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Research Article

OPTIMIZATION OF PIGMENT AND BIOMASS PRODUCTION FROM FUSARIUM MONILIFORME UNDER SUBMERGED FERMENTATION CONDITIONS

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ABSTRACT

Objective: This study aimed at improving the medium composition for efficient and economical production of pigment and mycelial biomass from the native isolates of *Fusarium moniliforme* by submerged culture.

Methods: The effect of cultural conditions like different production medium, temperatures, pH, incubation period, carbon sources, nitrogen sources, amino acids and metal salts on pigment and biomass production were studied.

Results: The optimum productivity of the pigment and biomass was achieved with optimized process parameters containing potato dextrose broth (PDB 2% w/v), temperature (28ºC), pH (5.5), incubation period (8 days), carbon source (glucose 2% w/v), nitrogen source (peptone 1% w/v), amino acid (methionine 0.5%) and metal salts (KH_2PO_4 0.5%). Conclusion: We hope that the intensive study on the pigment constituents from *Fusarium moniliforme* will lead to the discovery of a novel pharmaceutical and insecticidal property.

Keywords: *Fusarium moniliforme,* Secondary metabolites, Pigment production, Submerged culture, Biological activities.

INTRODUCTION

Microbial secondary metabolites have provided numerous pharmaceutical agents ranging from antibiotics to immunosuppressive compounds. Synthesis of these low molecular weight compounds is not required for normal growth of the microbe; however these compounds may provide several benefits to the organism. Fungi have the ability to produce a plethora of secondary metabolites, typically dependent on the stage of development and environmental factors ranging from nutrient concentrations to light and temperature [1, 2]. *Fusarium moniliforme* Sheldon, a field fungus common on many crops like corn, rice, wheat, barley, millet, sorghum and citrus fruits produces a number of interesting, biologically active secondary metabolites. *Fusarium moniliforme* strains are able to produce two pigment group, bikaverins and the karotenoids, the last of which has existing activity against *Leishmania brasiliensis.* They may produce one or more range of secondary metabolites, such as gibberellins (GAs) [3], the red pigment bikaverin and its minor coproduct nor-bikaverin [4, 5] and several mycotoxins such as moniliformin, beauvericin [6], fumonisins [7], fusaric acid [8], fusarin C [9] and naphthoquinones [10,11].

The culture filtrate was purified by solvent extraction, partition and absorption chromatography and yield four new pigments 8-O-methyl derivatives of bostrycoidin, javanicin, solaniol and bikaverin. Although the functions of most secondary metabolites are unknown, it is generally recognized that pigmented materials likely protect fungi from exposure to environmental stress like UV light [12]. This class of compounds is of interest due to the broad spectrum of their biological activities, such as antibacterial [13], antifungal [14], phytotoxic [15] insecticidal [16] and cytotoxic properties [13,14]. Secondary metabolites can be grouped into four different classes depending on their structural properties: polyketides, terpenes, nonribosomal peptides and amino acid-derived compounds. Among the four secondary metabolites, polyketides form the most abundant group [2], including most of the green and red fungal pigments, all of which belong to the group of naphthoquinones. So far, the structures of more than 100 naphthoquinone metabolites have been elucidated [17], indicating the structural diversity of this group. The ability to produce naphthoquinones is widespread among fungal organisms, especially among members of the genus *Fusarium.* Submerged culture gives rise to potential advantages of higher mycelial production in compact space and shorter time with lesser changes of contamination [18]. The synthesis of many secondary metabolites is regulated by environmental conditions, such as carbon and nitrogen sources, pH and light [19]. Supplement of carbon and nitrogen sources [20] amendment of amino acid, sodium chloride, mineral salts [21, 22] and various culture conditions like temperature, pH and incubation period [23] play a major role on growth and production of antimicrobial agent [24] by microorganisms. In this study, to improve the pigment and biomass production by *Fusarium moniliforme*, factors such as carbon sources, nitrogen sources, pH, various minerals and amino acids were evaluated in submerged flask culture technique.

MATERIALS AND METHODS

Isolation, Screening and Identification

Fusarium moniliforme was isolated from Agricultural fields and identified according to Saremi [25]. The stock culture was maintained on a Potato Dextrose Agar (PDA) slant. For inoculum preparation, the fungus was initially grown at 28°C on a PDA plate for 7 days. A 0.7 cm^2 plug from the outer zone of the colony was punched with a sterile well cutter and transferred to 100 mL Potato Dextrose Broth (PDB) medium in a 250 mL Erlenmeyer flask and grown at 28°C under basal conditions (static) or on a rotary shaker at 200 rpm for 7 days.

Extracellular pigments

The pigment production was indirectly evaluated by measuring the absorbance of the culture filtrate at 500 nm in UV spectrophotometer (Shimadzu) [10, 11].

Dry cell weight

The culture broth was centrifuged at 16,000 rpm for 20 min and the supernatant fluid was filtered through a filter paper (Whatman No.1). The mycelial biomass yield was estimated by washing with deionized water and dried at 50°C for 48 h [26].

Production on Various Kinds of Complex Media

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 submerged culture

flasks method using 250 mL Erlenmeyer flask [10,11]. The seed culture (*Fusarium moniliforme* KUMBF1201, 1202, 1206, 1207 grown in PDB) was inoculated into various flasks containing different media of 100 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 shake 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 dried at 80°C for 48 h. Total fungal pigments in the broth was quantified by double beam spectrophotometer at 500 nm absorbance [27].

Incubation period, temperature and pH

The effect of cultural conditions like different incubation temperatures (25-40ºC), pH (4.5-7.5) and incubation period (1-12 days) on growth and pigment production was studied separately by inoculating 3 mL of the spore suspension of each four *Fusarium moniliforme* isolates into the above improved medium and then kept at 200 rpm on a rotary shaker. The growth as well as the pigment production was determined separately in the similar manner as mentioned above.

