

PRODUCTION AND CHARACTERIZATION OF NAPHTHOQUINONE PIGMENT FROM FUSARIUM MONILIFORME MTCC6985

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ABSTRACT

Production and characterization of naphthoquinone pigment by the fungus *Fusarium moniliforme* MTCC6985 was investigated in this study. Cultivation conditions, including various medium, temperature, pH of the medium, time of incubation, carbon source, nitrogen source and mineral salts were optimized to improve the yield of total naphthoquinones in shake flask culture of *Fusarium moniliforme* MTCC6985. The highest yield of total naphthoquinones was obtained in Potato dextrose broth (PDB) supplemented with 2% glucose, yeast extract, potassium dihydrogen phosphate with a pH of 5.5 at 28°C. The purified and crude pigment extracted from the biomass was analyzed by TLC, UV absorption spectrum and FTIR spectroscopy.

Keywords: Naphthoquinone, *Fusarium moniliforme* MTCC6985, Potato dextrose broth.

INTRODUCTION

Naphthoquinones are widespread in nature and have been found in higher plants, fungi and actinomycetes. The interest of many investigators to this class of compounds is due to their broad-range biological action: phytotoxic, insecticidal, antibacterial and fungicidal. Besides, some of them also have cytostatic and anticarcinogenic properties ^[16]. The utilization of natural pigments in food stuff, cosmetic and pharmaceutical manufacturing processes has been increasing in recent years. This is due to the concern about the harmful effects of synthetic pigments and their industrial by-products on humans and the environment. Although there are many kinds of natural pigment, only few are available in a sufficient quantity to be used in industry ^[4]. In this way, the pigments from microbial sources are a

good alternative that could easily be produced in high yields and are capable of producing different colored pigments. The production of naphthoquinone pigments was mostly studied 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. Naphthoquinone derivatives both simple and condensed are produced by a number of *Fusaria*. Different types of naphthoquinones are produced by *Fusarium* sp; they are juglone, plumbagone, flavioline, mollisin, trichione, gunacin, cryptosporin, herbarin, homomentosin, lambertellin *etc* ^[16]. This work was designed to study factors that initiate pigment biosynthesis and determine the nature of naphthoquinones synthesized by *Fusarium moniliforme* MTCC6985.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Fusarium moniliforme MTCC6985 was obtained from the IMTECH, Chandigarh, India. The culture used throughout the experiment was maintained on a potato dextrose agar slant. Slants were inoculated, followed by incubation at 28°C for 5 days; the tubes were stored at 4°C. For inoculum preparation, the fungus grown on potato dextrose agar medium was transferred to the culture broth by punching out 4 mM² of the agar plate culture with a sterile cutter. The inoculum was grown in 250mL flask containing 50mL of potato dextrose broth at room temperature on a rotary shaker at 190 rpm for 5 days.

Determination of naphthoquinone pigment

Samples collected from shake flasks were centrifuged at 15,000 rpm for 30 minutes and the mycelial biomass yield was estimated. Total naphthoquinone in the broth were quantified by the spectrophotometric method determining absorbance at 500 nm using a double beam spectrophotometer. Total naphthoquinones was calculated by the equation, $A = \epsilon L \text{conc}^n$, where A is absorbance at 500 nm, L is length of cell (1 cm), ϵ is average molar absorptivity of total naphthoquinones (6,456 L/mol.cm²) and conc^n is concentration (mol/L) of pigment ^[13].

Production on Various Kinds of Complex Media

Five different 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 (HiMedia, India), malt extract broth (MB: 20 g/L glucose; 20 g/L malt extract; 1 g/L peptone), Sabouraud broth (SB: 10 g/L

peptone; 40 g/L glucose) were used in this study. The flask culture experiments were performed in 250 ml Erlenmeyer flask containing 100 ml of medium after inoculating with 10% (v/v) of the seed culture and cultivated at room temperature on a rotary shaker at 190 rpm for 5 day. The best complex media for production of naphthoquinone pigment was used to screen for the better carbon source, nitrogen source, mineral salts and initial medium pH for flask the culture experiment.

Influence of temperature

Investigations on the effect of cultivation temperatures on naphthoquinone pigment production have been carried out by incubating the Potato dextrose broth (PDB) at different temperatures. The production was carried out at 22, 25, 28, 31, 34, 37°C and then assayed for naphthoquinone pigment production. The optimum temperature achieved by this step was fixed for subsequent experiments.

Effect of time of incubation

To determine the optimum incubation period for pigment production, Potato dextrose broth (PDB) were incubated for different time durations (24, 48, 72, 96, 120 hours) and then assayed for naphthoquinone pigment production.

Effect of carbon source

Different carbon sources such as glucose, fructose, sucrose, maltose and lactose were supplemented separately to a final concentration of 2.0% (W/V) in the Potato dextrose broth (PDB). After incubation in an optimal condition the naphthoquinone pigment was quantified.

Influence of pH

In order to study the effect of pH, Potato dextrose broth (PDB) was inoculated and incubated. While optimizing the pH of the basal medium, the pH of aqueous solution was varied from 5.0 to 9.0 with 2M HCl and 1M NaOH and then assayed for naphthoquinone pigment production. The optimum pH achieved by this step was fixed for subsequent experiments.

Effect of nitrogen source

Different nitrogen sources such as sodium nitrate, sodium nitrite, urea, yeast extract and peptone were supplemented separately to a final concentration of 2.0% (W/V) in the Potato dextrose broth (PDB). After incubation in an optimal condition the naphthoquinone pigment 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 2.0% (W/V) was added to Potato dextrose broth (PDB). After incubation in an optimal condition the naphthoquinone pigment was quantified.

