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Contributed Paper

Optimization of Submerged Culture for the Production of Naphthoquinones Pigment by *Fusarium verticillioides*

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ABSTRACT

A locally isolated fungus genus, *Fusarium verticillioides*., was evaluated as a potential producer of naphthoquinone pigment. Five variable growth media (type of complex media, carbon source, nitrogen source, mineral salt and initial pH of the medium) were systematically manipulated in shake flask culture to improve the yield of total naphthoquinone. The naphthoquinone production was supported by addition of peptone and yeast extract but malt extract exhibited the opposite result. Substantially, the mineral salt of K^+, Na^+, Mn^{2+}, Cu^{2+} and Zn^{2+}, less than 50 mg/l, were necessary for the efficient naphthoquinone production by *F. verticillioides*. In addition, efficiency of pigment production at various values of initial medium pH showed that the biosynthesis of naphthoquinone was regarded by initial pH and changing of medium pH. Taken together, the results indicated that the naphthoquinone pigment production for this particular strain could be maximized by using a basal growth medium consisting of 20% (w/v) of boiled white potatoes; 20 g/l glucose; 2.5 g/l yeast extract, supplemented with 5 mg/l of KH_2PO_4 and adjust initial medium pH at 8.0. The pigment production under the optimum condition was 13.61 mg/g-cell when cultured at 30°C with agitation rate at 200 rpm for 7 days.

Keywords: *F. verticillioides*, naphthoquinone metabolites, pigment production, submerged culture.

1. INTRODUCTION

The utilization of natural pigments in foodstuff, cosmetic and pharmaceutical manufacturing processes has been increasing in recent years. This is due to concerning about the toxicity problems caused by synthetic compounds and their industrial by-products on human and the environment [1]. Although there are many kinds of natural pigment, only few are available in a sufficient quantity to be used in the industry because they are usually extracted from plants [2]. In this way the pigments from microbial sources are a good alternative that could easily be produced in high yields and capability of producing different colored pigments [3].

F. verticillioides. (teleomophrph *Gibberella*

moniliformis) is a phytopathogenic filamentous fungus which has a cosmopolitan distribution and colonizes a wide range of host plants producing serious diseases in a number of agriculturally important plant species [4]. It has been exploited to produce naphthoquinone pigments in culture, several of which have phytotoxicity, antimicrobial activity and cytotoxicity [5,6]. The known pigments o-methyl-bostrycoidin, o-methyl-fusarubin and bikaverin have been identified as major product in these fungi [7]. Gatenbeck and Bentley [8] suggested that synthesis of naphthoquinones by fungi proceeded via the polyketide route by formation of a common precursor, acetyl Co A and malonyl Co A, from primary metabolism. The production of naphthoquinone pigments was mostly studied on nutrient-rich laboratory media for maximal amounts of various pigments and to study their structure [9,10]. Most investigators have exerted their efforts to cultivate these organisms on submerged culture than solid artificial media [11]. Submerged culture gives rise to potential advantages of higher mycelial production in compact space and shorter time with lesser changes of contamination [12]. Biosynthesis of secondary metabolites is regulated by medium ingredients such as carbon sources, nitrogen sources and other environmental factors [13]. Demain and Fang [14] reviewed that the formation of secondary metabolites and spores are regulated by similar factors. This similarity could insure secondary metabolite production during sporulation. The culture requirements are traditionally optimized by the one-factor-at-a-time method, i.e. varying one factor whole keeping all others at certain levels. The method is simple and easy, without the need for statistical analysis, but it involves a relatively large number of experiments and the interactions amongst factors are often ignored [15].

In this paper we present an investigation on the effect of medium ingredients on the production of naphthoquinone pigment from a locally isolated strain of *F. verticillioides*. We have performed the optimization of carbon source, nitrogen source, addition of metal ions and initial pH of the culture medium in order to evaluate the potential for growth and pigment production.