Effect of supplementary Carbon and Nitrogen source

Various carbon sources such as glucose, fructose, sucrose, maltose and lactose, and nitrogen sources such as sodium nitrate, sodium nitrite, urea, yeast extract and peptone respectively were amended separately into the basal medium (PDB) at a concentration of 2% (W/V). Each four *Fusarium moniliforme* isolates was inoculated to the respective media and incubated at 25ºC for 8 days at 200 rpm on a rotary shaker. After incubation in an optimal condition the pigment and biomass production was quantified.

Effect of mineral salts

Magnesium (Mg^{2+}), zinc (Zn^{2+}), and copper (Cu^{2+}) ions in the form of Sulfate salts ($MgSO_4$, $ZnSO_4$, $CuSO_4$), Potassium (K^+) ions in the form of Dihydrogen phosphate (KH_2PO_4), Ferric (Fe^{2+}) ion in the form of Ferric chloride ($FeCl_2$) were used. Each metal ion at concentration of 0.5% (W/V) was added to basal medium (PDB). Each four *Fusarium moniliforme* isolates were inoculated to the respective media and incubated at 25ºC for 8 days at 200 rpm on a rotary shaker. After incubation in an optimal condition the pigment and biomass production was quantified.

Effect of Amino acids

Various amino acids such as leucine, cysteine, methionine, histidine, glutamic acid and proline were amended at a concentration of 0.5% (W/V) in combination with glucose as a carbon source. The effect of pigment and biomass production was estimated after incubation at optimum conditions. The biomass accumulation and pigment production for each amino acid concentration were estimated as stated above.

RESULTS AND DISCUSSION

Isolation, Screening and Identification of *Fusarium moniliforme*

Twenty six isolates of *Fusarium sp.* (KUMBF1201 – KUMBF1226) (Table-1) were obtained from different cultivation of agricultural fields from various locations in Trichy and Coimbatore district, Tamil Nadu, India. The selected four potential pigment producing *Fusarium moniliforme* KUMBF1201, KUMBF1202, KUMBF1206, KUMBF1207 isolates were microscopically identified and confirmed by Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, G.G. Agharkar Road, Pune, India.

Pigment and biomass production by four *Fusarium moniliforme* isolates on liquid broth medium

Six liquid broth media (PDB, SDB, PGB, MB, YEB and NB) were tested for quantification of pigment and biomass production was recorded [10]. The results revealed that among the six liquid media tested, for the four fungal isolates, *Fusarium moniliforme* KUMBF1201 showed maximum biomass production observed in PDB (7.37±0.15 g/L) whereas the lowest concentration was in the NB (2.77±0.07 g/L) (Fig. 1). Total pigment production in the broth was quantified by determining at 500 nm (absorbance) using a UV spectrophotometer (Shimadzu). Of the four isolates, *Fusarium moniliforme* KUMBF1201 showed the maximum pigment production in PDB medium (1.363±0.03 nm) whereas the minimum pigment concentration was in NB medium (0.236±0.03 nm) (Fig.2). The main difference between PDB and other nutrient media was that PDB contains starch and the others did not.

Table 1: Pigment (PDA Medium) producing *Fusarium* species in various sources

S. No.	Sample No	Sampling site	Name of the fungi	Sources	Pigment producers	Pigment color
1	KUMBF1201	Manachanallur	*Fusarium moniliforme*	Paddy field soil	++++	Pinkish violet
2	KUMBF1202	Thuraiyar	*Fusarium moniliforme*	Infected paddy grains	++++	Pinkish violet
3	KUMBF1203	Sirugamani	*Fusarium sp*	Infected banana tissue	+++	Brown
4	KUMBF1204	Lalgudi	*Fusarium semitectum*	Infected cotton seed	+++	Yellow
5	KUMBF1205	Somarasampettai	*Fusarium sp*	Infected banana root	+++	Pink
6	KUMBF1206	Malumichampatti	*Fusarium moniliforme*	Corn field soil	++++	Pinkish violet
7	KUMBF1207	Eachanari	*Fusarium moniliforme*	Infected corn root	++++	Pinkish violet
8	KUMBF1208	Vadavalli	*Fusarium semitectum*	Infected cotton root	+++	Yellow
9	KUMBF1209	Pollachi	*Fusarium oxysporum*	Banana field soil	++	Light brown
10	KUMBF1210	Sulur	*Fusarium sp*	Coconut field soil	++	White cottony
11	KUMBF1211	Thuraiyar	*Fusarium sp*	Infected banana tissue	++	Light brown
12	KUMBF1212	Sirugamani	*Fusarium oxysporum*	Infected banana tissue	++	Pink
13	KUMBF1213	Eachanari	*Fusarium sp*	Corn field soil	++	White cottony
14	KUMBF1214	Eachanari	*Fusarium bevicompactum*	Banana field soil	++	Light brown
15	KUMBF1215	Sulur	*Fusarium sp*	Corn field soil	++	Brown
16	KUMBF1216	Somarasampettai	*Fusarium oxysporum*	Culitivated soil	++	White cottony
17	KUMBF1217	Sirugamani	*Fusarium sp*	Cultivated soil	++	Light yellow
18	KUMBF1218	Pollachi	*Fusarium sp*	Coconut field soil	++	Yellow
19	KUMBF1219	Malumichampatti	*Fusarium oxysporum*	Cultivated soil	++	White cottony
20	KUMBF1220	Malumichampatti	*Fusarium sp*	Cultivated soil		White cottony
21	KUMBF1221	Vadavalli	*Fusarium semitectum*	Cotton field soil	++	Yellow
22	KUMBF1222	Sulur	*Fusarium sp*	Cultivated soil	++	White cottony
23	KUMBF1223	Thuraiyar	*Fusarium sp*	Cultivated soil	++	White cottony
24	KUMBF1224	Lalgudi	*Fusarium semitectum*	Cultivated soil	++	Light yellow
25	KUMBF1225	Manachanallur	*Fusarium sp*	Cultivated soil	++	Brown
26	KUMBF1226	Somarasampettai	*Fusarium sp*	Coconut field soil	++	Light brown