Pigment production, extraction and concentration

The culture flasks inoculated with 5% of old inoculum and incubated at 28°C on a rotary shaker at 190 rpm. The maximum pigment production was found in the 5th day of incubation (i.e., after 72 hours). The medium were brownish yellow in color. The extraction process was modified based on the information that erythrostominone could be chemically transformed to 3, 5, 8-TMON by heating it under acidic conditions. One liter of culture grown under optimized conditions was filtered to separate the mycelium, the broth acidified to pH 3 with 2 M HCl, and concentrated to 200 ml in a water bath at 60°C instead of rotary evaporator. The concentrated culture broth was heated at 100°C for 1 hour, cooled down, and the pH adjusted to 7 with 1 M KOH. The neutral broth was kept at 4°C for further analysis ^[21].

Column chromatography

The dried extract was dissolved in methanol and then applied to a silica gel column (200 X 20 mm; silica gel 60; SD Fine Chemicals, Mumbai). Compounds were separated using a methanol /ethyl acetate (95:5) solvent system. Fractions (5 mL / 10 min) containing visible pigments were collected and concentrated ^[18].

Thin layer chromatography (TLC)

Silica gel plates were prepared by coating a uniform layer of silica to a clean glass slide with the help of an applicator. These plates were then activated by heating in an oven at 60 °C for about half an hour. The extracts (10µl) dissolved in methanol were applied to silica gel thin layer chromatography (TLC), and developed with methanol/ ethyl acetate (95:5 by vol) as solvents ^[18]. Colour bands were to be observed and the R_f values are noted.

FT-IR

A FT-IR spectrum of the pigment was recorded with a Thermo Nicolet, Avatar 370; Spectral range: 4000 - 400 cm^{-1} ; Resolution: 0.9 cm^{-1} ; KBr beam splitter; DTGS Detector (7800 – 350 cm^{-1}); HATR assembly for measurement.

RESULTS AND DISCUSSION

Naphthoquinone production on various kinds of complex medium

The pigment formation in the fungi on the composition of the medium used have been obtained in studies of naphthoquinone biosynthesis in *Fusarium moniliforme* MTCC 6985. Most of reports aimed to enhance the amount of pigment by cultivation in rich complex media ^[11] ^[16]. The mineral salt liquid medium with carbon sources, including glucose or sucrose or glycerol, was favorably applied and often with the amendment of metals (Mg, Zn, Cu) to enhance metabolite production ^[1].

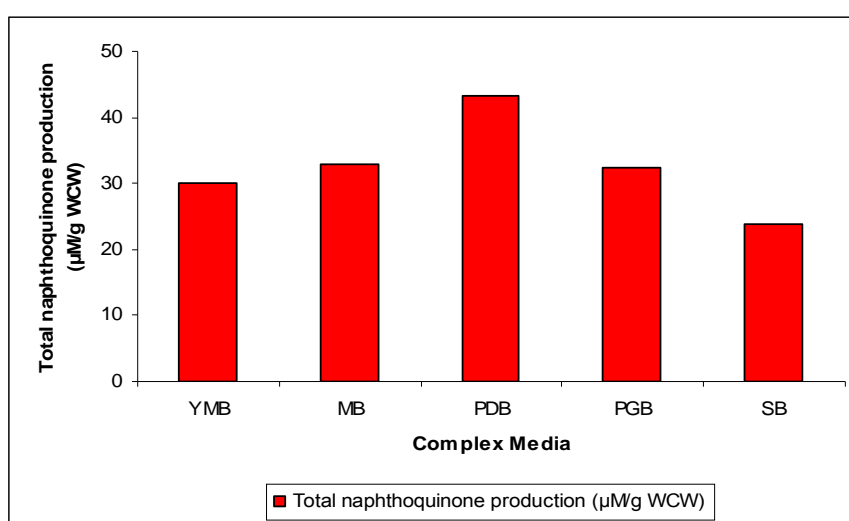


Fig.1. Effect of various complex media on the total naphthoquinone from *Fusarium moniliforme* MTCC6985.

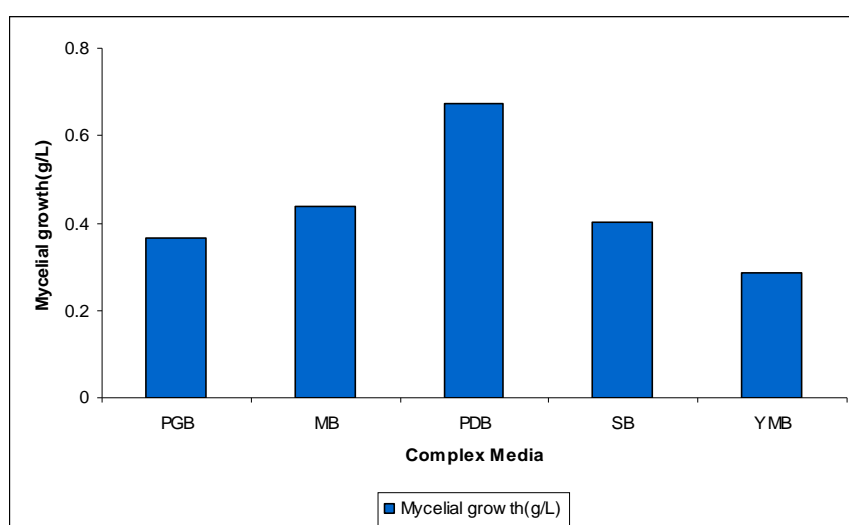


Fig.2 Effect of various complex media on the mycelial growth from *Fusarium moniliforme* MTCC6985.