2. MATERIALS AND METHODS

2.1 Microorganism and Inoculums Preparation

F. verticillioides selected from a culture collection in our laboratory was isolated from soil in Chiang Mai, Thailand. The culture used throughout the experiment was maintained on a potato dextrose agar (PDA) slant. Slants were inoculated, followed by incubation at 30°C for 7 days and then stored at 4°C for inoculum preparation, the fungus grown on PDA medium in Petri dish and then transferred to the seed culture medium by punching out 5 mm^2 of the agar plate culture with a sterilized cutter. The seed culture was grown in 250 ml flask containing 50 ml of potato dextrose broth (PDB: the broth from 200 g/l boiled white potatoes; glucose 20 g/l) medium at room temperature on a rotary shaker (Innova 4080, New Brunswick Scientific, U.S.A.) at 200 rpm for 1 day.

2.2 Naphthoquinone Production on Various Kinds of Complex Media

Six different media: potato dextrose broth (PDB), peptone glycerol broth (PGB: 5 g/l peptone (Difco laboratories, USA); 10 g/l glycerol), sabouraud broth (SB: 10 g/l peptone; 40 g/l glucose), yeast extract malt extract broth (YMB: 10 g/l glucose; 5 g/l peptone; 3 g/l yeast extract (Scharlau, Germany); 3 g/l malt extract (Scharlau, Germany)), malt extract broth (MB: 20 g/l glucose; 20 g/l malt extract; 1 g/l peptone)

and nutrient broth (NB: 8 g/l nutrient medium (Merck, Germany)) were used in this study. The flask culture experiments were performed in 250 ml Erlenmeyer flask containing 50 ml of medium after inoculating with 10% (v/v) of the seed culture and cultivated at room temperature on a rotary shaker at 200 rpm for 7 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.

2.3 Effect of Carbon Sources

Various kind of sugars including glucose, fructose, sucrose, maltose, galactose, mannose, xylose, arabinose, lactose and glycerol at 20 g/l were provided instead of dextrose as the carbon source in PDB medium. An optimal carbon source at 10-50 g/l was used to determine their effect on fungus growth and pigment production. No more nitrogen source was added to PDB which was a complete medium in itself.

2.4 Effect of Nitrogen Sources

Two groups of nitrogen sources, organic and inorganic compounds were tested. Organic nitrogen sources including peptone (2.5, 5.0 and 10 g/l), yeast extract (2.5, 5.0 and 10 g/l) and inorganic nitrogen sources including 5 g/l of sodium nitrite, sodium nitrate, ammonium sulfate or urea were used. PDB media containing 0 g/l nitrogen source were used as basal control.

2.5 Effect of Mineral Salts

Magnesium (Mg^{2+}), zinc (Zn^{2+}) and copper (Cu^{2+}) ions in the form of sulfate salts ($MgSO_4$, $ZnSO_4$ and $CuSO_4$), manganese (Mn^{2+}) and calcium (Ca^{2+}) ions in the form of chloride salts ($MnCl_2$ and $CaCl_2$), potassium (K^+) and sodium (Na^+) ions in the form of dihydrogen phosphate (KH_2PO_4 and NaH_2PO_4), ferric (Fe^{2+}) ion in the form of chloride ($FeCl_2$) were used. Each metal ion at concentration of 5, 50 and 500 mg/l was added to PDB medium containing 20 g/l glucose and 2.5 g/l yeast extract to investigate the effect of these ions on production yield.

2.6 Effect of Medium pH

The most favorable initial medium pH for mycelial growth and pigment production was investigated. Based on 5 experimental results, the PDB medium containing optimal mineral salt concentration was adjusted only prior to inoculation over a pH range of 3.0 – 11.0. The final medium pH after culture was determined for all the media in order to compensate for any pH-pigment interactions.

2.7 Determination of Naphthoquinone

Samples collected at various interval times from shake flasks were centrifuged at 16,000 rpm for 30 min, and the final pH of the resulting supernatant was measured (model 350; Coring, USA). The mycelial biomass yield was estimated by washing with water and drying at 80°C for 48 hr.