++ - Poor pigmentation, +++ - Medium pigmentation, ++++ - Good pigmentation

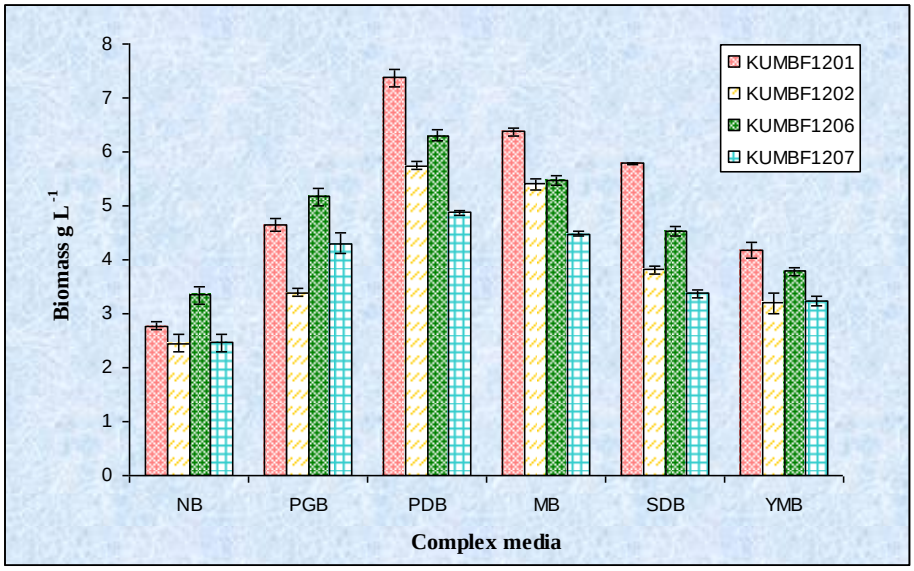

Fig. 1: Impact of six liquid medium on biomass production by four *Fusarium moniliforme* isolates; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

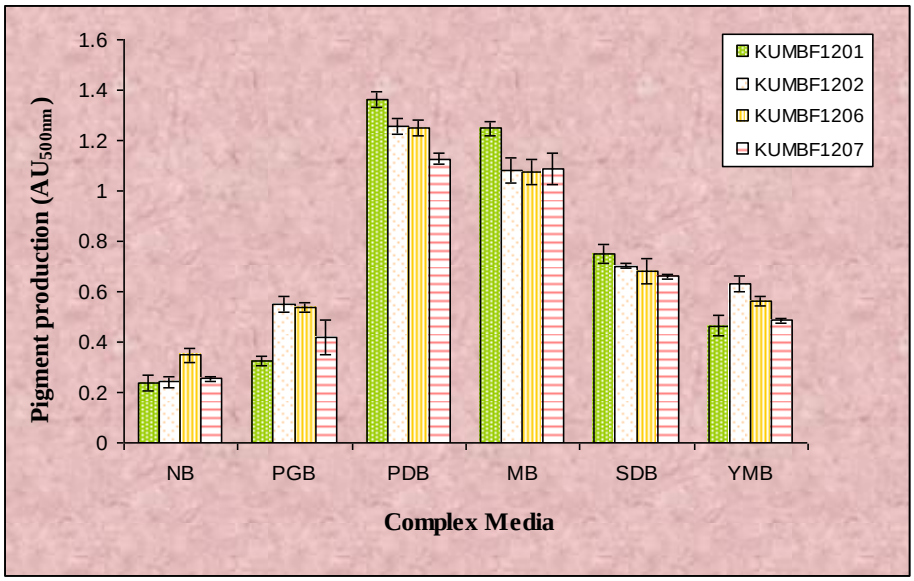

Fig. 2: Impact of six liquid medium on biosynthesis of pigment by four *Fusarium moniliforme* isolates; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

The main reason suggested that PDB might have components such as metal ions/or other micronutrients appropriate for enzymes to work effectively and enhanced the growth metabolites and pigment production [10, 11]. Yeast extract, malt extract and peptone are the important nitrogen source in the medium of YMB, MB and NB, whereas in SDB and PGB medium contain peptone only acts as the source of nitrogen [28]. These results indicated that yeast extract and peptone were effective for pigment and biomass production by *Fusarium moniliforme* isolates.

Impact of culture conditions

Temperature is another important factor as it influences the metabolic activity of fungi and subsequently, their growth. To test the optimal temperature for biomass and pigment production of *Fusarium moniliforme* isolates (KUMBF1201, KUMBF1202, KUMBF1206, KUMBF1207) were cultivated at various temperatures (25–40°C). Of all the temperatures tested at 28ºC gave the highest biomass production (8.11±0.04 g/L) (Fig.3) of *Fusarium moniliforme* KUMBF1201, while the maximum pigment production (1.498±0.02 nm) was obtained in PDB medium (Fig.4). Therefore, the temperature 28ºC was considered as optimum for the future studies.

