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NATURAL RED COLORANT VIA SOLID-STATE FERMENTATION OF OIL PALM FROND BY *MONASCUS PURPUREUS* FTC 5356: EFFECT OF OPERATING FACTORS

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Abstract

Nowadays, natural colorants are valuable in many industries as an alternative to potentially harmful synthetic colorants. Synthetic colorants may cause serious side effects such as cancer and allergies to the human. The aim of the study is to identify the effects of operating factors on the red pigment produced by *Monascus purpureus* FTC5356 fermented on the treated agro-biomass. The agro-biomass used is oil palm frond (OPF). The study was conducted under the solid-state fermentation using one-factor-at-a-time (OFAT) approach. Five operational factors such as the initial moisture content of OPF (% IMC) (w/w), initial pH, supplementation of nitrogen source (w/w), the percentage of petiole to leaflet and inoculums size (spores/ml) were investigated. The highest production of red pigment was reached at day 8 (2.68 AU/g dry matter). The optimal red pigment production with the treated OPF substrate was achieved at the following operational factor values: 50% (w/w) IMC, pH 6 (3.68 AU/g dry matter), 2% (w/w) peptone, 100% (w/w) petiole, and inoculums size of 10^8 spores/ml. The environmental and nutritional conditions of the substrate have proven to play a significant role in producing the red pigment.

Keywords: Colorants, *Monascus purpureus*, Oil palm fronds, Red pigments.

1. Introduction

Colorants are found in natural and synthetic, and have characteristic of importance to many industries including food, textiles, and pharmaceutical. Colorants are compounds that transmit colour to a substance. Colours offer an appealing appearance to many consumer products. Recently, there is an increased global interest in the process development colorants from the natural products as the use of synthetic colorants are associated with safety issues. Furthermore, the use of synthetic colorants has been decreased due to increasing awareness of its toxicity level. Moreover, it has also been associated with long-term harmful effects [1-3]. For instance, among the synthetic food colorants commercially used, the red dye amaranth (FD and C Red No. 2, E123), erythrosine (FD and C Red No. 3, E127) and tartrazine (FD and C Yellow No. 5, E102), induced DNA damage in the glandular stomach, urinary bladder, colon and gastrointestinal organs; even when used at a low dosage (10 mg/kg) [1]. Thus, there is a growing demand for natural colorants, which resulted in emerging investigations to explore the potential sources of natural colorants [4].

Pigments or natural dyes are compounds with a wide range of colours. Said [5] mentioned that pigments are made from various sources including extraction from microorganisms, plants and animals . However, pigments extracted from plants and animals are insufficient to be used in industries due to limitation of sources [6]. Hence, pigments using microorganism are widely preferred as they offer several advantages. The main reason is due to the nature of microorganism, which has the ability to grow rapidly under a controlled condition [5], thus, resulted in a higher production. There are many common microorganisms that are capable to produce pigments including microorganisms from genus *Monascus*. The *Monascus* species widely studied are *Monascus purpureus*, *Monascus ruber*, *Monascus paxi* and *Monascus anka* [7-13]. *Monascus* species caught special attention due to the edible pigments they produced [14-16]. Among the pigments, true sustainable natural red pigments that are suitable to be used in foods are difficult to obtain.

Several studies indicated that the production of natural colorants is expensive, since the growth media used for culturing the microbes is expensive. Thus, investigation on pigment production using agro-industrial and domestic residues as a growth media is suggested to be a good strategy as it incurs lower cost and reduces the environmental pollution [17, 18]. Agro-industrial residues such as oil cake (from coconut, sesame, palm kernel and groundnut), jackfruit seed powder and corn cob are useful as substrates for pigment production [19-21].

The waste products from the palm oil plantations are well known as one of the largest agro-industrial residues in Malaysia. Based on studies by Malaysian Palm Oil Board (MPOB), approximately 80 million tonnes of oil palm biomass were produced in 2011. The biomass wastes from oil palm, include the frond, trunk, shell, kernel, empty fruit bunch (EFB) and palm pressed fibre (PPF). The abandoned residues are commonly used for ruminant feedstock [23]. In some cases, these residues are dumped into the ground to be decomposed or disposed by burning, thus, creating environmental pollution. Oil palm frond (OPF) has the most unpleasant parts among the oil palm residues [24]. OPF is composed of three main components such as petiole, rachis, and leaves. Evidences indicated that the petiole contains approximately 70% (w/w) of dry matter in the OPF while the rest are from leaves and rachis [25]. The pre-treatment of OPF substrate could be applied to enhance the release of cellulose from the OPF. Few studies are being

conducted to manage the OPF, such using OPF in the bioethanol production [15, 26-28]. In this study, OPF was used as a carbon source in solid-state fermentation to produce pigments.