Total naphthoquinones in the broth were quantified by the colorimetric method determining absorbance at 500 nm using a double beam spectrophotometer (Lambda 25 UV/VIS Spectrometer, Perkin-Elmer, USA). Total naphthoquinones was calculated by the equation, $A = \varepsilon L conc^n$, 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.cm^2) and $conc^n$ is concentration (mol/L) of pigment [16].

3. RESULTS AND DISCUSSION

3.1 Naphthoquinone Production on Various Kinds of Complex Media

The data on dependence of pigment formation in the fungi on the composition of the medium used have been obtained in

studies of naphthoquinone biosynthesis in *F. verticillioides* Most of reports aimed to enhance the amount of pigment by cultivation in rich complex media [7,17]. 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 [9,18]. In our work, we desired to select a suitable complex media for the naphthoquinone production by locally isolated strain of *F. verticillioides* which was cultivated in 6 different types of nutrient media, including PDB, SB, YMB, MB, PGB and NB (Figure1). The maximum production of naphtho-quinone pigment was reached in PDB medium (463.78 μM/g dry cell weight (DCW)) whereas the lowest concentration was MB medium. The major difference between PDB and other media was that PDB contained potato starch and the others did not. This suggested that PDB might have components such as metal ions/or other micronutrients appropriate for enzymes to work effectively and enhanced naphthoquinone production. Tatum *et al.* [9] remarked that potato dextrose medium was suitable medium for cultivation of *F. oxysporum* isolated from citrus tree.

Moderate concentration of pigment was observed in SB, PGB and YMB at 198.23, 366.16 and 198.72 μM/g (DCW), respectively. The important nitrogen sources in various culture media was considered, SB and PGB containing with peptone meanwhile YMB and MB consisting of yeast extract, malt extract and peptone. These results indicated that peptone and yeast extract were effective for naphthoquinone production of *F. verticillioides*. Repression of pigment production was occurred when culture media supplemented with malt extract. In MB and SB medium, *F. verticillioides* had efficiently growth in high yields due to plenty of carbon sources and nitrogen sources in both mediums.

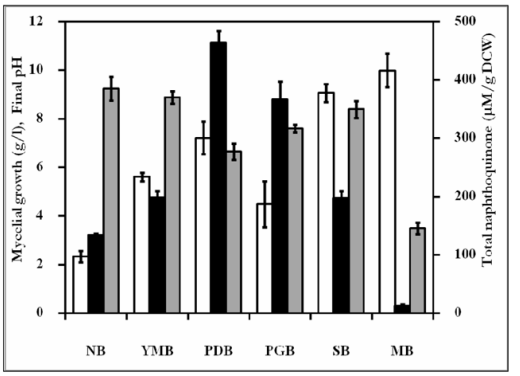

Figure 1. Mycelial growth (white bar), final pH (gray bar) and Total naphthoquinone (black bar) of *F. verticillioides* when cultured in various types of complex media at room temperature, 200 rpm for 7 days.

3.2 Effect of Carbon Sources

Influence of carbon sources on mycelial growth and naphthoquinone pigment production was examined. *F. verticillioides* was cultured for 7 days in PDB medium, in which various kinds of carbon sources were added at 20 g/l. As shown in Table 1, among the 10 kinds of carbon sources examined, the result indicated that *F. verticillioides* 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 [19]. *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 [20]. The maximum pigment production was obtained in glucose medium (478.62 μM/g (DCW)) while pigment is rarely produced in glycerol, arabinose and lactose. Glucose, usually an excellent carbon source for growth, interfered with the biosynthesis of many secondary metabolites [13]. Brandao *et al.* [21] reported that the activation of the plasma membrane H(+)-ATPase in *F. oxsporum* was induced by

Table 1. Effect of carbon source on the mycelia growth, final pH and total naphthoquinone of *F. verticillioides*.

