Journal of Agricultural Chemistry and Biotechnology

Journal homepage: www.jacb.mans.edu.eg
Available online at: www.jacb.journals.ekb.eg

Factors Affecting Red Pigment Production by Local Fungal Isolate of *Penicillium* sp.

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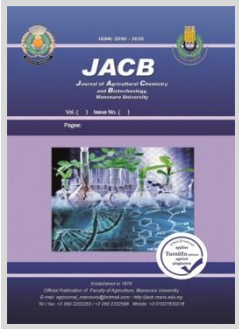

ABSTRACT

There are a great interest in replacing synthetic pigments with another healthier which obtained from natural sources, especially microbial pigments. In this study *Penicillium* sp. was isolated from soil sample and noticed its capability to produce red pigment on potato dextrose agar plates. The factors affecting pigment production in submerged culture were studied to determine the best conditions using potato dextrose broth as a basal medium. Mannitol was found to be the best carbon source which folded the production about 5 times comparing with dextrose. Nitrogen sources reduced pigment production although they increase growth yield. Biomass and red pigment production were found to be not directly associated. The highest production of red pigment (21.08 OD_{490}) by *Penicillium* Sp. was obtained at 24° C, pH 6.5 with 1% (w/v) mannitol as a carbon source inoculated with 1 ml of 10^6 spore/ml after 6 days of incubation.

Keywords: Red pigment, water-soluble pigment, *Penicillium.*

INTRODUCTION

The harmful side effects of chemically synthetic dyes led to increase the demand of natural colorants. Also, using natural colorants in the industries of food, cosmetic, textile and pharmaceutical instead of synthetic colorants is an international trend because the negative perceptions and scrutiny of synthetic pigments by many consumers (Mendez *et al*., 2011). There are different sources for natural pigments such as plants, insects, algae and microorganisms. The preference of plants and microorganisms for pigments biotechnological production is due to the easy understanding of its proper cultural techniques and processing (Aberoumand, 2011). Different pigments could be produced in large scale by filamentous fungi such as melanins, phenazines, flavins, quinines, carotenoids, violacein, indigo and monascins (Dufosse *et al*., 2014; Rao *et al*., 2017).

Although the natural pigments can be produced from various sources as mentioned above, microbial pigments have some advantages. Seasonal changes does not affect their production (Mapari *et al*., 2006). They are easy to produce - even on a large scale-, inexpensive and the high stability of their structures. Filamentous fungi are a potential producer of numerous pigments with different colors ranging from yellow, orange, red, brown, bronze, and chestnut (Dufossé, 2018; Bouhri *et al*., 2020).

The production of pigments by *Monascus* genus is the oldest recorded (Dufosse *et al*., 2005), which have been widely used in medicine and food industry especially in Eastern Asia countries (Zahan *et al*., 2020). *Monascus* sp. (especially *M. purpureus*, *M. pilosus*, and *M. ruber*) is one of the well-known filamentous fungi which produce various pigments such as orange-colorant (rubropunctatin), yellow-colorant (xanthomonascin and ankaflavin) and red-colorant (monascorubin, rubropunctamine and monascorubramine) (Carvalho *et al*., 2005; Charalampia *et al*., 2017). Additionally, many other microorganisms have been reported their ability in producing natural pigments such as *Penicillium* sp. *Eremothecium ashbyii*, *Haematococcus pluvialis Dunaliella salina* and *Spirulina* sp. (Zahan *et al*., 2020).

Carvalho *et al*. (2005) reported that *Penicillium* sp. use the similar metabolic pathways of *Monascus* fungi in producing *Monascus*-like pigments.

Biotechnological processes are affected by many variables, among them environmental conditions like pH and temperature which have a great effect on metabolites biosynthesis such as pigments. Also the composition of the culture media including carbon sources and nitrogen sources has a great effect. Manipulating and optimization of the medium composition can improve the production of biopigments (Pombeiro-Sponchiado *et al*., 2017).

The aim of the present study is isolation high pigment producer from local area and investigation the factors affecting pigment production.

MATERIALS AND METHODS

Isolation of pigment producing fungi:

The fungal isolate was isolated from a soil sample taken from the farm of Faculty of Agriculture, Mansoura University using potato dextrose agar (PDA) plates. These plates were inoculated with 1 ml of appropriate dilution of the soil sample and incubated at 28±1° C for 5 days. A single colony exhibited red coloration was purified by single hyphal tip method and subcultured on PDA slants. The slants were incubated for 5 days at 28±1° C. The isolate was maintained on PDA slants at 4° C and subcultured periodically.