In our work, we desired to select a suitable complex media for the naphthoquinone production by *Fusarium moniliforme* MTCC6985 which was cultivated in five different types of nutrient media, including PDB, PGB, YMB, MB, and SB. The maximum production of naphthoquinone pigment was reached in Potato dextrose broth (PDB) medium (43.2 $\mu\text{M/g}$ wet cell weight (WCW), whereas the lowest concentration was SB medium (23.8 $\mu\text{M/g}$ wet cell weight (WCW). Moderate concentration of pigment was observed in PGB, YMB and MB at 32.5, 30.0 and 32.8 $\mu\text{M/g}$ (WCW), respectively. The important nitrogen sources in various culture media was considered, SB and PDB containing with peptone mean while YMB and MB consisting of yeast extract, malt extract and peptone. The major difference between PDB and other media was that PDB contained components such as metal ions/or other micronutrients appropriate for enzymes to work effectively and enhanced naphthoquinone production. These results indicated that peptone and yeast extract were effective for naphthoquinone production of *Fusarium moniliforme* MTCC 6985. The effect of complex medium on fungal growth and pigment production is shown in Fig.1 and 2.

Effect of temperature

Temperature had little effect on the growth of the fungus. However, naphthoquinone production was affected by temperature; with an increase in yield from 22°C to 28°C.

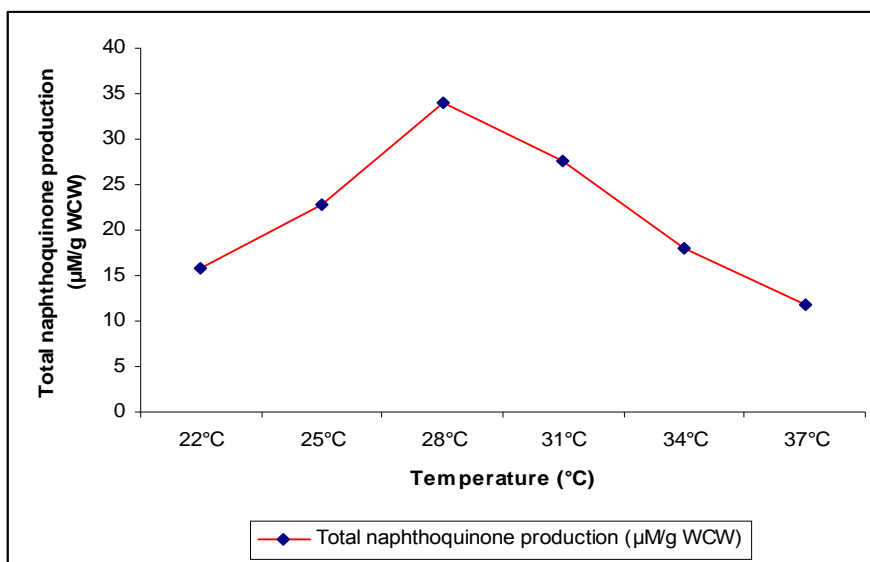


Fig.3. Effect of temperature on total naphthoquinone from *Fusarium moniliforme* MTCC6985.

The highest yield (34.1 μ M/g (WCW) of naphthoquinones were detected at 28°C (Fig. 3). This is similar to naphthoquinone production by *Fusarium solani* [19] and the red pigment/citrinin produced by *Monascus ruber* [5].

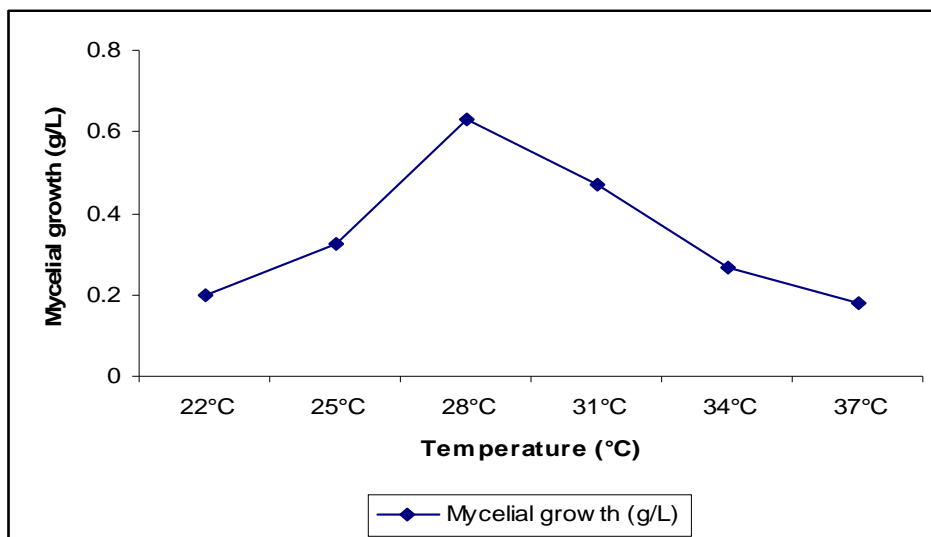


Fig.4. Effect of temperature on the mycelial growth from *Fusarium moniliforme* MTCC6985.

The optimum temperature for pigment production by these fungi was at 22-28°C, with an optimal temperature for growth of 28°C. These correspond with secondary metabolite production in other microorganisms.

