fig. 5: Effect of pigment production from six liquid media

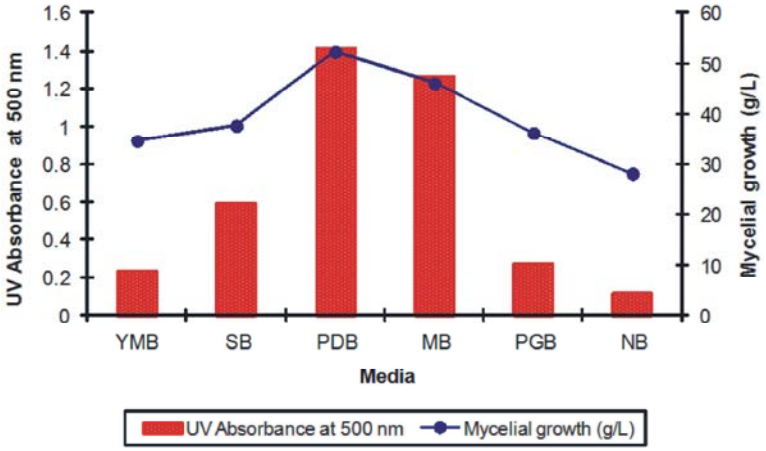

Fig. 6: Effect of various complex media on the mycelial growth and pigment production from *Fusarium moniliforme* KUMBF1201.

Pigment Production and Mycelial Growth of *Fusarium moniliforme* KUMBF1201 Isolate on Liquid Broth Medium: Six liquid broth media (PDB, SDB, PGB, MB, YEB and NB) (Figure 5) were tested for quantification of pigment production and mycelial growth was recorded [18]. The results revealed that among the liquid media tested, maximum moisture content of mycelial growth was obtained in PDB (52.3 g/L mycelial growth) whereas the lowest concentration was in the NB (28.13 g/L mycelial growth (Figure 6). Total pigment production in the broth was quantified by determining at 500 nm (absorbance) using a double beam spectrophotometer. The maximum production of pigment was reached in PDB medium (1.48 nm) whereas the lowest concentration was NB medium (1.12 nm). The main difference between PDB and other nutrient media was that PDB contained starch and the others did not. The main reason suggested that PDB might have components such as metal ions/or other micronutrients appropriate for enzymes to work effectively and enhanced growth metabolites and pigment production. The important nitrogen sources in various culture media was considered, YMB, MB and NB consisting of yeast extract, malt extract and peptone meanwhile SB and PGB containing with peptone. These results indicated that yeast extract and peptone were effective for growth and pigment production of *Fusarium moniliforme* KUMBF1201. These findings revealed that *Fusarium moniliforme* KUMBF1201 grew well on PDA and PDB media. Radial growth was maximum at a temperature of 28°C (74.96±0.35 mm) followed by a temperature of 30°C (71.88±0.21 mm). Therefore, optimum temperature for the best growth was 28°C, which was close to room temperature. The results revealed that a suitable pH for pigment and maximum mycelial mass production was 5.5 followed by 5.0 for *Fusarium moniliforme* KUMBF1201. Several species of *Fusarium* have been reported to grow and sporulate in pH ranges of 5.0 to 6.0 [19].

In most studies of pigment biosynthesis, cultivation of fungi involved rich organic media of complex or indefinite composition [18, 20, 21]. PDA is one of the most commonly used culture media, because of its simple formulation and its ability to support mycelial growth and pigment production for wide range of fungi.

Several workers stated that PDA and PDB to be the best culture media for mycelial growth and pigment production [22-24]. Most fungi thrive on PDA, but this can be too rich in nutrients, thus encouraging the mycelial growth with ultimate loss of sporulation [25]. Conventionally, starch based media, such as PDA or MA are good substrates for the species of *Fusarium* and dematiaceous hypromycetes to grow rapidly and produce abundant aerial mycelia. Glucose, usually an excellent carbon source for growth, interfered with the biosynthesis of many secondary metabolites. *Fusarium moniliforme* KUMBF1201 was able to utilize a variety of sugars as carbon source where as peptone and yeast extract were appropriate nitrogen sources for pigment production. Exploration of fungal biodiversity is still going on, with special interest in water-soluble pigments [26-29]. Aspects of this work should be directly applicable in the food colorants, biosurfactant, pharmaceutical and also cottage industries with potential future economic benefits.

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