The main objectives of the present study were to determine the effects of the pre-treatment of OPF and operational factors such as initial moisture content (IMC), initial pH, supplementation of nitrogen source (w/w), ratio of petiole to leaflet and inoculums size (spores/ml) on the red pigments production. The one-factor-at-a-time (OFAT) approach was applied to achieve the objectives of this study.

2. Methodology

2.1. Strain culture and substrate preparation

The strain of *Monascus purpureus* FTC 5356 was acquired from Mardi, Serdang, Malaysia. The stock culture was kept on potato dextrose agar (PDA) media and incubated at 30 ^{o}C for 7 days. Sterile distilled water was added to fully sporulated agar slant culture and the concentration of the suspension was adjusted to approximately 10^5 spores/ml, unless otherwise stated. The number of spores were counted using Neubauer hemacytometer (Cole-Parmer 79001-00).

The fresh oil palm fronds (OPF) were acquired from the Federal Land Development Authority (FELDA) Bukit Goh, Kuantan, Pahang. The OPF samples were cut into small pieces, washed and dried at 60 ^{o}C in an oven (Memmert UFB-500) for 3 days. The dried OPF was grounded into particle size smaller than 1mm using a commercial grinder (Retsch ZM-200, Germany). Later, the grounded OPF was soaked in distilled water in a ratio of 1:18 (w/v) at 121 ^{o}C for 15 minutes, which is known as autohydrolysis process [29]. The treated OPF was later washed with distilled water and oven-dried at 45 ^{o}C for 24 hours. The treated OPF was used for the solid-state fermentation process.

2.2. Solid-state fermentation (SSF)

The One Factor at a Time (OFAT) method was used to investigate the operating factors that influence the pigment production. The effects of initial moisture content (IMC) (45% to 65%) (w/w), percentage petiole to leaflet (0% to 100%) (w/w), initial pH (pH 4 to 8), inoculum size (10^5 to 10^9 spores/ml) on the pigment production were determined. During the experiments, the factors specified above were changed separately. While, the other factors such as IMC (55%) (w/w), concentration of the peptone (2%) (w/w), percentage of the petiole (50%) (w/w), initial pH (pH 6) or inoculum size (10^5 spores/ml) were fixed. The ranges and the fixed factors were set after several runs during preliminary experiments (data not published). After the completion of inoculation, the contents of the flask were mixed thoroughly and incubated at 30 ^{o}C. The flasks were harvested at day 8. All experiments were performed with three replicates, and the means ± standard deviations were reported.

2.3. Pigment extraction and determination

The harvested solid was oven-dried at 60 ^{o}C for 24 hours using oven (Memmert UFB-500). The dried solid was extracted with 95% ethanol in a ratio of 1:10 (w/v), shaked at 200 rpm (Infors AG-CH-4103 Bottmingen) for one hour. The mixture was filtered using Whatman No.1 filter paper. The red pigment was estimated by measuring the absorbance at 500 nm using UV-Vis

spectrophotometer (Hitachi U-1800). Pigment yield was expressed as absorbance units (AU) per gram of dried solids [30, 31].

2.4. Fermentable sugar determination

According to Miller [32], the concentration of crude fermentable sugar was analyzed by dinitrosalicylic acid (DNS) method at 575 nm using UV-Vis spectrophotometer (Hitachi U-1800).

2.5. Microscopy analysis

The morphological study of the treated and non-treated OPF were performed using the scanning electron microscope (SEM). Firstly, the samples were dried and grounded using a commercial grinder to obtain the 1 mm particle size. Prior to the microscopy analysis, an amount of treated and non-treated OPF were attached to the aluminium sample stubs and sputter coated with a thin layer of gold [33]. The OPF samples were then scanned with the focused beam of electrons at 1000x magnification using a Zeiss EVO-50 scanning electron micrograph (SEM).