Morphological identification of the isolated fungus:

The isolate was cultured on PDA plates for 5 days at 28° C to observe the colonial morphology. Macroscopic characteristics such as color, size, shape and surface texture were described according to the method of Pitt (1979).The microscopic features of the isolate were studied from isolate grown on PDA plates 2–3 days. Olympus SZ61 and CX41 microscopes with Tucsen digital camera using Motic software was employed to capture images. The method of morphological identification was established by Pitt (1979).

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DOI: 10.21608/jacb.2020.123892

Inoculum preparation:

To prepare the spore suspension, 10 ml of sterilized 0.9% (w/v) sodium chloride solution containing 0.1% (v/v) Tween-80 were aseptically added to 5 days old PDA slant culture. With a sterile wire loop, the culture surface was gently scraped and homogenized. The spore suspension was counted using a hemocytometer (Fuchs-Rosenthal counting chamber, bright-line Marienfeld, Germany), and adjusted to obtain a concentration of 10^6 spores/ml and used as the inoculum.

Production of red pigment:

One ml of the inoculum was aseptically added to 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth (PDB) as a basal medium. PDB was prepared by adding 10 g dextrose to 1 litter potato extract and autoclaved at 121° C for 15 min. Potato extract was prepared by adding appropriate amount of tap water to 200 g of little diced potato, boiled for 30 min then filtered through cheesecloth and tap water was added to complete 1.0 litter. Fermentation was carried out at 30±1° C for 10 days at 150 rpm in an orbital shaker.

Pigment extraction and estimation

At the end of incubation period, the fermented broth was transferred into a 50 ml centrifuge tube, centrifuged at 6000 rpm for 20 min then filtered throw Whatman No. 1 filter paper (Whatman, Kent, England). The supernatant was used to measure the absorbance – after dilution if needed- using spectrophotometer after considering the dilution factor. The sensitivity for the presence of the pigment was scanned at five different wavelengths (400, 490, 520, 540 and 550 nm) to determine the appropriate wavelength for maximum light absorption by produced pigment. The pigment absorbs maximum light at 490 nm (data not shown) and this is in agreement with Santos-Ebinuma *et al.,* (2013). Uninoculated liquid broth was used as a blank. The yield of the pigment was expressed as optical density at 490 nm (OD_{490}) of the extract. In this study only extracellular pigments were considered.

Mycelial dry weight

The mycelial biomass yield was collected by filtration throw Whatman No. 1 filter paper, washed with distilled water then dried in the oven at 50° C for 48 h. Dry weight of fungal biomass was determined (Olsson and Nielsen, 1997).

Factors affecting red pigment production:

In an attempt to maximize red pigment production seven variables were studied in this work including incubation time, initial pH, carbon sources, nitrogen sources, incubation temperature, inoculum density and carbon source concentration. First of all incubation time was carried out using PDB (1% dextrose) at 30±1° C for ten days in an orbital shaker at 150 rpm. The pigment production and growth yield were estimated by taking the complete content of the flask every day.

To determine the optimum initial pH for pigment production and mycelia growth six pH values from 5 to 9 were studied. Effect of carbon sources were studied by replacing dextrose in the basal media with an equal amount (1% w/v) of different carbon sources (starch, mannitol, maltose, fructose, galactose, sucrose and lactose).

Supplementation with different nitrogen sources and its effect on pigment production and mycelia growth was also studied using eight different nitrogen sources include four inorganic nitrogen sources (ammonium sulfate, sodium nitrate, ammonium chloride and ammonium nitrate), and four organic nitrogen sources (malt extract, beef extract, yeast extract and peptone) in concentration of 1% (w/v) comparing with the control (without nitrogen sources). To estimate the effect of incubation temperature on pigment production and mycelia growth, five temperature degrees (21, 24, 27, 30 and 33° C) were studied.

Effect of inoculum density was studied by preparing inoculums with different density ranging from 10^3 to 10^8 spore/ml and the fermentation media were inoculated with 1 ml from the inoculum dilution.

Finally the effect of optimum carbon source concentration - which was determined from previous experiments - was studied using different concentrations from 0.5 to 5.0% at intervals of 0.5%.

Statistical analysis:

All experiments were carried out in triplicate. The standard deviations and the least significant difference were calculated using the statistical software program CoStat at a significance level (P) ≤ 0.05. The results are expressed as mean values ± standard deviation (SD). The data were compared using the mean Dunkan's test and different letters above indicates significant differences between means.

RESULTS AND DISCUSSION

Isolation and identification of the red pigment producing fungus:

Among the fungal isolates getting from PDA plates after 5 days of incubation, the isolate which exhibited red coloration was chosen. Its colonies reached 3-4 cm diameter after 5 days of incubation on PDA. It formed irregularly spreading, velvety, greyish green, conidiogenesis abundant, heavy sporing colonies with white to madder red mycelium, reverse was greyish red and the media was pigmented with red color as shown in Fig (1A and B).