Effect of pH

The different morphology of fungal mycelia under a different initial pH value was the critical factor in biomass accumulation and pigment formation [6] [8] [17]. The medium pH may affect cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis. In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by pH [14]. In order to investigate the initial pH effect on mycelial growth and naphthoquinone pigment production, *Fusarium moniliforme* was cultivated in PDB medium with 20g/L of glucose, 20 g/L of yeast extract and 20 g/L of FeCl₂ over a pH range of 5 -9 for 5 days. The highest biomass yield (62.9 μ M/g (WCW) was found at pH 5.5.

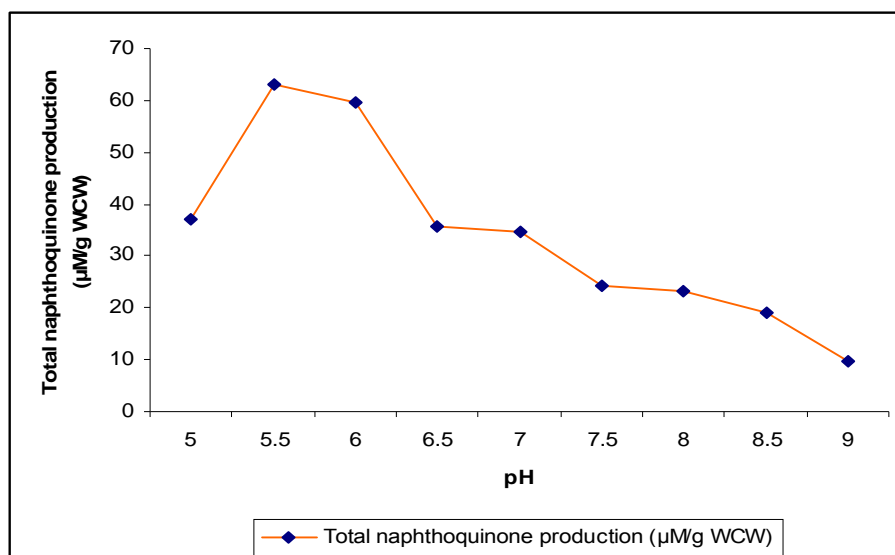


Fig.5. Effect of pH on the total naphthoquinone from *Fusarium moniliforme* MTCC6985.

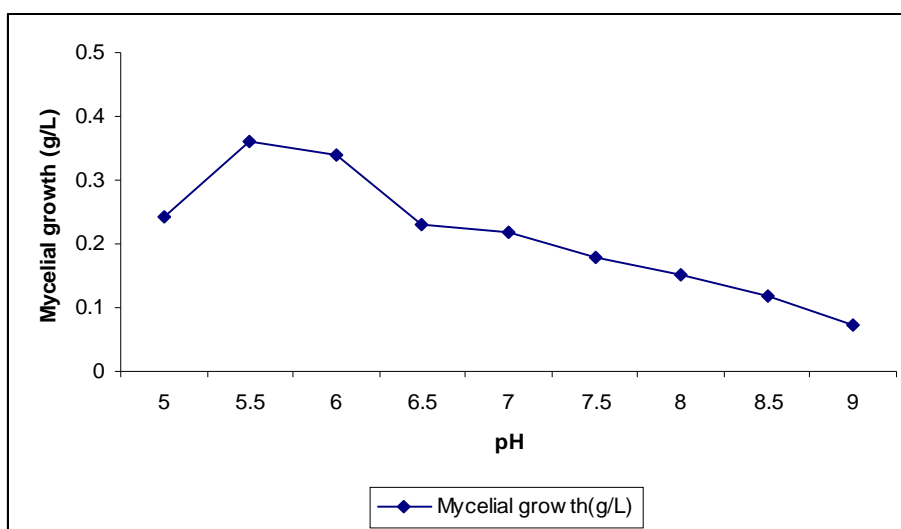


Fig.6. Effect of pH on the mycelial growth from *Fusarium moniliforme* MTCC6985.

The effect of pH fungal growth and pigment production is shown in Fig.5 and 6. In either case, the relationship between pigment production and initial pH for *Fusarium moniliforme* MTCC6985 between pH 5 and 9 were observed. This result suggested the involvement of pH in pigment synthesis. The optimal pH for pigment production (62.9 μM/g (WCW) was observed when the pH of the culture medium was set at 5.5. In related experiment have found that synthesis of naphthazarins (fusarubin, javanicin, bostricoidin etc.) during the inhibition of fungal growth correlated with high concentration of hydrogen ions in the medium (pH 4.0

and lower) and excess carbon. On the other hand, fungal growth was inhibited when the pH of culture medium increased to 8 and was accompanied by the formation of only the dimeric naphthoquinone, aurofusarin^[17].

Effect of incubation time

The optimization of the naphthoquinone production parameter was initiated by incubating the selected strain at different time interval (24, 48, 72, 96, 120 hours). To determine the optimum incubation time for naphthoquinone production, conical flasks were incubated for different time durations of 24 hours equal intervals.