3. Result and discussion

In solid-state fermentation (SSF), moisture level, nitrogen level, pH, and inoculum size, are among the important factors for microbial growth and activity [34-36]. The selection of the 500 nm wavelength in the study is due to the maximal spectra absorption to detect the colour of the red pigment [5, 30]. The improvement on the agro-biomass (oil palm frond; OPF) composition after the treatment process, favored the red pigment production. The availability of the fermentable sugar, in the form of monosaccharide, in the treated OPF might support the fungal growth and the red pigment development. The colour of the pigments produced changed according to the variation in the pH.

3.1. Effect of treatment on the OPF substrate

Studies indicated the use of oil palm substrates such as OPF leaflet and petiole as the substrate for red pigment production. Autohydrolysis process was applied where the substrates (OPF) were initially treated with liquid hot water using autoclave machine at 121 ^{o}C. On the other hand, no prior treatment was performed on the non-treated substrates.

Analysis of the internal structure of OPF content was conducted to investigate the efficiency of the treatment in improving the hydrolysis process. The structural changes and surface characteristics of the non-treated and treated OPF substrates were examined using the SEM method. SEM images of the non-treated and treated OPF substrates (leaflet and petiole) are illustrated in Figs. 1 and 2. In terms of structure, Figs. 1(a) and (b) display that the non-treated OPF (leaflet and petiole) had solid, flat, full of impurities surfaces, with many silica components. In contrast, treated OPF (leaflet and petiole) had many pores and uneven cracks on its surface as shown in Figs. 1(b) and 2(b). This was due to the disruption of the internal structure of holocellulose following the removal of lignin and hemicellulose of the OPF during the liquid hot water treatment. This was supported by Hong et al. [29], where they found that the OPF treated with liquid hot water formed many pores and the coated matrix silica component was separated during the treatment.

The effectiveness of the non-treated and treated OPF in producing red pigment by SSF process has been investigated in this study. Evidence indicated that the treated OPF produced a higher amount of red pigment (3.817 AU/g) compared to the non-treated OPF (0.047 AU/g) (Figs. 1 and 2). The non-treated OPF had a lower yield of red pigment due to the existence of lignin, cellulose, and hemicellulose. These elements are known to protect the cellulose from enzymatic degradation of the plant cell walls [37]. Contrarily, the liquid hot water damaged and cracked the solid surface of treated OPF, Figs. 1(b) and 2(b). Thus, this exposed the internal structure of the cells through the pores [38]. As such, the treated OPF is more susceptible to enzymatic hydrolysis due to the disruption of the internal structure [39]. In the fermentation step, the fungus released enzymes that could degrade the cellulose into reducing sugar or fermentable sugar [40, 41]. As a result, a higher production of red pigment on the treated OPF was noticed.

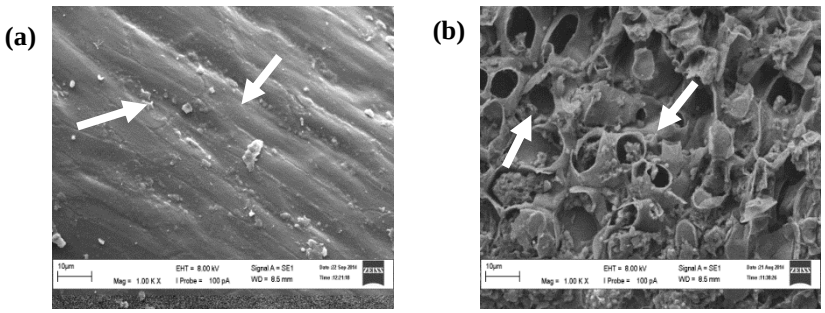

Fig. 1. SEM micrograph of OPF leaflet under 1000x magnification. (a) Non-treated, (b) Treated.

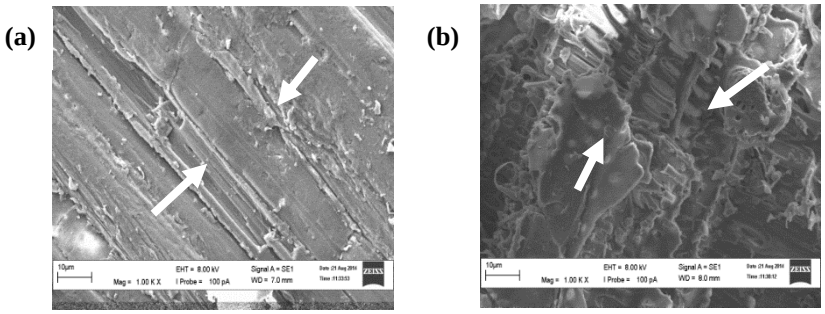

Fig. 2. SEM micrograph of OPF petiole under 1000x magnification. (a) Non-treated, (b) Treated.