Microscopic examination showed that the isolate forms long, smooth biverticillate conidiophores bearing narrow funnel-shaped conidial heads with heavy-walled elliptical conidia (Fig.1 C and D). Based on the characteristics described above, the fungus is most likely identified as *Penicillium* Sp. depending on phenotypic characteristics described by Pitt (1979).

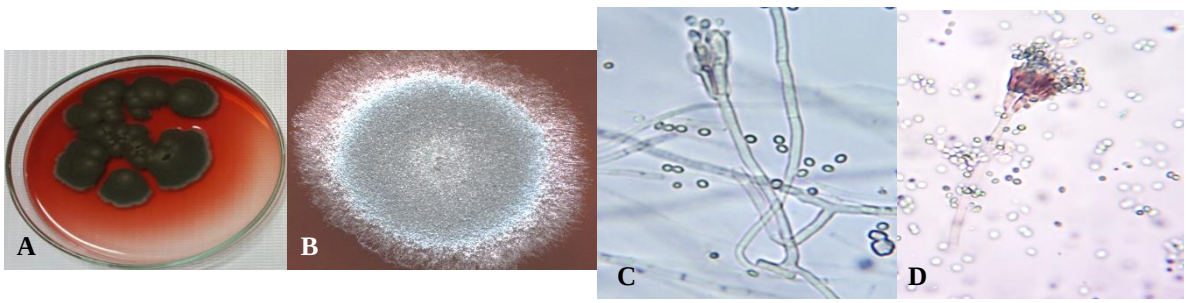

Fig. 1. Cultural and morphological characterization of *Penicillium* Sp. on PDA, A) The top view of petri dish at seventh day B) *Penicillium* colony C) and D) *Penicillium* structure and conidiophores.

Factors affecting pigment production:

Effect of incubation time

Results illustrated in Fig. (2) show that there were not observed growth or pigment after 24 h of incubation while red pigment production began after 2 days of incubation and it gradually increased with further incubation to reach a peak of 2.866 OD_{490} after 8 days, then the production significantly decreased with further incubation. Santos-Ebinuma *et al.* (2013) obtained maximum pigment production by *Penicillium purpurogenum* DPUA 1275 after 12 days of incubation, while Chadni *et al.* (2017) obtained maximum yield of the pigment by *Talaromyces verruculosus* after 24 days of incubation. On the other hand, shorter incubation periods were reported by many authors, Méndez *et al.* (2011) obtained the highest yield of red pigment by *P. purpurogenum* GH2 after 150 h (6.25 days) of incubation, Babitha *et al.* (2006) obtained maximum pigment production by *Monascus purpureus* after 6 days of incubation. Also Velmurugan *et al.* (2011) and Silbir and Goksungur (2019) obtained maximum pigment production by *Monascus purpureus* after 7 days of incubation. Previous studies reveal that the optimum incubation time for maximum pigment production varies from one strain to another. In general, strains which produce high yield of pigments in relatively short time of incubation are more effective than the others because they reduce the cost of the production.

Also, the growth yield increased with further incubation and reached a peak of 4.52 g/L after 5 days and it slightly decreased with further incubation although increasing in red pigment yield was observed till the eighth day. These results are in harmony with that reported by Santos-Ebinuma *et al.* (2013) who obtained maximum growth yield from *Penicillium purpurogenum* DPUA 1275 after 5 days of incubation. Also Silbir and Goksungur (2019) observed maximum biomass of *Monascus purpureus* after 7 days of incubation and then it decreased. Results revealed that there is a relation between growth and pigment production.