Desai *et al.* [29] reported that *Fusarium sp.* showed maximum growth and sporulation at 27±2°C. These findings KUMBF1201 revealed that favourable pH for maximum biomass production (8.88±0.04 g/L) (Fig.5) was reached at pH-5.5, whereas maximum synthesis of pigment (1.011±0.03 nm) recorded at pH-5.5 (Fig.6).

Numerous studies have reported that most of the filamentous fungi need an acidic pH (5.0–6.0) as optimum for growth and pigment production in submerged culture [30, 31]. Sharma *et al.,* [32] studied the effect of pH on the growth and sporulation of *F. oxysporum* f. sp. *lini* and reported that tested *Fusarium* spp. could sporulate and grew well at 5.5 pH. To study the effect of incubation period on pigment production, four isolates were cultivated in the optimal medium

with different incubation periods from 2 to 12 day old culture at 28°C in shake flask cultures.

The mycelial dry weight and pigment production of the fungus remains almost constant after eight to ten days of incubation, respectively. The inoculum age of eight day was observed to be optimum for maximum biomass (8.38±0.06 g/L) (Fig.7) and pigment production (1.339±0.01 nm) of *Fusarium moniliforme* KUMBF1201. (Fig.8). Amongst several fungal physiological properties, the inoculum age usually plays an important role in fungal development [31].

Impact of carbon and nitrogen sources

To determine the suitable carbon source for the pigment production of *Fusarium moniliforme,* isolates (KUMBF1201, KUMBF1202, KUMBF1206, KUMBF1207) were cultivated in the basal medium containing various carbon sources.

Carbon sources like glucose, fructose, lactose, maltose and sucrose have got remarkable influence on enhancement of biomass and pigment production. Of all the carbon sources tested, glucose gave the highest biomass production (11.33±0.07 g/L) (Fig.9) of *Fusarium moniliforme* KUMBF1201, while the maximum pigment production (1.405±0.07 nm) was obtained in PDB medium (Fig.10). Glucose, usually an excellent carbon source for growth, interfered with the biosynthesis of many secondary metabolites [33]. In this study, peptone, yeast extract, sodium nitrate, sodium nitrite, urea had a positive effect on pigment production, whereas other nitrogen sources strongly inhibited red pigment synthesis.

Of all the nitrogen sources tested, peptone gave the highest yield for biomass production (11.66±0.1 g/L), (Fig.11) while the maximum pigment production (1.449±0.04 nm) was obtained in PDB medium (Fig.12). It has been reported that various types of peptone supported greater pigment production in many kinds of pigment-producing filamentous fungi [30].

Impact of metal salts

The bio-elements are one of the important factors affecting pigment production in several microorganisms [34]. Some of them such as K^+, Mg^{2+} and Zn^{2+} ions played a significant role in the increase of biomass and pigment production. To determine the suitable metal salts for the pigment production of *Fusarium moniliforme* isolates (KUMBF1201, KUMBF1202, KUMBF1206, KUMBF1207) were cultivated in the basal medium containing various metal salts.

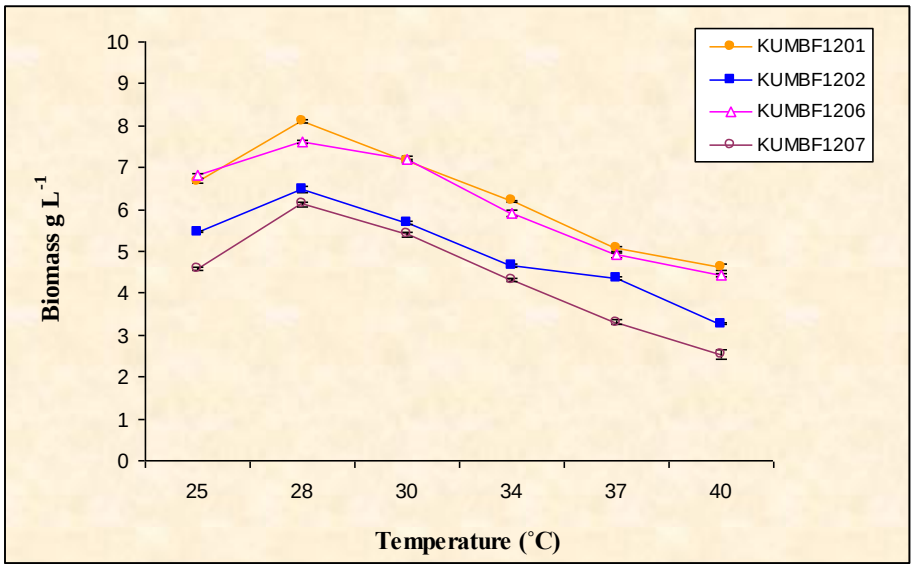

Fig. 3: Impact of temperature on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

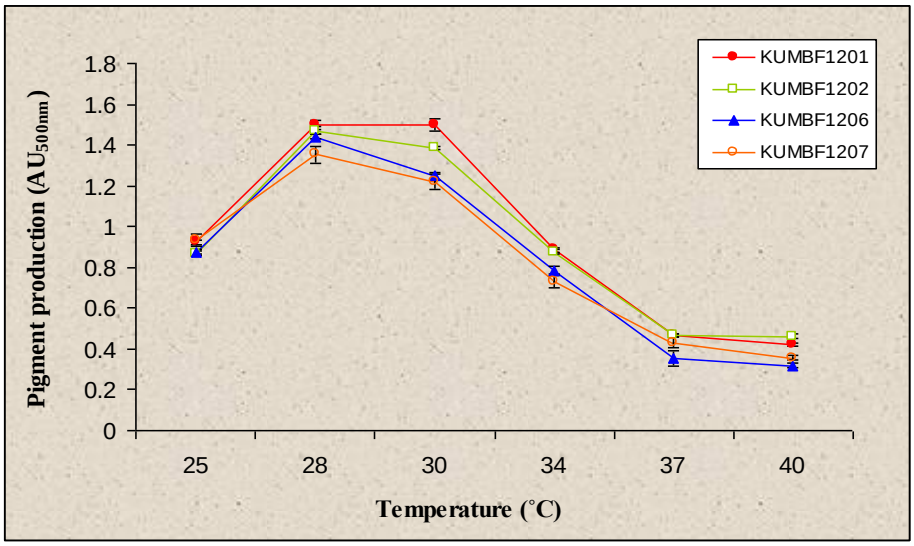

Fig. 4: Impact of temperature on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