Carbon source (20 g/l)	Mycelial growth (g/l)	Total naphthoquinone (μM/g (DCW))	Final pH
Glycerol	9.85	30.98	4.56
Maltose	10.55	147.15	7.16
Fructose	9.68	306.69	5.93
Sucrose	9.97	398.08	6.24
Glucose	8.97	478.62	6.89
Mannose	8.84	429.06	7.24
Xylose	9.49	340.77	8.11
Galactose	9.52	331.47	7.93
Arabinose	10.31	30.98	7.31
Lactose	10.05	21.69	7.46

glucose while lactose had lesser effect. The effect of glucose concentration on fungal growth and pigment production is shown in Figure 2. The amount of mycelia increased proportionally to the concentration of glucose up to the 50 g/l. On the contrary, the final medium pH was slightly decreased. These results suggested that the suitable concentration of glucose for pigment production was 20 g/l.

3.3 Effect of Nitrogen Sources

It is well known that utilization of different nitrogen sources in fermentation had effects on micro-organism growth and pigment production [22,23]. 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 [7]. In this experiment, the effect of nitrogen source for pigment production was also studied in the PDB medium containing various nitrogen sources. As shown in Figure 3, 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 org anic nitrogen sources are essential for s econdary metabolite biosynthesis [22,24]. 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

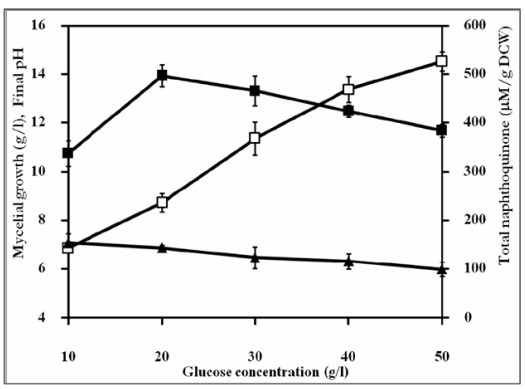

Figure 2. Effect of glucose concentration on mycelial growth (□), final pH (▲) and total naphthoquinone (■). Fermentation was carried out in a shake flask for 7 days at room temperature 200 rpm.

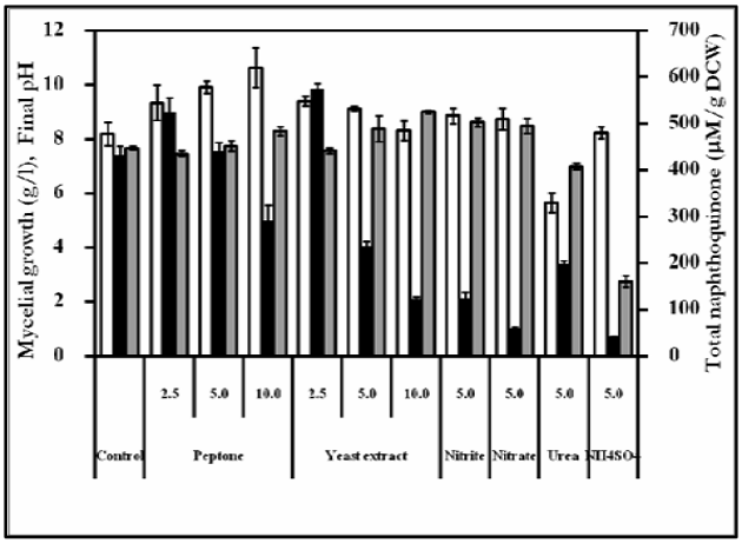

Figure 3. Effect of nitrogen sources concentration on mycelial growth (□), final pH (▲) and total naphthoquinone (■). Fermentation was carried out in a shake flask for 7 days at room temperature 200 rpm.

presence of different amino acids [24]. Demain [13] 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 [13]. 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. [6].