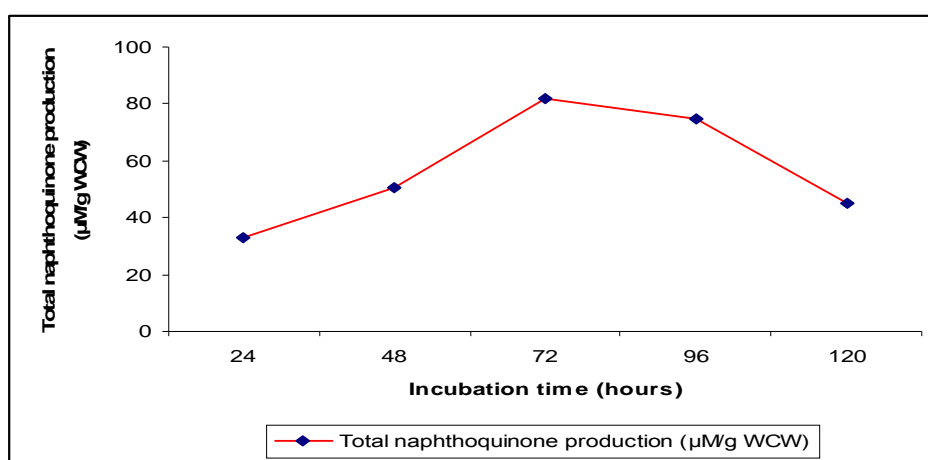


Fig.7. Effect of incubation time on the total naphthoquinone from *Fusarium moniliforme* MTCC6985.

The maximum mycelia pigment production was obtained in 72 hours of incubation (81.8 μM/g (WCW) (Fig.7 and 8).

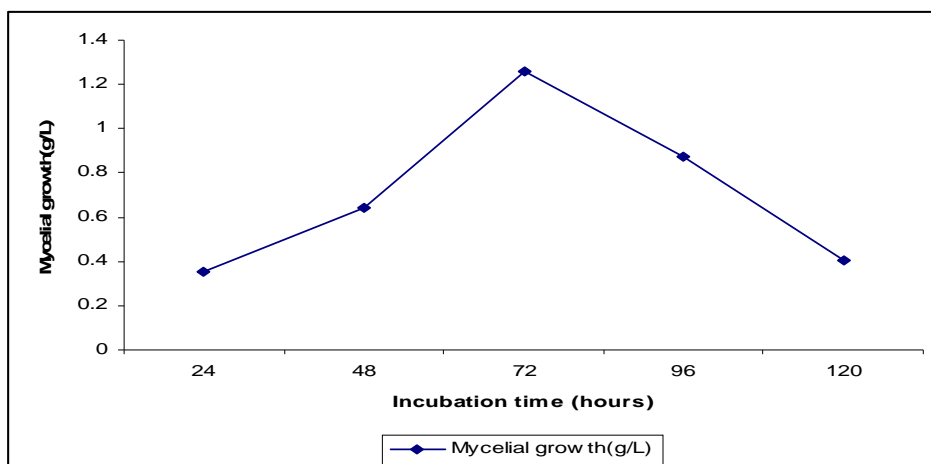


Fig.8. Effect of incubation time on the mycelial growth from *Fusarium moniliforme* MTCC 6985.

Effect of carbon source

Influence of carbon sources on mycelial growth and naphthoquinone pigment production was examined. *Fusarium moniliforme* MTCC 6985 was cultured for 5 days in PDB medium, in which various kinds of carbon sources were added at 20 g/L. As shown in Fig. 9, among the 5 kinds of carbon sources examined, the result indicated that *Fusarium moniliforme* MTCC 6985 potentially used various carbon sources for mycelial biomass and pigment production. Several hexose, pentose or disaccharide sugars were strengthened for macroconidial germination of *F. solani* related with secondary metabolite production^[10]. *F. equiseti* (Corda) Sacc. 1886 produced various tones of brown pigment when cultivated in media containing glucose, maltose, galactose, mannitol, sucrose, fructose, lactose and starch was observed^[2]. The maximum pigment production was obtained in glucose medium (31.9 $\mu\text{M/g}$ (WCW)) while pigment is rarely produced in fructose and sucrose. Glucose, usually an excellent carbon source for growth, interfered with the biosynthesis of many secondary metabolites^[7]. The effect of carbon source on fungal growth and pigment production is shown in (Fig.9 and 10).

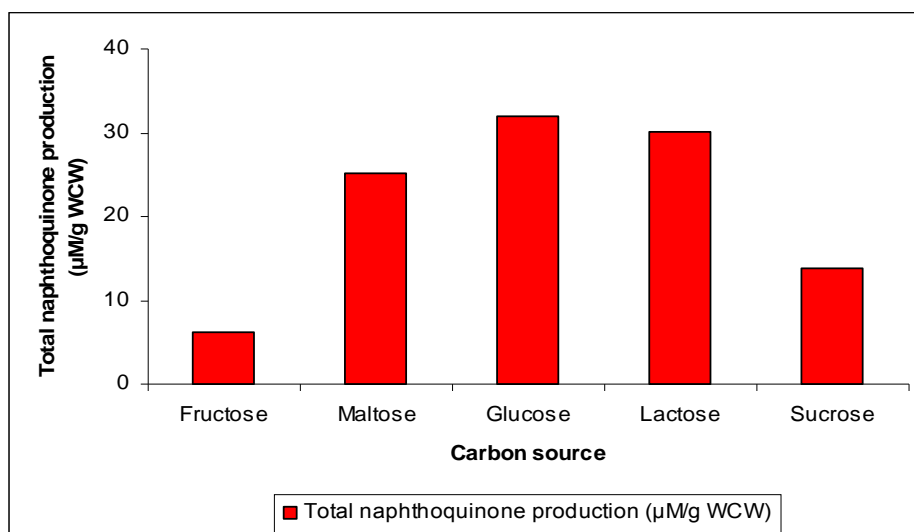


Fig.9. Effect of carbon source on the total naphthoquinone from *Fusarium moniliforme* MTCC 6985.