3.2. Effect of initial moisture content (IMC) on red pigment

The moisture content is one of the crucial factors to assess the effectiveness of the SSF process. The IMC of the substrate may directly affect the fungal growth and product formation [42]. The effect of IMC on the red pigment was determined by exposing the treated OPF to different percentages of IMC ranging from 45% to 65% (w/w) with supplementation of 2% (w/w) peptone. Each substrate was adjusted with 50% (w/w) OPF petiole and 10^5 spores/ml of *Monascus purpureus* FTC 5356. The initial pH was fixed at pH 6. The results demonstrated that the red

pigment production was maximal at 50% (w/w) IMC with the yield of 2.96 AU/g dry matter, with maximal biomass concentration, as shown in Fig. 3.

The results are consistent with Babitha et al. [19], who reported poor production of red pigment at higher IMC (>50%) (w/w) [19]. In addition, excessive water contents (≥80%) (w/w) at a higher IMC, converts the SSF to liquid state fermentation (LSF), which the later supports the growth of bacterial culture [5, 43]. Instead, too low moisture content (<40%) (w/w) leads to poor dispersion and solubility of the nutrients in the substrate. This resulted in reduced fungal growth, thus, decreased pigment production [30, 42, 44, 45].

The increase in IMC percentage from 45 to 50% (w/w) (Fig. 3) proportionally increased the solubility of nutrients in the substrate. Hence, it promoted the fungal growth, and consequently increased the production of the red pigment. An adequate water content in the substrate facilitates the oxygen transport process [46] in the substrate, which promotes the fungal growth. Nonetheless, excessive water content in the substrate (>50%) (w/w), may lead to a reduction in the oxygen transfer and diffusion in the substrate due to the agglomeration of substrate. This eventually lowered the porosity of the substrate and promoted the risk of contamination [5, 42, 45, 47, 48]. Hence, the fungal growth was terminated, which affected the red pigment formation (Fig. 3).

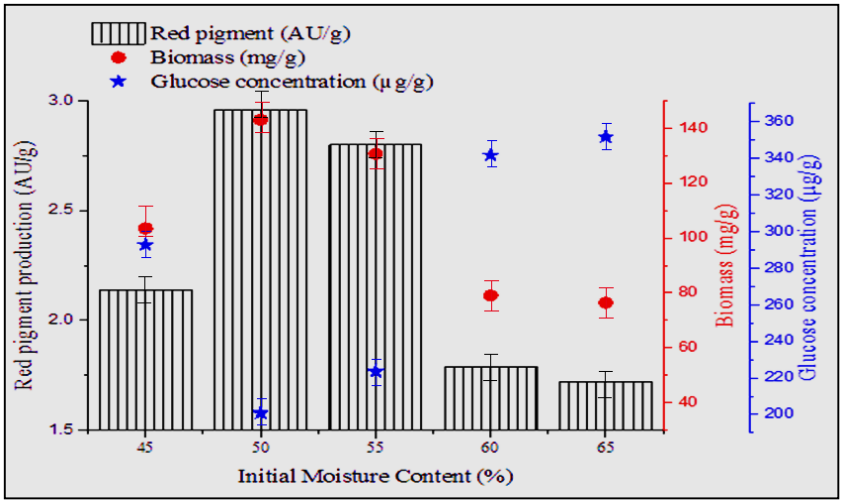

Fig. 3. Effect of initial moisture content on red pigment production.

3.3. Effect of nitrogen source on red pigment

The effect of nitrogen source (peptone) on the red pigment was investigated using the treated OPF with different concentrations of peptone ranging from 1% to 5% (w/w). The IMC (55%) (w/w), initial pH (pH 6), OPF petiole (50%) (w/w) and inoculum sizes (10^5 spores/ml) were fixed as constant factors. The results indicated that supplementation of peptone on the treated OPF had a significant effect on the quantity of the pigment production (Fig. 4). The figure depicts that 2% (w/w) peptone resulted in the highest pigment production and a biomass concentration with the yield of 2.93 AU/g dry matter and 138.99 mg cell dry weight/g dry matter respectively.