Moderate concentrations (2.5 and 5.0 g/l) of peptone and 2.5 g/l of yeast extract were critical for high production of naphthoquinone by *F. verticillioides*, but higher concentration of these nutrients reduced pigment production. Ueno *et al.* [25] reported that the effect of peptone and yeast extract as shown to increase T-2 toxin production by *F. solani* M-1-1. The best pigment production (2.5 g/l) was achieved when yeast extract was employed as nitrogen source in which its optimal concentration was at 573.41 μM/g (DCW). The mycelial growth increased proportionally to the increase of peptone concentration and decreased when yeast extract concentration increased. Optimal concentrations of glucose and yeast extract were used in subsequent experiments.

3.4 Effect of Metal Salts

It has been reported that bio-elements are one of the important factors affecting pigment production in several micro-organisms [26]. Some of them such as Fe^{2+}, Mg^{2+} and Zn^{2+} ions played a significant role in the increase of naphthoquinone pigment formation [7]. The effect of different bio-elements on mycelial growth and pigment production of *F. verticillioides*. Experiment 5 was done in the above optimized culture medium and the result shown in Figure 4. Each kind of trace element was added to the culture medium at final concentrations of 5,

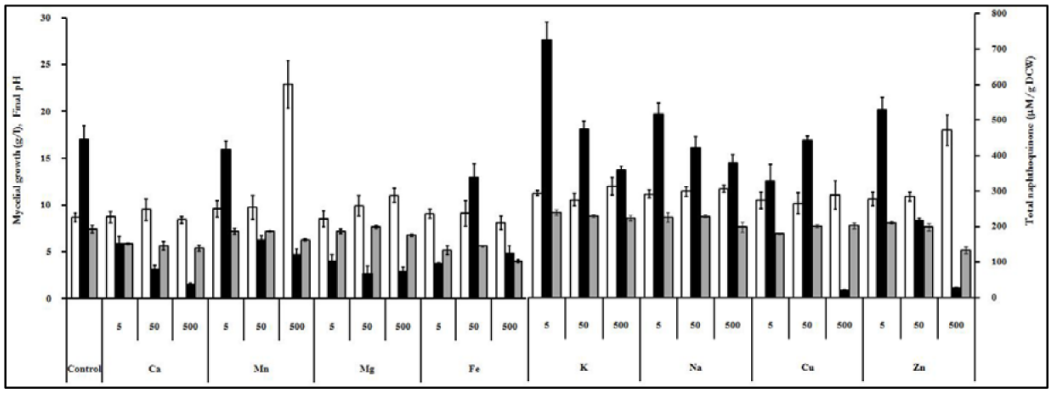

Figure 4. Effect of mineral salts concentration (5, 50 and 500 mg/l) on total naphthoquinone. Fermentation was carried out in a shake flask for 7 days at room temperature 200 rpm.

50 and 500 mg/l. The pigment production of media supplemented with metal ions were higher than those obtained from the unsupplemented media except of which Ca^{2+}, Mg^{2+} and at 500 mg/l of Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}. Naphthoquinone yielded from the addition of K^+, Na^+, Mn^{2+}, Mg^{2+} and Zn^{2+} decreased with increasing of metal ion concentration while those from Fe^{2+} and Cu^{2+} were highest at 50 mg/l. The maximum pigment production was found in the medium containing 5 mg/l of K^+ (725.67 μM/g (DCW)) while the maximum mycelial growth appeared in medium supplemented with 500 mg/l of Mn^{2+} (data not shown). This result suggested that small amount of metal ions such as K^+, Na^+, Mn^{2+}, Cu^{2+} and Zn^{2+} less than 50 mg/l were necessary for efficient