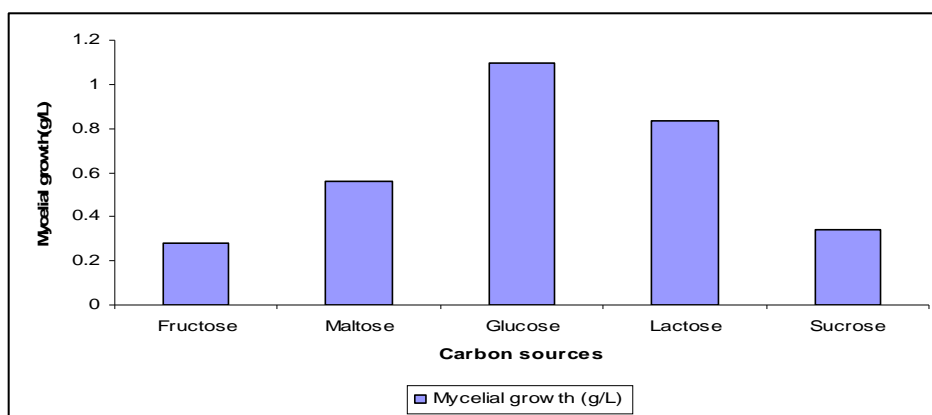


Fig.10. Effect of carbon source on the mycelial growth from *Fusarium moniliforme* MTCC 6985.

Effect of nitrogen source

It is well known that utilization of different nitrogen sources in fermentation had effects on microorganism growth and pigment production [5] [15].

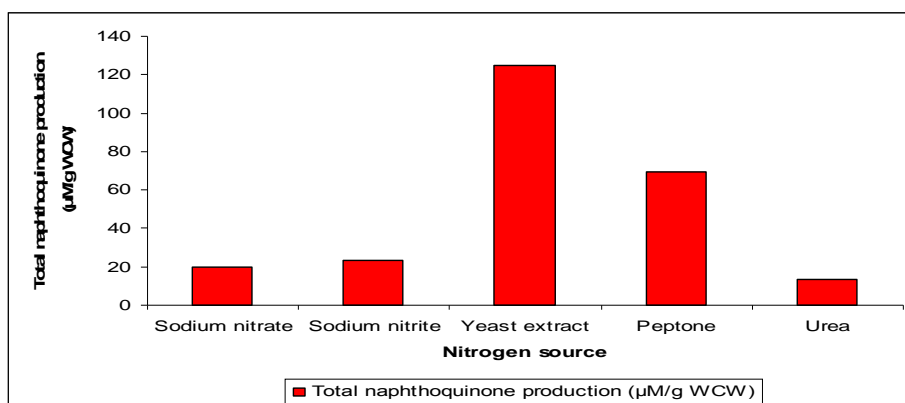


Fig.11. Effect of nitrogen source on the total naphthoquinone from *Fusarium moniliforme* MTCC6985.

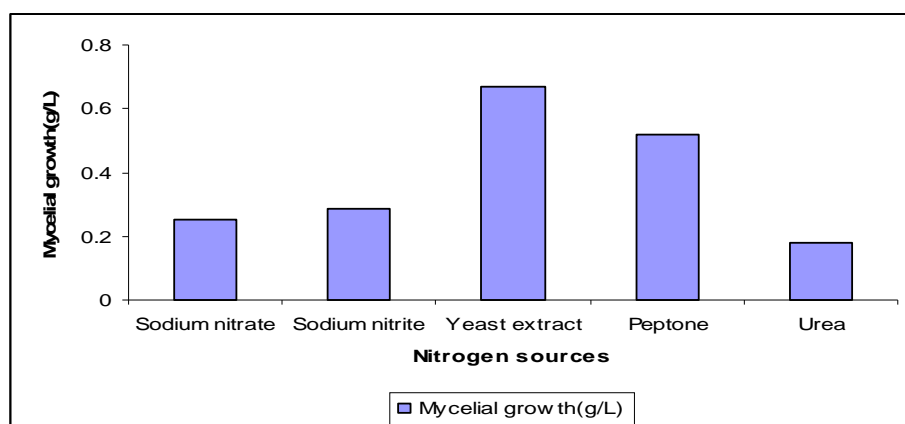


Fig.12. Effect of nitrogen source on the mycelial growth from *Fusarium moniliforme* MTCC6985.

Thus, when grown on maltose (at a concentration of 20-50 g/L) the fungus *F. solani* synthesized dihydrofusarubins and javanicin if 4.6 g/L of ammonium tartate was added. An increase in the concentration of the nitrogen source to 6.9 g/L led to the synthesis of bostrycoidin, the molecule of which contains a nitrogen atom ^[16]. In this experiment, the effect of nitrogen source for pigment production was studied in the PDB medium containing various nitrogen sources. As shown in Fig.11, organic nitrogen sources yielded higher mycelial growth compared with the other inorganic nitrogen sources. It has been reported that various kinds of amino acids containing in organic nitrogen sources are essential for secondary metabolite biosynthesis ^{[5] [12]}. In fact, various pigment derivatives with improved functional properties in the color range of orange-red to violet-red can be produced by *Monascus* fermentations in the presence of different amino acids.

Demain *et al* ^[7] reviewed that L-asparagine and L-arginine were much better nitrogen sources for antibiotic formation in *Cephalosporium acremonium* than ammonium compounds. Similarly, penicillin production by *Penicillium chrysogenum* was stimulated by glutamate analogs such as L-glutamic acid, γ -monohydroxamate and γ -benzyl-L-glutamate but this secondary metabolite production was negatively affected by inorganic nitrogen source such as ammonia ^[7]. However, the presence of nitrogen in both forms (NO_3 and NH_4^+) and salts containing zinc ions are regarded as essential for production of toxin (naphthazarins and fusaric acid) in *Fusarium* sp ^[8].