Contrarily, the glucose concentration was the lowest at 2% (w/w) of peptone. On the other hand, the formation of the red pigment was interrupted with 5% (w/w) peptone. This may be due to the excessive nutrient in the fermentation medium, where the medium became toxic to the fungal. Thus, it inhibited the growth of the *Monascus purpureus*. The findings were in agreement with a study conducted by Dikshit and Tallapragada [49], which reported that the pigment production by *Oryza* sp with additional 2% (w/w) peptone on the local polished rice, increased the pigment formation. It should be noted that the molecules of red pigment produced by *Monascus* sp. contains nitrogen in their structure. Several studies demonstrated that nitrogen source affected the production of orange and yellow pigments by *Monascus* sp. [50, 51]. The orange pigment transformed into the red pigment as the yellow pigment was unable to react with the amino group. This eventually resulted in the production of corresponding amine of the red pigment. The capability of the reaction is also influenced by the carbon-to-nitrogen (C:N) ratio, where the red pigment was promoted when the C:N ratio > 20 [51, 52].

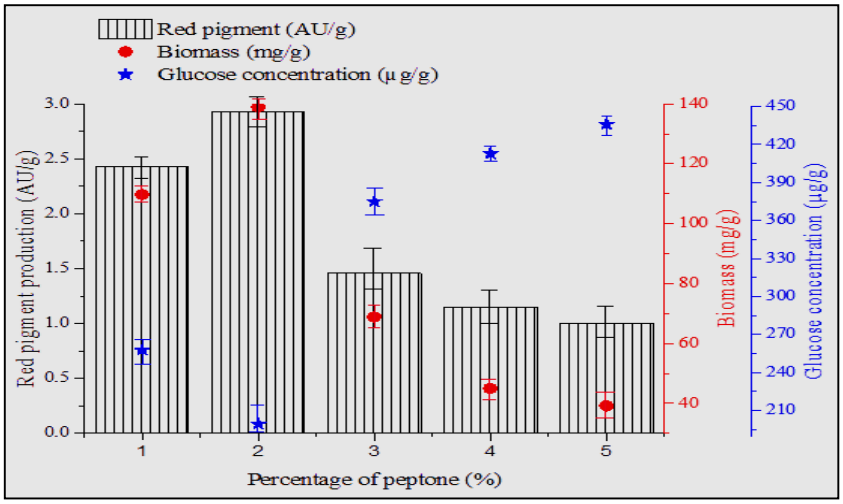

Fig. 4. Effect of peptone concentration on red pigment production.

3.4. Effect of percentage of petiole to the leaflet on the red pigment

The entire leaflet and petiole tested were able to produce the red pigment. Fig. 5 demonstrates the effect of various percentages of petiole to leaflet ratio on the red pigment production. Figure 5 indicated that 100% (w/w) of petiole constituent was found to be the best substrate to synthesize the red pigment, with a yield of 4.87 AU/g dry matter. Moreover, the biomass and glucose concentrations were of 225.13 mg cell dry weight/g dry matter and 80.93 µg/g, respectively for 100% (w/w) of petiole constituent (Fig. 5). In contrast, poor red pigment production was observed at 100% (w/w) leaflet (0.62 AU/g dry matter). This might be due to the effect of nutritional factor of the OPF, where the cellulose content of the leaflet (168 g/kg) was lower than the petiole (317 g/kg) [53].

The total carbohydrate in petiole was relatively higher compared to the leaflet with values of 946 g/kg and 750 g/kg, respectively [53]. Therefore, the insufficient amount of fermentable sugar in the leaflet might affect the medium. This

subsequently influences the fermentation feedstock and red pigment formation. Although the leaflet is recorded to compose the most nutrient contents, but petiole was rich in cellulosic material and sugar [18], which are important for the fermentation feedstock.