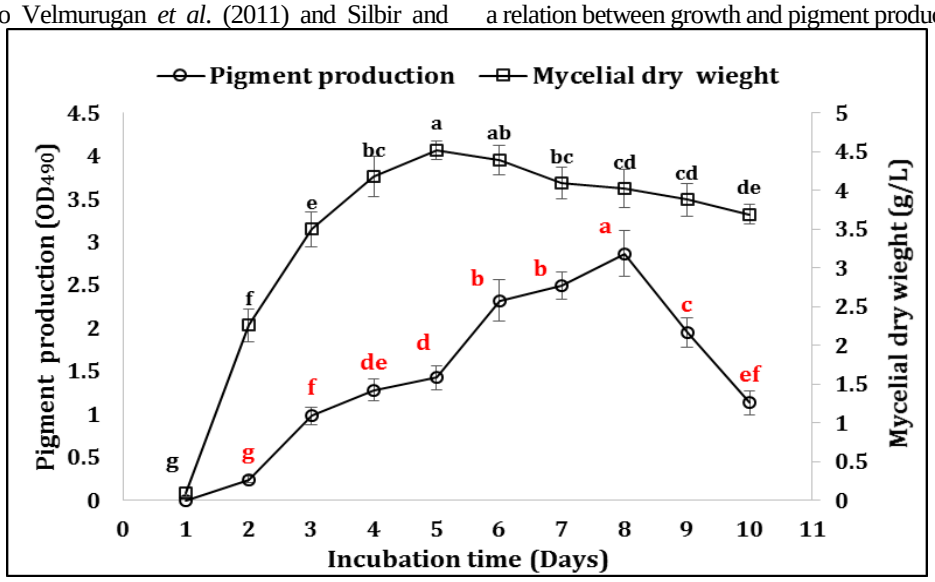

Fig. 2. Effect of incubation time on red pigment and biomass production by *Penicillium* sp. on PDB at 30±1° C.

Effect of initial pH:

Results illustrated in Fig. (3A) show that *Penicillium* sp. gave good growth at different pH values ranged from 5 to 8 with small differences in growth yield, but the growth yield strongly decreased at pH 9, the best growths were observed at pH 6 and 6.5 after 4 and 6 days, respectively, as shown in Fig. (3A), while red pigment production strongly decreased at pH 5, 8 and 9 with pigment production of 1.396, 1.094 and 0.162 OD_{490}, respectively after 8 days of incubation. The highest yields of red pigment were observed at pH 6, 6.5 and 7 after 8 days of incubation which reached 2.604, 2.965 and 2.46 OD_{490}, respectively with non-significant difference between them (LSD at 0.05 = 0.55). Results reveal that the initial pH has a respectable effect on pigment production even though the growth rate did not affected in the same way. These results are in harmony with that reported by Sethi *et al.* (2016) who reported that maximum production of red pigment by *Penicillium purpurogenum* BKS9 was obtained at pH 6.0. Also Afshari *et al.* (2015) obtained the highest production of yellow pigment by *Penicillium aculeatum* ATCC 10409 at pH 6.5. The results obtained by Méndez *et al.* (2011) are in close proximity with the present study as they reported that the highest yield of red pigment by *P. purpurogenum* GH2 was at pH 5 and 24 °C.. On the other hand Gunasekaran and Poorniammal (2008) observed that the highest biomass and pigment production by *Penicillium* sp. was with initial pH 9.0.

The pH is related to the characteristics of the cell wall and membrane permeability, therefore it influences either uptake or loss of ions from or to the medium. The pH may affect the function of cell membrane, cell structure and morphology, the ionic state of substrates, the solubility of salts, nutrients uptake and the biosynthesis of the product. Generally, microorganisms can only grow within a certain pH range, and the formation of metabolite is also affected by the pH value (Merlin *et al.*, 2013). Also initial pH of the medium can affect the activities of enzymes involved in pigments biosynthesis, but the effect depends on the particular microorganism used (Méndez *et al.*, 2011).

Effect of carbon sources:

To estimate the effect of different carbon sources on growth and red pigment production by *Penicillium* sp., dextrose has been replaced by seven different carbon sources. For growth yield, there were no significant differences between dextrose and both of starch, mannitol and sucrose (LSD at 0.05 = 1.18) but each of maltose, fructose, galactose and lactose decreased growth yield comparing with dextrose as a control as shown in Fig. (4A). As for pigment production, results illustrated in Fig. (4B) show that among the used carbon sources, mannitol proved to be superior for pigment production, it folded the production about 5 times to reach

15.12 OD_{490} after 6 days of incubation instead of 3.009 OD_{490} after 8 days using dextrose, while lactose totally inhibited red pigment production. Also the other carbon sources slightly enhanced the production comparing with control. By using mannitol as a carbon source, although growth yield decreased about 16.72% after 6 days of incubation comparing with the fourth day, pigment production increased about 51.65%. This may be due to release the pigment from lysed mycelia.