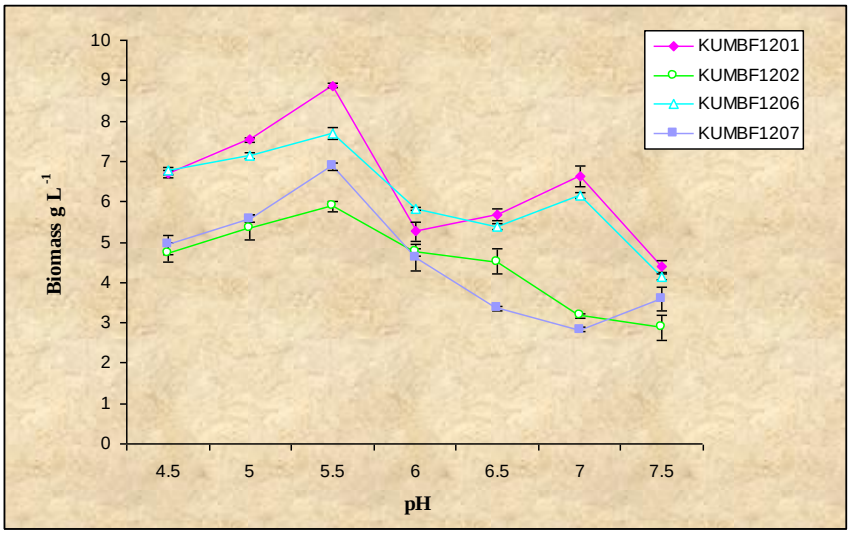

Fig. 5: Impact of pH on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

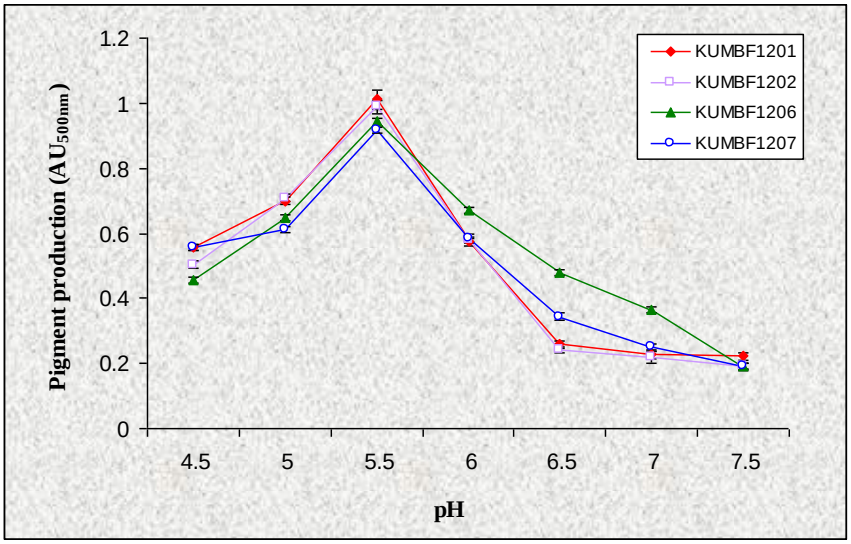

Fig. 6: Impact of pH on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

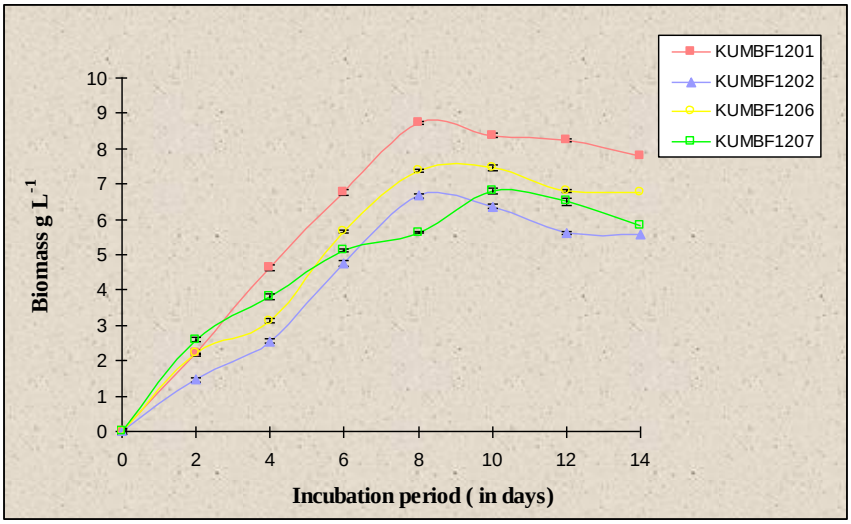

Fig. 7: Impact of incubation period on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