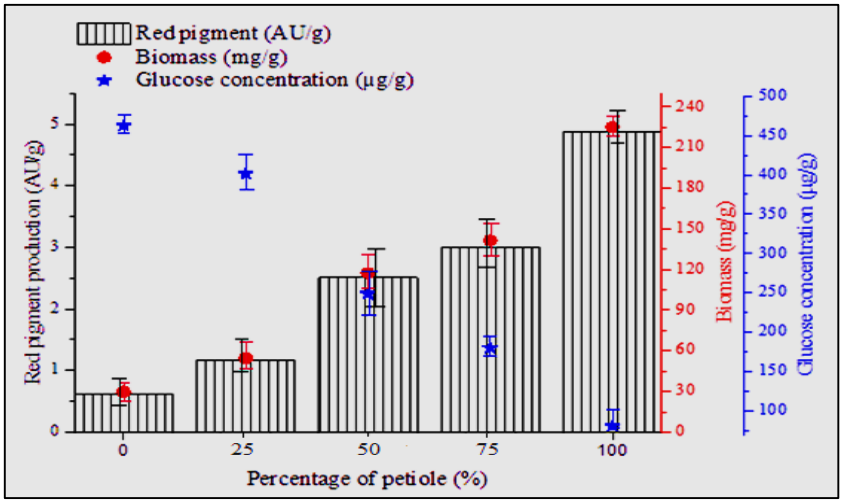

Fig. 5. Effect of percentage petiole to leaflet on red pigment production.

3.5. Effect of Initial pH

The initial pH of the substrate is crucial in the physiological functions of fungi, conidial development and red pigment production [54]. The effect of initial pH on the red pigment was investigated by cultivating the treated OPF at different pH values (pH 4-pH 8.0), as the red pigment is a growth associated product. The IMC (55%) (w/w), peptone 2% (w/w), percentage of OPF petiole (50%) (w/w) and inoculum size (10^5 spores/ml) of *Monascus purpureus* FTC 5356 were fixed as the constant factors.

Figure 6 depicts the effect of different initial pH on the red pigment. The results demonstrated that the yield of red pigment production (3.21 AU/g dry matter) and biomass concentration (150.3 mg cell dry weight/g dry matter) were optimal at pH 7.0 compared to the other pH tested (Fig. 6). Similar findings were reported by Lee et al. [54, 55], where the studies reported that the red pigment production was prominent at a pH range of 5.50 to 8.5. Nevertheless, poor production of the red pigment was observed (0.96 AU/g dry matter) at pH 4 (Fig. 6). At pH 4, the amino acid or ammonia content of the OPF substrate was decreased rapidly due to the chemical degradation of the protein compound. Thus, this led to the production of mostly yellow and orange pigments [56, 57].

Several studies were in agreement with the findings of the current study, where the pigment synthesis was shifted from red to yellow pigment at lower pH (pH 4 and 5) [19, 58, 59]. At higher initial pH (pH >5), a higher concentration of amino acids or ammonia was maintained in the substrate. This enhanced the modification of orange to the red *Monascus* pigments [56]. The results suggested that pH affected the biosynthesis of the pigment produced by *Monascus* sp.

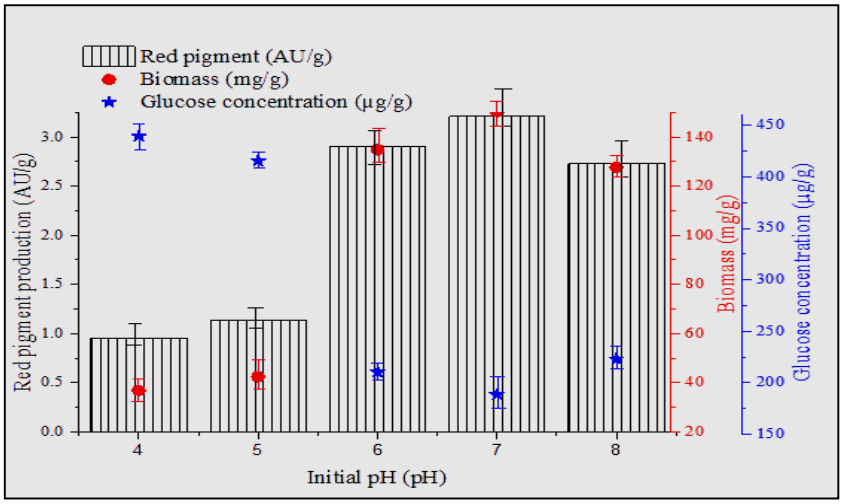

Fig. 6. Effect of pH on the red pigment production.