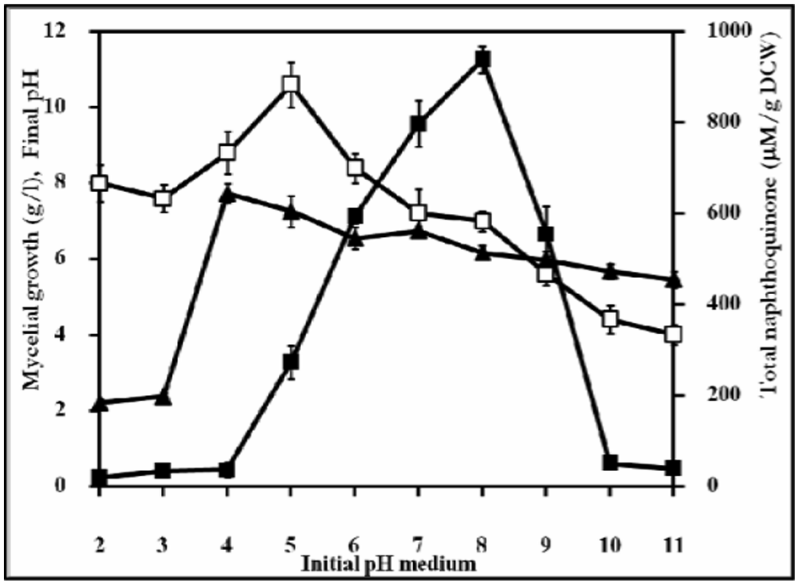

Figure 5. Effect of initial pH on mycelial growth (□), final pH (▲) and total naphthoquinone (■). Fermentation was carried out in a shake flask for 7 days at room temperature 200 rpm.

naphthoquinone production by *F. verticillioides*. Toropova and his coworkers [27] have reported the importance of Mg^{2+}, Mn^{2+} and Fe^{2+} ion for antibiotic and pigment formation by *Hypomyces rosellus* 94/77. 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 [28].

3.5 Effect of Initial pH

Many investigators claimed that the different morphology of fungal mycelia under a different initial pH value was the critical factor in biomass accumulation and pigment formation [6, 16, 29, 30]. The medium pH may affect cell membrane function, cell morphology and structure, t he 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 [31]. In order to investigate the initial pH effect on mycelial growth and naphthoquinone pigment production, *F. verticillioides* was cultivated in PDB medium with 2.5 g/l of yeast extract and 5 mg/l of KH_2PO_4 over a pH range of 3-11 for 7 days. The result in Figure5 shows that the fungus does grow in broad range of initial pH although decreasing in mycelial growth is obtained when increasing of initial pH. The highest biomass yield (10.60 g/l) was found at pH 5.0 while at pH 11 it was 4.02 g/l. Measurement of naphthoquinone pigment was obtained in particularly range of pH 5-9 whereas at pH 3, 4, 10 and 11, the production was inhibited. In either case, the inverse relationship between pigment production and initial pH for *F. verticillioides* between pH 4 and 8 were observed. However, the final pH of culture medium at day 7 became approximately 5-6. This result suggested the involvement of decreasing pH in pigment synthesis, consistently with the necessary of decreasing pH on naphthoquinone synthesis earlier reported by Baker *et al*. [32]. The optimal pH for pigment production (938.53 μM/g (DCW)) was observed when the initial pH of the culture medium was set at 8. Similar result of pigment production by *F. solani* f. sp. *piperis* might be stimulated by a higher initial pH of the culture medium was also shown [6]. 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 [7, 30].

4. CONCLUSIONS

The production of naphthoquinone metabolite by *Fusarium* strain was regulated by many factors in the culture media. The levels of induction, repression or even inhibition were depended on various types and amount of carbon source, nitrogen source, mineral salt and pH of the culture media. In conclusion, *F. verticillioides* was able to utilize a variety of sugars as carbon source whereas peptone and yeast extract were appropriate nitrogen sources for naphthoquinone pigment production. There might be a great advantage in adding K^+ metal ion to the medium for enhancing the pigment production. Mycelial growth favored the acidic pH range and reached maximum yield at pH 5 while the pigment production was suitable for the alkali pH range. From our results, the highest naphthoquinone production of *F. verticillioides* was achieved when cultured in the modified PDB (20 g/l glucose; 2.5 g/l

yeast extract containing 5 mg/l of K^+ and adjusted the initial medium pH to 8). This pigment production was two times higher than those cultured in the common PDB medium.

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