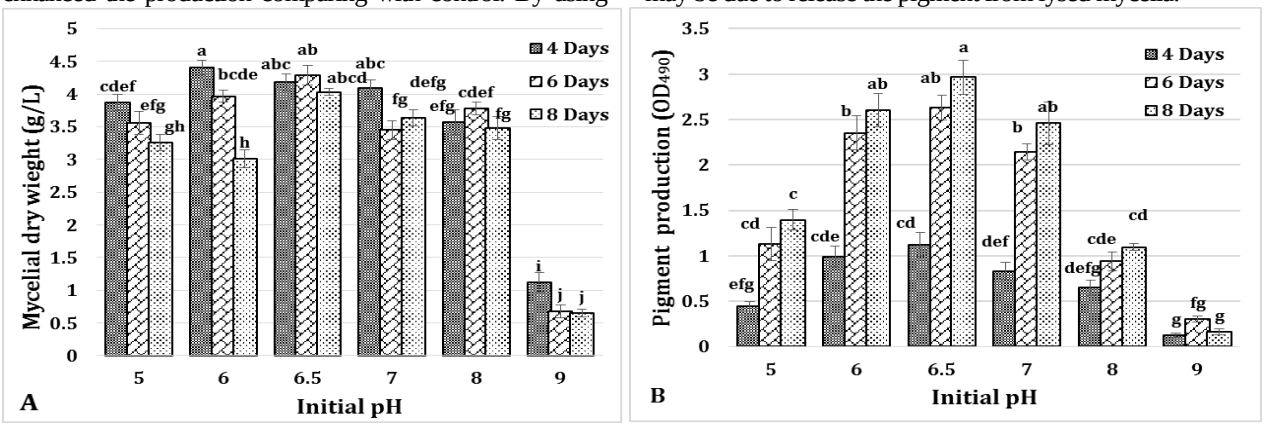

Fig. 3. Effect of initial pH on growth yield (A) and red pigment production (B) by *Penicillium purpurogenum* on PDB at 30±1ºC.

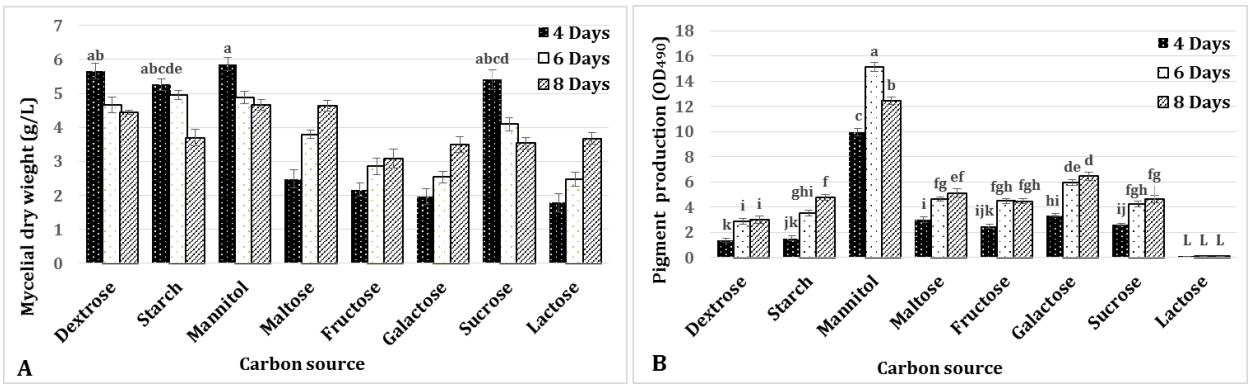

Fig. 4. Effect of different carbon sources on growth yield (A) and red pigment production (B) by *Penicillium* sp. at 30±1º C and pH 6.5.

These results are in close to previously reported by Santos-Ebinuma *et al.* (2013), They found that *P. purpurogenum* has the capability to utilize different carbon. They also observed that there is no direct relation between growth yield and pigment production, while glucose and fructose promoted the highest growth, starch and sucrose were the best for pigment production.

Also, among 11 carbon sources tested by Cho *et al.* (2002) starch was the best for red pigment production by *Paecilomyces sinclairii.* Sankhyayan *et al.* (2019) found that lactose and fructose strongly decreased pigment production and these results are similar to the results with lactose in our research.

Gunasekaran and Poorniammal (2008) examined 10 carbon sources for growth yield and pigment production by *Penicillium* sp. and they found that the favorable for mycelial growth were glucose, fructose, mannose and sucrose while starch was the best for pigment production.

From the obtained results, mannitol gave a great yield and folded red pigment production by *Penicillium* sp.. So, for subsequent experiments mannitol was used as a carbon source with potato extract (Potato mannitol broth (PMB)).

Effect of nitrogen sources:

Results illustrated in Fig. (5A) show that addition of most examined nitrogen sources significantly enhanced *Penicillium* sp. growth to reach an increase of 71.54, 64.8, 57.7, 56.5, 49.4 and 36.36% in growth yield using malt extract, yeast extract, sodium nitrate, ammonium sulfate and ammonium chloride, respectively comparing with control (PMB without addition of nitrogen source). On the other hand, data illustrated in Fig. (5B) show that red pigment production decreased by addition of nitrogen sources than control. It is obvious from results that ammonium chloride totally inhibited pigment production, while beef extract and malt extract decreased pigment production about 19.33 and 67.73%, respectively.

Regarding to *Penicillium* sp. growth, a favorable biomass was obtained with most nitrogen sources, however, the production of pigments not only depends on the utilization of the nitrogen source, but also on the metabolism related to a favorable metabolic pathway Santos-Ebinuma *et al.* (2013).