The best pigment production was achieved when yeast extract was employed as nitrogen source (124.8 $\mu\text{M/g}$ (WCW)). While pigment is rarely produced in urea (13.5 $\mu\text{M/g}$ (WCW)), sodium nitrate (20.1 $\mu\text{M/g}$ (WCW)), sodium nitrite (23.1 $\mu\text{M/g}$ (WCW)). The effect of nitrogen source on fungal growth and pigment production is shown in Fig.11 and 12.

Effect of mineral salts

The bio-elements are one of the important factors affecting pigment production in several microorganisms ^[9]. Some of them such as K^+ , Mg^{2+} and Zn^{2+} ions played a significant role in the increase of naphthoquinone pigment formation ^[16]. The effect of different bioelements on mycelial growth and pigment production of *Fusarium moniliforme* MTCC6985. Experiment was done in the above optimized culture medium and the result shown in Fig. 14. Each kind of trace element was added to the culture medium at final concentration of 20 (g/L). The maximum pigment production was found in the medium containing 20g/L of potassium dihydrogen phosphate (100.7 $\mu\text{M/g}$ (WCW)).

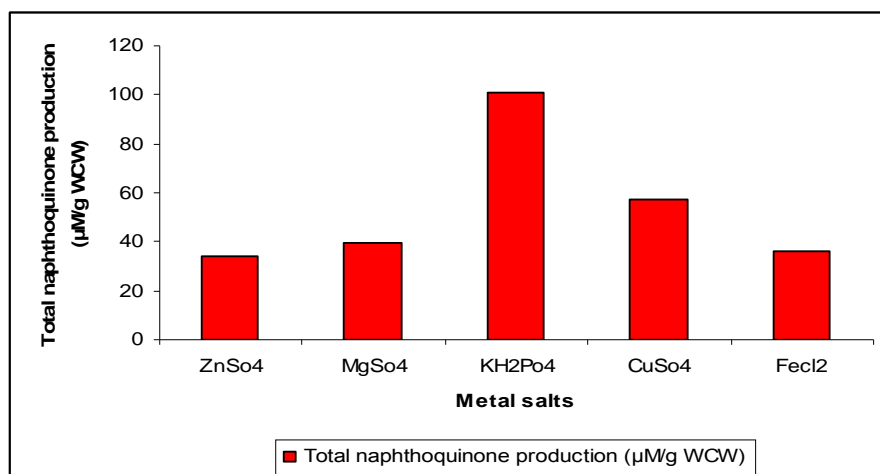


Fig.13. Effect of metal salts on the total naphthoquinone from *Fusarium moniliforme* MTCC 6985.

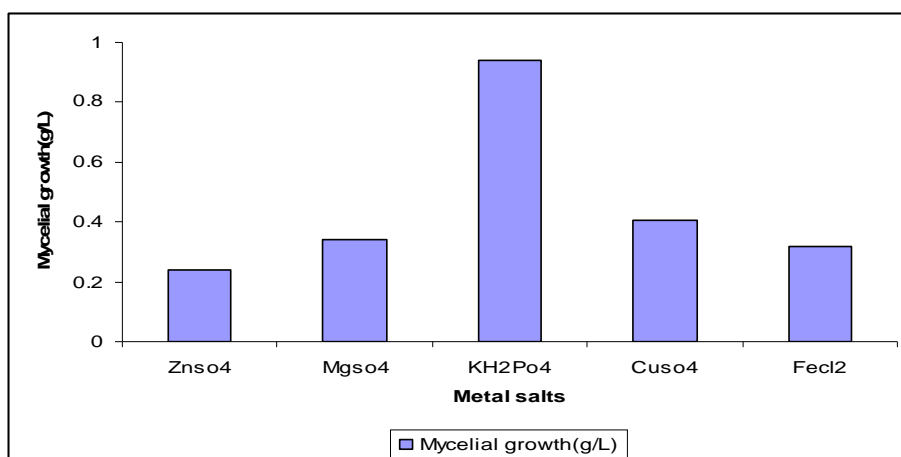


Fig.14. Effect of metal salts on the mycelial growth from *Fusarium moniliforme* MTCC 6985.

This result suggested that small amount of metal ions such as K^+ , Na^+ , Cu^{2+} and Zn^{2+} 20 g/L were necessary for efficient naphthoquinone production by *Fusarium moniliforme* MTCC6985 (Fig.13). Toropova and his coworkers^[20] have reported the importance of K^+ , Mn^{2+} and Fe^{2+} ion for antibiotic and pigment formation by *Hypomyces rosellus*. Contrastingly, media supplemented with Zn^{2+} ion had negative effect on pigment production of fungi. In addition, the detrimental effect of Zn^{2+} ion on *Monascus* pigment production was also reported^[3].

Thin layer chromatography

The crude pigments from the *Fusarium moniliforme* MTCC6985, TLC showed two distinct bands of dark yellow (R_f value – 0.9) and light brown (R_f value – 0.62). (Fig.15)



Fig.15. Chromatogram of crude extract from *Fusarium moniliforme* MTCC6985 on TLC plate

UV absorption spectrum

The UV absorption spectra in ethyl acetate were recorded using a UV spectrophotometer. UV $\lambda_{\max}^{\text{EtOH}}$ at 260, 265, 273 and 508 nm^[18].