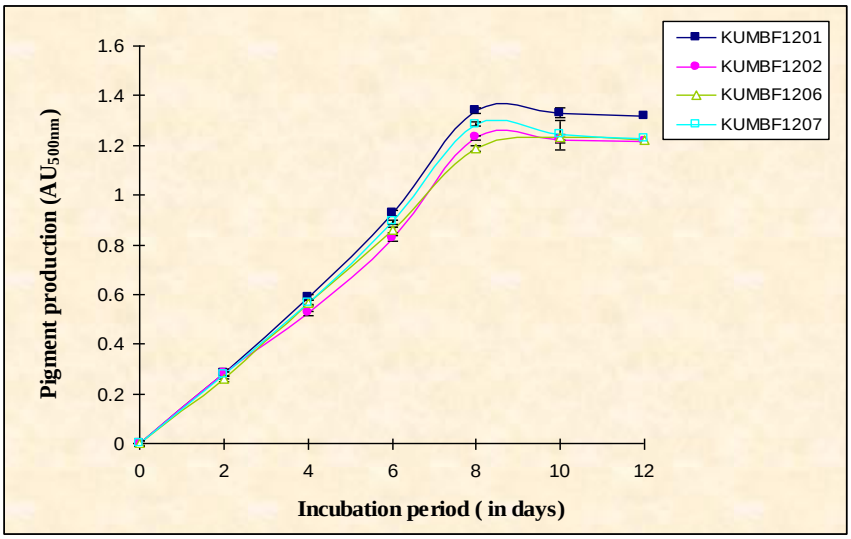

Fig. 8: Impact of incubation period on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

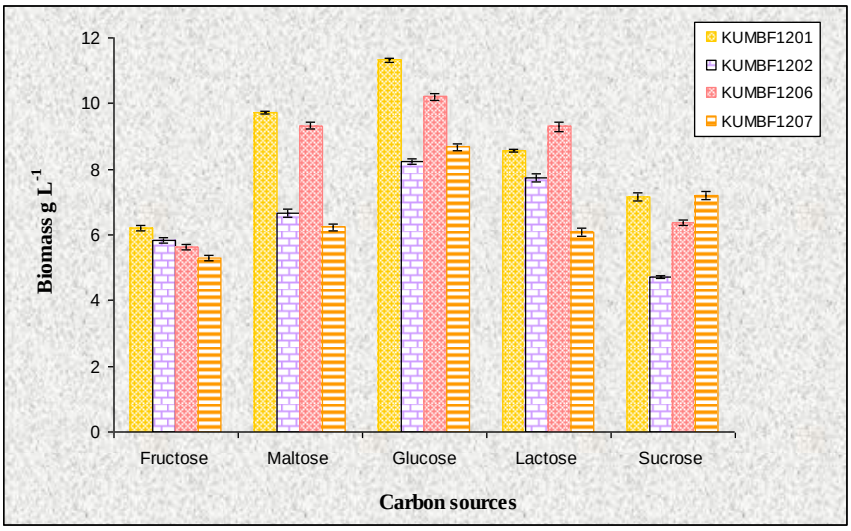

Fig. 9: Impact of carbon sources on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

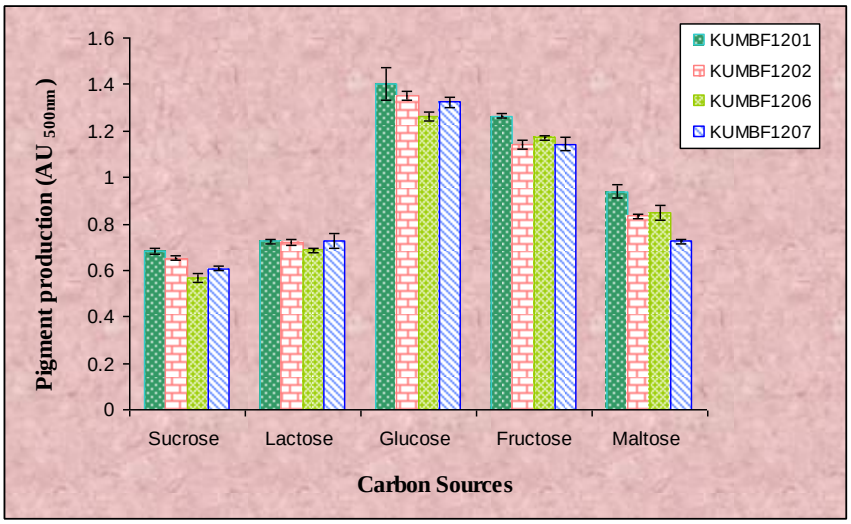

Fig. 10: Impact of carbon sources on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

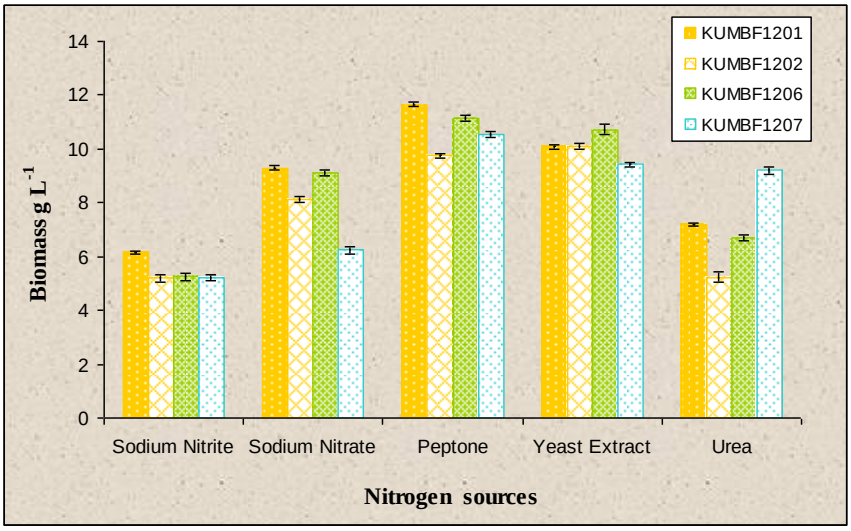

Fig. 11: Impact of Nitrogen sources on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Glucose 2%; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