Previous studies have proved that the effect of a nitrogen source on pigment production is strain dependent, while using one of nitrogen sources could promote pigment production, using another nitrogen source could inhibit pigment production by the same isolate. Cho *et al.* (2002), reported that red pigment synthesis by *Paecilomyces sinclairii* strongly inhibited by using soy peptone or malt extract. Also Gunasekaran and Poorniammal (2008) fount that red pigment synthesis by *Penicillium* sp. strongly inhibited by using soy peptone, beef extract or potassium nitrate as a nitrogen source. Furthermore Santos-Ebinuma *et al.* (2013) reported that ammonium sulfate strongly inhibited the production of pigments by *P. purpurogenum.*

On the other hand, many authors reported peptone as the best nitrogen source for pigment production (Cho *et al.*, 2002; Gunasekaran and Poorniammal, 2008; Mousa *et al.*, 2018). While Santos-Ebinuma *et al.* (2013) reported that yeast extract was the best for pigment production by *P. purpurogenum* followed by malt extract.

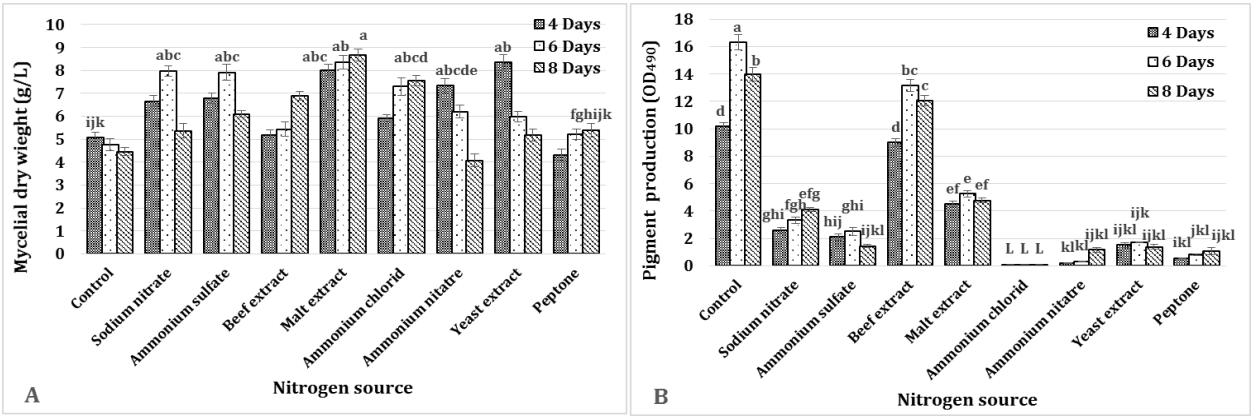

Fig. 5. Effect of different Nitrogen sources on growth yield (A) and red pigment production (B) by *Penicillium* sp. on PMB at 30±1° C.

Effect of incubation temperature:

As shown in Fig. (6 A and B), incubation temperature affected growth yield and pigment production by *Penicillium* sp.. Although the effect on growth yield was insignificant at 24, 27 and 30° C (LSD at 0.05 = 0.81), it was significant for pigment production. *Penicillium* sp. produced good yield of red pigment at temperature range between 24 and 30°C. Increasing or decreasing incubation temperature above or below that range negatively affected pigment production. Thus, it is noticed that temperature plays a vital role in the metabolism of the fungal cells and therefore affects their red pigment production.

Maximum production of red pigment was at 24 °C which reached 19.71 and 20.02 OD_{490} after 6 and 8 days of incubation, respectively. These results are similar to previously reported by Mendez *et al.*, (2011), they obtained highest production of red pigment by *P. purpurogenum* at 24° C after 150 h (6.25 days) of incubation. Also Cho *et al.* (2002) reported that 25° C was the optimum temperature for both growth and pigment production by *Paecilomyces sinclairii* and Ahn *et al.* (2006) found that pigment production by *Monascus* sp. J101 folded 10 times at 25° C compared with 30° C. Zahan *et al.* (2020) reported that incubation temperature should be maintain around 30° C for pigment production by *Penicillium minioluteum* ED24. Similar information had been reported by Afshari *et al.* (2015) as they obtained the highest production of yellow pigment by *Penicillium aculeatum* ATCC 10409 at pH 6.5, and 30° C. Also Gunasekaran and Poorniammal (2008) found that 30° C was the optimal temperature for growth and pigment production by *Penicillium* sp. after 5 days of incubation. Patil *et al.* (2015) obtained maximum pigment production by *P. purpurogenum* at 27° C, and they had good biomass production at 30° C but the pigment production decreased.