FTIR Spectroscopy

FTIR spectrum of the crude pigment was recorded; spectral range 4000-500 1/ cm. FTIR absorption in KBr exhibits, cm^{-1} : 2947, 2887, 2845 (C–H), 1627 (C=O), 1541 (C=C). These indicate that this pigments pattern is similar to that of naphthoquinone^[18].

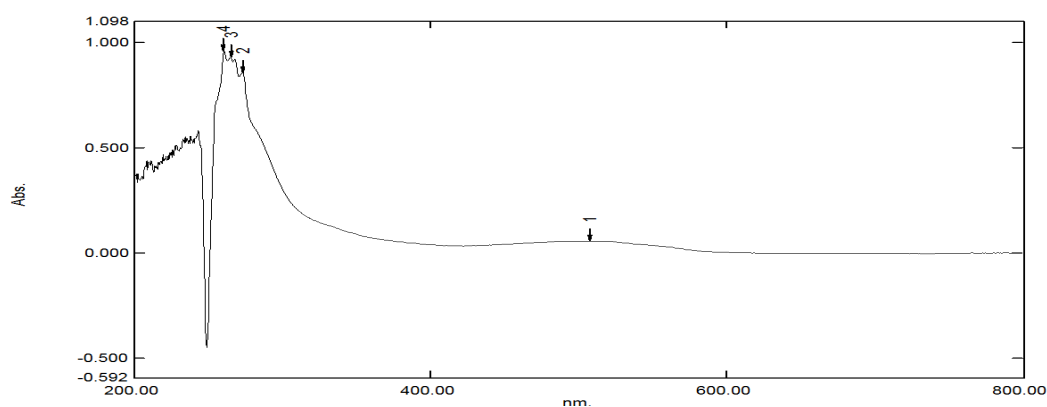


Fig. 16. UV absorption spectrum of purified compound.

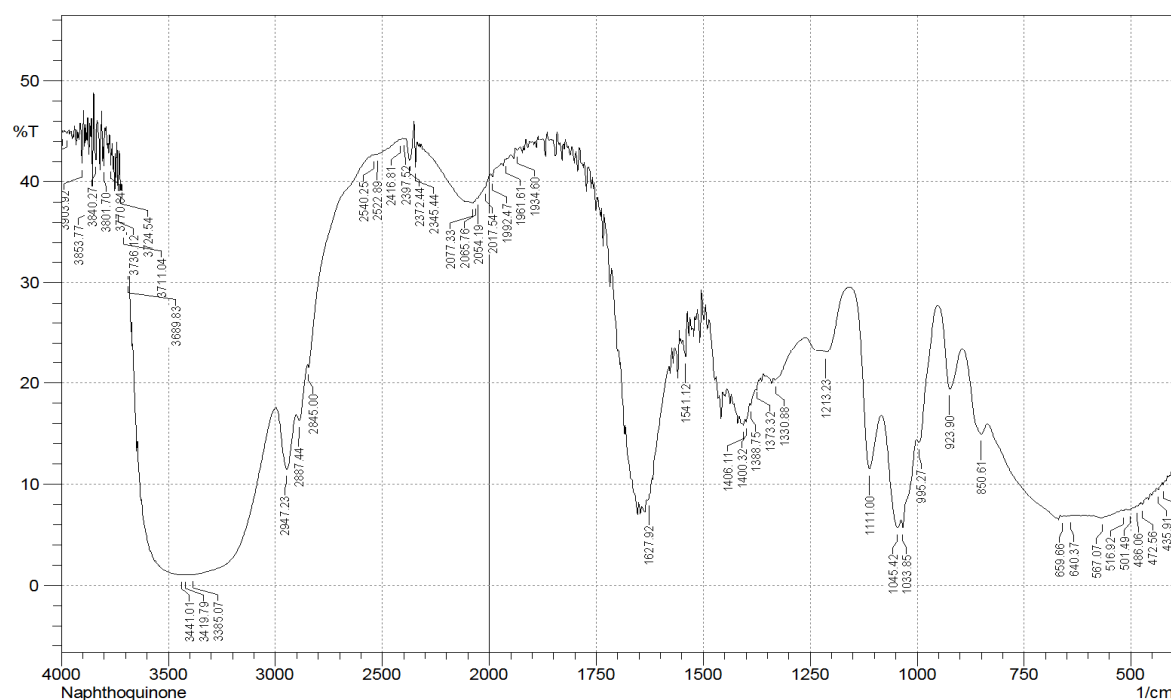


Fig.17. FTIR spectra of crude extract from *Fusarium moniliforme* MTCC6985

CONCLUSION

Fungi have the ability to produce a plethora of secondary metabolites, typically dependent on the stage of development of the fungus and environmental factors ranging from nutrient concentrations to light and temperature. Biosynthesis of secondary metabolites is regulated by medium ingredients such as carbon sources, nitrogen sources and other environmental factors. The highest yield of total naphthoquinones was obtained in Potato dextrose broth (PDB) supplemented with 2% glucose, yeast extract with a pH of 5.5 at 28°C. There might be a great advantage in adding K^+ metal ion to the medium for enhancing the pigment production. The purified and crude pigment extracted from the biomass was analyzed by TLC, UV absorption spectrum and FTIR spectroscopy. Submerged culture has potential advantage for higher mycelial production in a compact space and for a shorter incubation time with a lesser chance of contamination. Further optimization of the culture medium composition and physicochemical conditions of growth allows regulation of naphthoquinone in order to obtain standardized nutraceutical substances in higher yield.

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