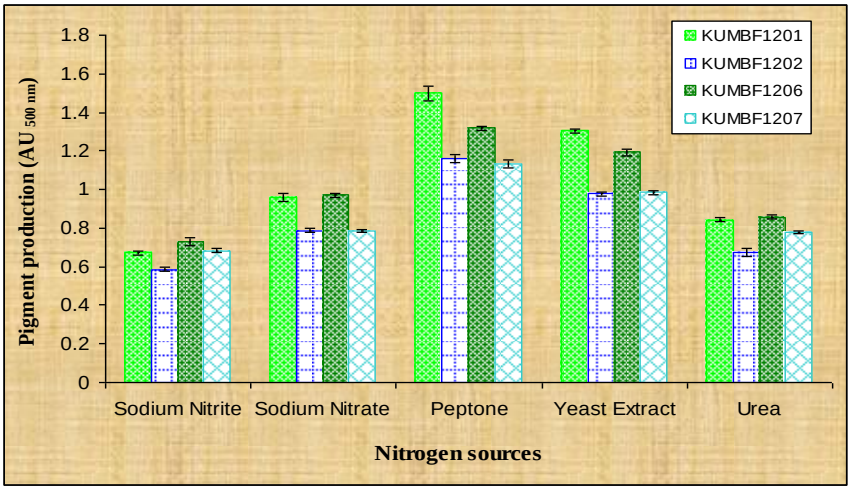

Fig. 12: Impact of Nitrogen sources on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Glucose 2%; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

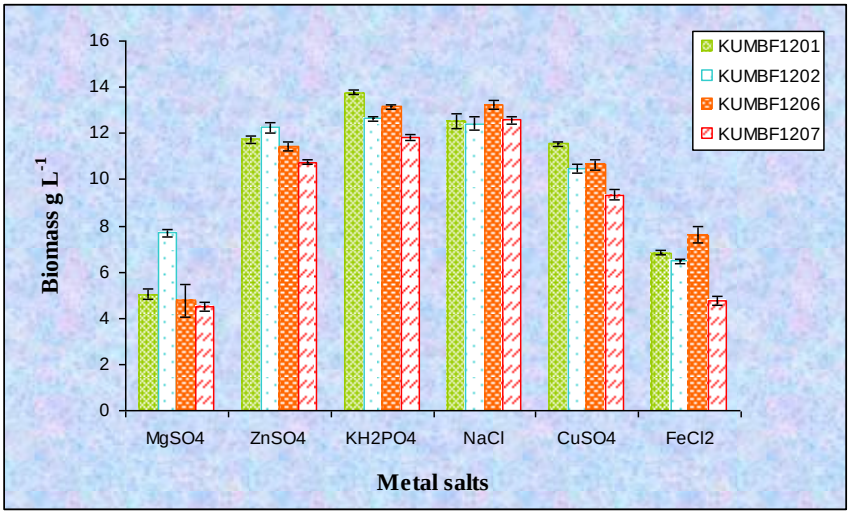

Fig. 13: Impact of metal salts on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth 2%; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Glucose 2%; Peptone 1%; Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

Out of the five metal salts screened, KH_2PO_4, $MgCl_2$, $ZnSo_4$, $FeCl_2$ and $CuSo_4$ have got remarkable influence on enhancement of biomass and pigment production. Of all the metal salts tested, KH_2PO_4 gave the highest yield for biomass production (13.77±0.1 g/L) (Fig.13) of *Fusarium moniliforme* KUMBF1201, while the maximum pigment production (1.524±0.02 nm) (Fig.14) was obtained in PDB medium. It has been reported that various types of potassium ions supported greater biomass and pigment production in many kinds of pigment-producing filamentous fungi [10, 11]. Toropova and his coworkers [35] have reported the importance of Mg^{2+}, Mn^{2+} and Fe^{2+} ion for antibiotic and pigment formation by *Hypomyces rosellus* 94/77. The negative effect of ferrous and cobalt ions on the pigment production may be explained by an indirect contribution of the metabolite to energy production in the cell [36].

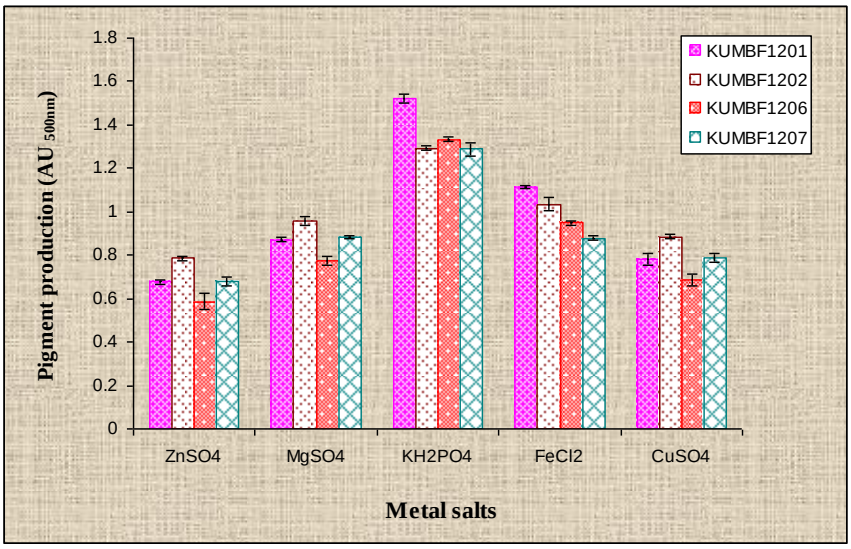

Fig. 14: Impact of metal salts on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Glucose 2%; Peptone 1%; Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

Amino acid amendment

Out of six amino acids screened, aspartic acid, alanine, glutamic acid and methionine have got remarkable influence on enhancement of biomass and pigment production by *Fusarium moniliforme* KUMBF1201, KUMBF1202, KUMBF1206, KUMBF1207.