Afshari *et al.* (2015) reported that temperature influences the metabolic activity of fungi and, subsequently, their growth. Incubation temperature may be involved in enzymatic processes regulation within the fungal cells. At optimum temperature the enzymatic activity seems to be enhanced for pigment production; consequently the maximum production of pigment was observed at this temperature.

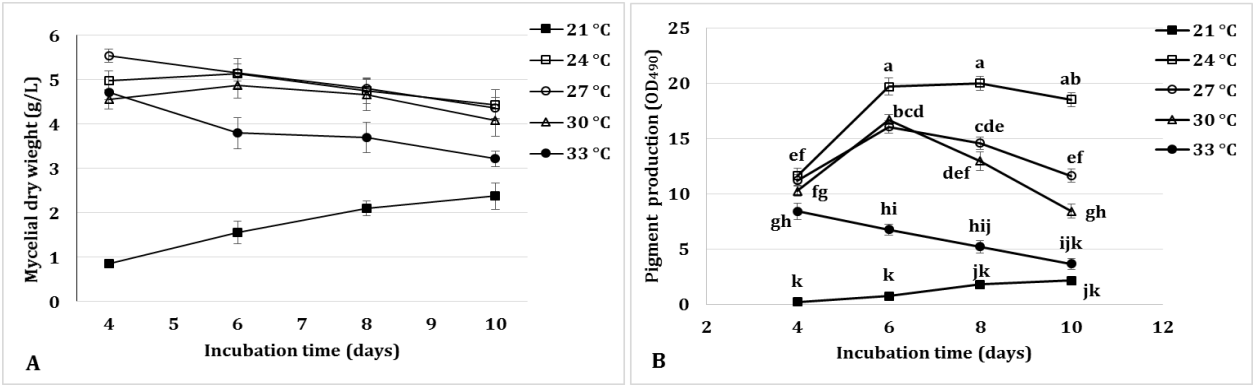

Fig. 6. Effect of incubation temperature on growth yield (A) and red pigment production (B) by *Penicillium* sp. on PMB at pH 6.5.

Effect of inoculum density:

Results illustrated in Fig. (7) show that increasing of inoculum density led to gradually increase in red pigment production to reach a peak of 20.53 OD_{490} at concentration of 10^6 spore/ml after 8 days of incubation, but further increasing of inoculum density significantly decreased pigment production, although increasing of growth yield with further increasing of inoculum density till 10^8 spore/ml.

Babitha *et al.* (2007) found that the best inoculum size for pigment production was 3 ml of $9x10^4$ spore/ ml and using more inoculum size decreased pigment production. Velmurugan *et al.* (2011) found that the best inoculum size for pigment production was 4 ml of spore suspension and using more inoculum size decreased pigment production. Santos-Ebinuma *et al.* (2013) found that the higher production of red pigment by *Penicillium purpurogenum* DPUA 1275 was achieved with 10^8 spore/ml after 12 day of incubation.

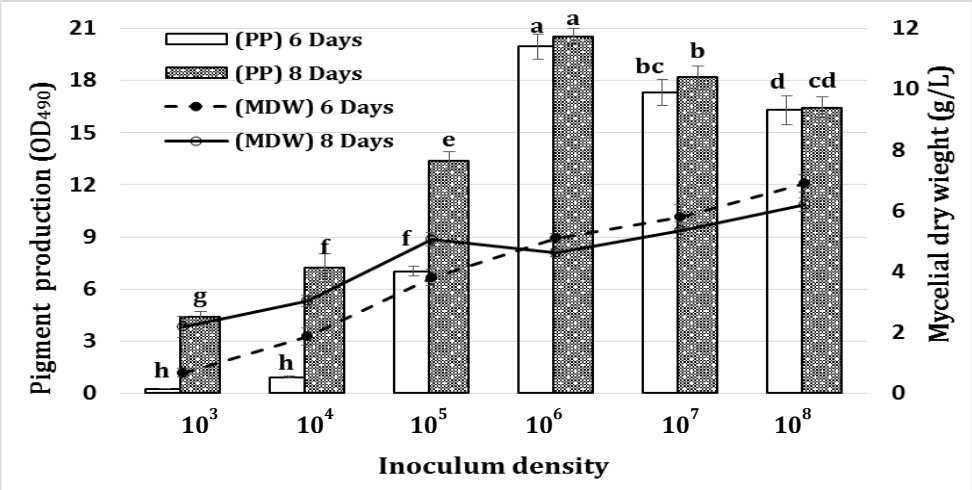

Fig. 7. Effect of inoculum density on growth yield and red pigment production by *Penicillium* sp. on PMB after 6 and 8 days of incubation at 24° C and pH 6.5.