3.6. Effect of inoculum size on the red pigment production

An adequate amount of inoculum size is essential for the production of the higher amount of red pigment. The treated OPF was inoculated with different inoculum sizes ranged from 10^5 to 10^9 spores/ml to determine the effect of inoculum size of *Monascus purpureus* FTC 5356 on the red pigment production. The IMC (55%) (w/w), peptone (2%) (w/w), the percentage of OPF (50%) (w/w) and initial pH (pH 6) of the substrate were fixed as constant factors. Figure 7 demonstrates the effect of different values of inoculum size on the red pigment, where maximal red pigment production (3.24 AU/g dry matter) was observed with treated OPF and the inoculum size of 10^8 spores/ml. In contrast, poor red pigment production (2.85 AU/g dry matter) was observed with the inoculum size of 10^5 spores/ml.

There was a progressive increase in the growth of the fungus inoculated with 10^5 to 10^8 spores/ml. The final biomass concentration of the sample was maximal (179.49 mg cell dry weight/g dry matter) with the inoculum size of 10^8 spores/ml. The multiplication of the fungal cells was slow at a lower inoculum size (10^5 spores/ml), where a longer time was required to produce the desired products. In contrast, larger inoculum size (10^6 to 10^8 spores/ml) would promote the rapid proliferation of the cell. This condition increased the biomass synthesis [60], in which, the formation of the red pigment was enhanced. Furthermore, the balance between the proliferation of the cell and the availability of the nutrients in the substrate would yield an optimum red pigment formation.

Nonetheless, very large inoculums size (>10^8 spores/ml) (Fig. 7) in the substrate would deplete the oxygen availability in the substrate. As a result, poor fungal growth is being obtained [46]. This led to poor red pigment formation. Conversely, less amount of red pigment production was observed with the very small size of the inoculum (i.e., <10^5 spores/ml), due to insufficient biomass to form mycelia. In addition, very small size of inoculum might promote the growth of undesirable organisms on the substrates [55]. Therefore, inoculums size of 10^8 spores/ml was appropriate for a maximal production of red pigment on the OPF as a substrate (Fig. 7).

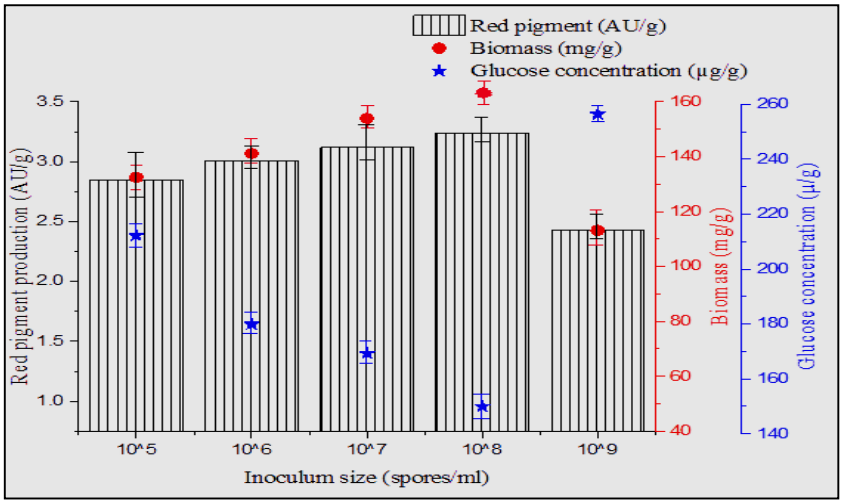

Fig. 7. Effect of inoculum size on red pigment production.

4. Conclusion

This study demonstrated that the nutritional and environmental conditions, such as nitrogen source, IMC, pH, and inoculums size influenced the production of red pigments by *Monascus purpureus* FTC 5356. The maximal red pigment production was observed with 50% (w/w) IMC, 2% (w/w) peptone, 100% (w/w) petiole, pH 7 and 10^8 spores/ml. Importantly, the fungal strain of *Monascus purpureus* FTC 5356 offers a cost-effective solution for the production of red pigment, especially the utilization of abandoned residues of OPF as a substrate. In short, the treated OPF is highly feasible as a potential source for commercial production colorants, particularly red pigments in Malaysia.

Acknowledgement

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Abbreviations

AU	Absorbance Unit
DNS	Dinitrosalicylic Acid
EFB	Empty Fruit Bunch
FELDA	Federal Land Development Authority
IMC	Initial Moisture Content
OFAT	One-Factor-At-A-Time
OPF	Oil Palm Frond
PDA	Potato Dextrose Agar
PPF	Palm Pressed Fibre
SEM	Scanning Electron Microscope
SSF	Solid-State Fermentation

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