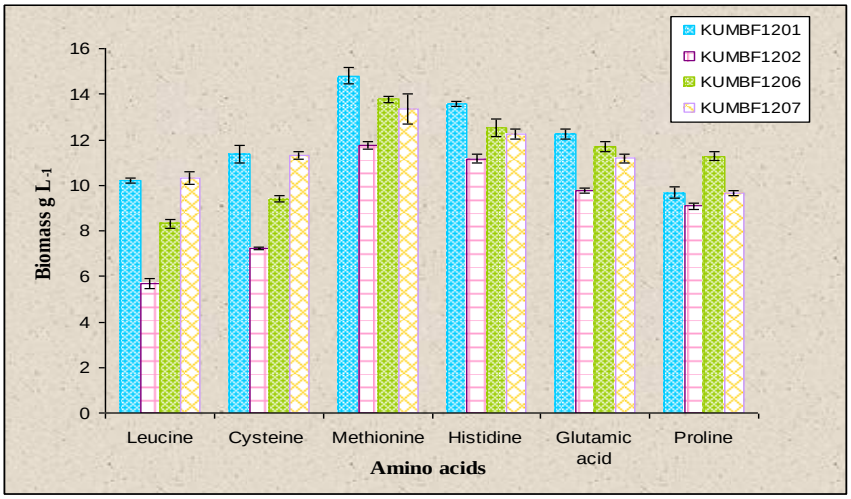

Fig. 15: Impact of Amino acids on biomass production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Glucose 2%; Peptone 1%; KH_2PO_4 0.5% Results are mean of independent experiment ± SD and are expressed as mycelial dry weight (g/L).

Amino acid supplement may have some role by sharing their carbon ring or both carbon and nitrogen skeleton into the primary or secondary metabolism processes of microorganisms [21]. Concerning the effect of amino acids amendment in combination with carbon sources, the result showed that methionine in combination with glucose promoted maximum biomass (14.8±0.19 g/L) (Fig.15) and pigment production (1.605±0.03 nm) of *Fusarium moniliforme* KUMBF1201 (Fig.16). Similarly, Herr [37] harvested a highest yield of *Aphanomyces cochlioides* mycelium grown in a combination of low glucose, high asparagine and high methionine. The utilization of amino acids as a source of carbon and energy by many of the fungi in the class Oomycetes [38].

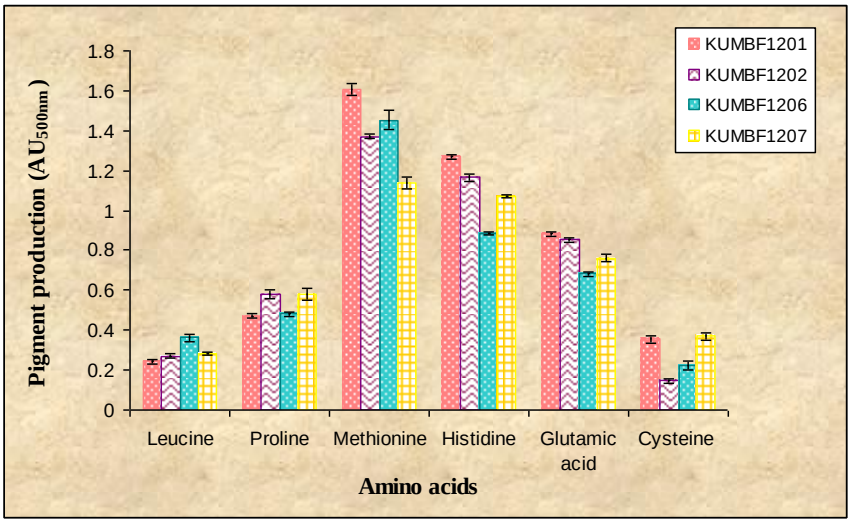

Fig. 16: Impact of Amino acids on pigment production by four *Fusarium moniliforme* isolates; Potato Dextrose Broth; Inoculum 2%; Temperature 28°C; pH 5.5; Incubation period 8 days; Glucose 2%; Peptone 1%; KH_2PO_4 0.5% Results are mean of independent experiment ± SD and are expressed as Absorbance Units.

]

CONCLUSION

Fungi have the ability to produce a plethora of secondary metabolites, typically dependent on the stage of development and environmental factors ranging from nutrient concentrations to light and temperature. The biosynthesis of pigment is directly related to cultural conditions that include biomass in the production phase and duration of the incubation periods. In this study, different carbon and nitrogen sources, mineral salts, amino acids supplement to the culture broth strongly influenced the growth and biosynthesis of pigment by *Fusarium moniliforme*. The basal medium, enriched with glucose (2%) as carbon source, peptone (1%) as nitrogen source and metal salts KH_2PO_4 (0.5%) promoted the pigment biosynthesis. Optimum temperature required for maximum production of pigment and biomass was 28ºC and pH 5.5, respectively, in an incubation period of eight days, while specific rate of product formation was at maximum on the 8th day. Amendment of amino acid methionine (0.5%) with glucose enhanced the pigment and biomass production. The above mentioned optimized culture conditions for the best production of pigment and biomass was achieved under fermentation conditions using submerged cultivation by *Fusarium moniliforme* isolates.

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