Results are in agreement with previously reported by numerous authors. These results suggest that addition of small amount of the inoculum into fermentation medium resulted in insufficient biomass which produced small amount of products, whereas addition of large amount of the inoculum produced too much amount of biomass which reduced necessary nutrients for product formation (Pandey *et al*., 2000; Babitha *et al*., 2007; Velmurugan *et al*., 2011, Sankhyayan *et al*., 2019 and Zahan *et al*., 2020).

Effect of mannitol concentration:

Results illustrated in Fig. (8) show the effect of different concentration of mannitol on growth and red pigment production by *Penicillium* sp.. Results reveal that growth yield gradually increased with gradual increasing in mannitol concentration, while pigment production decreased at concentrations below 1% and above 1.5% (w/v). 1% (w/v) mannitol was the best concentration for pigment production which obtained maximum production of 21.08 OD_{490}, and there is no significant difference in pigment production between 1 and 1.5%.

Cho, *et al*. (2002) reported that the best carbon source for red pigment production by the fungus *Paecilomyces sinclairii* was soluble starch at concentration of 1.5% (w⁄v). Also Gunasekaran and Poorniammal (2008) reported that the best carbon source for red pigment production by the fungus *Penicillium* sp. was soluble starch at concentration of 2% (w⁄v). While results reported by Mousa *et al.* (2018) reveal that maltose is the best carbon source for pigment production by *Monascus purpureus* at concentration of 1% and the production was almost steady by increasing maltose concentration till 5%.

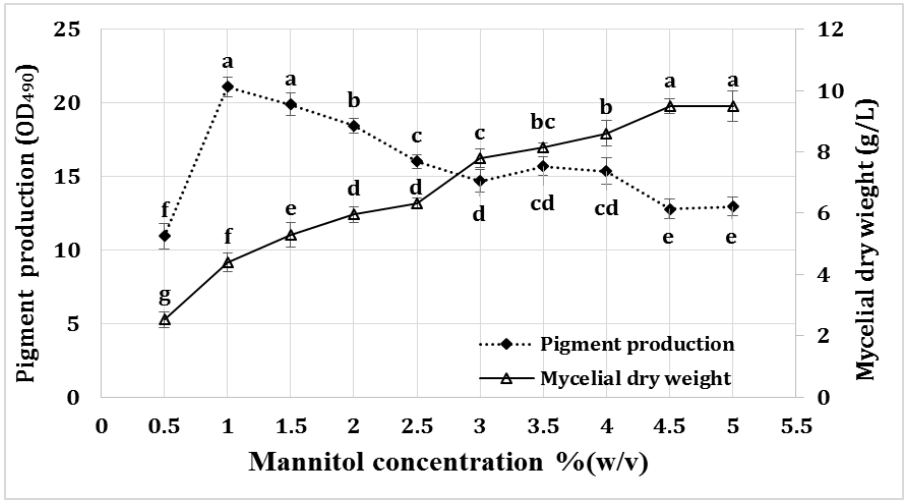

Fig. 8. Effect of mannitol concentration on growth yield and red pigment production by *Penicillium* sp. on PMB after 6 days of incubation at 24° C and pH 6.5.

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العوامل التي تؤثر على إنتاج الصبغة الحمراء بواسطة العزلة الفطرية المحلية *Penicillium* sp.

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هناك اهتمام كبير باستبدال الصبغات المخلقة كيماويا بأخرى طبيعية أكثر صحية والتي يمكن الحصول عليها من مصادر طبيعية خاصة الصبغات الميكروبية. في هذه الدراسة تم الحصول على عزلة من فطر البنيسيليوم من التربة حيث لوحظت قدرتها على إنتاج الصبغة الحمراء على أطباق بيئة أجار البطاطس والدكستروز. تمت دراسة العوامل التي تؤثر على إنتاج الصبغة في مزارع مغمورة لتحديد أفضل الظروف للإنتاج باستخدم بيئة البطاطس والدكستروز كبيئة أساسية. وقد وجد أن أفضل مصدر كربون هو المانيتول حيث ضاعف الإنتاج خمس مرات مقارنة بالدكستروز. من ناحية أخرى فقد قللت مصادر النيتروجين من إنتاج الصبغة بالرغم من زيادتها لمحصول النمو، كما وجد أنه ليس هناك ارتباط مباشر بين إنتاج الصبغة والكتلة الحيوية للفطر. وقد تم الحصول على أعلى إنتاج من الصبغة (21.08 كثافة ضوئية) بواسطة فطر البنيسيليوم بعد ستة أيام من التحضين على 24° م ، درجة حموضة 6.5 ، مع تدعيم بيئة التخمير بإضافة المانيتول كمصدر كربون بتركيز 1% مع حجم لقاح 2% من معلق جراثيم يحتوي على 10^6 جرثومة/مل.