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Research paper

Kinetic Characterization of Red Pigment of *Monascus Purpureus* at Different Level of Nitrogen Concentration.

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Abstract

Natural and synthetics colors are the two types of food coloring existed in the industries. Over time, most of synthetic colors were banned due to the clearly evident side effects and their possible carcinogenic effects. Pigment produced by *Monascus purpureus* is microbial origin and safe to use as food grade coloring. The aim of this research is to study the kinetic characterization of red pigment production throughout the solid state fermentation process at different level of nitrogen (peptone) concentration. The concentrations of nitrogen used are in between 1 to 5% of peptone. The highest μ_{max} (0.1182 day^{-1}), P_{cells} (119.7 g ml^{-1} day^{-1}), $Y_{X/S}$ (92.53), $Y_{P/S}$ (1.35), and $Y_{P/X}$ (0.016) are obtained at day 5 of fermentation period with the addition of 2% peptone. However, no further increase of the kinetic values with the increasing of peptone concentration from 2% to 5% was observed, except on $Y_{P/X}$ where the value was high with higher concentration. This indicates that with higher concentration the conversion of biomass to red pigment is enhanced.

Keywords: *Monascus purpureus; natural color; nitrogen source; red pigment; solid state fermentation.*

1. Introduction

Color is the signal that is immediately perceived by the optical sense of human and this attributes whether a certain food is appealing or not. Attractive food usually translates into increased consumption, which is a fundamental behavioral response. Requirements of colorants are increasing day by day in human lifestyle. The global demand for food pigments is estimated to reach up to US$27.5 billion in 2018 [1]. People always have argued on two types of colorants, natural and synthetic. Currently natural pigments comprise 31% of the market, as compared to 40% for synthetics ones while others are semi-synthetic [1]. Preference of natural colorants has been a high concern because by studying the harmful effect of synthetic colorants, it is found that prolong consume can caused health impairment abilities including their possible carcinogenic effects [2]. A good alternative for the food industries is the use of natural pigments produced by microorganism, plants and animals. Among the various pigments producing microorganisms, the genus of *Monascus* fungi stands out. *Monascus* pigments have been traditionally used in food application [3]. This filamentous fungus has been widely used in the production of fermented foods such as angkak, anka, beni koji and red yeast rice in Asian countries [4].

Pigments can be produced through solid state fermentation (SSF) or submerged fermentation (SmF). Lately, SSF has gained so much attention from researchers that produce primary and secondary metabolites. This is because SSF provides more adequate habitat for fungus, with high pigment productivity in a relatively low cost process by using agro-industrial residue as substrate [5][6]. Several factors affect the production of pigments such as the nitrogen sources, moisture content, and inoculum size [7][8][9][10]. Possibilities for applying kinetics techniques for efficient exploitation of biochemical reaction systems should always be sought [11]. Many factors, such as inoculum, substrate, minerals, nitrogen source, temperature and/or pH, can influence the red pigment production process as well as fungal growth.

Peptone, monosodium glutamate, yeast and ammonium sulfate were commonly used as nitrogen source for pigment production. Research done by [8] showed that peptone was more favorable nitrogen sources for growth of *Monascus* compared to ammonium and nitrate because peptone supports the formation of red pigments. However, addition of peptone may lead to a change of the pH of oil palm frond and this believed to contribute to different in composition of *Monascus* pigments.

Monascus is a filamentous fungus belonging to the genus *Monascus*, family monascaceae and class ascomyceta. This has power to synthesis secondary metabolites as the bio pigments with connected ascomyceta. *Monascus* produces three types of natural pigments, mainly red (monascorubramine and rubropunctamine), orange (monascorubrinand rubropunctatin) and yellow (monascin and ankaflavin) [12]. Among these colors, it has been known that red color is widely used in foods industries as food colorant.

Recently, research has been concerned on the studies on general culture conditions and substrate evaluation for pigments production. Hence, most of the studies have been performed at laboratory-scale. Pigment production in industrial scale consumes a very high cost, thus there are needs to develop a technology for low cost production as well as give high yield of product. At the end, the aim of this technology is to replace the synthetic colors in terms of cost and production can be attained by using cheaply available substrate through solid state fermentation (SSF).

Lately, SSF have gained much attention from researchers that produce primary and secondary metabolites. This is because SSF provides more adequate habitat for fungus, with high pigment productivity in a relatively low cost process by using agro-industrial residue as substrate [5][6]. Agro residues and crop such as oil palm frond [13], corn cob [14], sugarcane bagasse [3], and jack fruit seed [10] were used as substrate for pigment production.

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Utilizing these waste have resulted in lower price fermentation and high pigment output. So far, there are research focusing on utilizing oil palm frond as substrate but none of it highlights the kinetic study of a red pigment. Therefore, this research focuses on kinetic study of red pigment using OPF as substrate. The kinetic study will help to better understand the *Monascus* fermentation process. For this reason, studying the effects of operating conditions such as nitrogen source on red pigment production and fungal growth are of crucial importance, primarily because such studies can be used to optimize the performance and lowering cost of the process [15]. This research provides data that can be utilized for better understanding about *Monascus* fermentation product focusing on red pigments.

2. Material and Methods

2.1. Culture

Monascus purpureus FTC 5356 is obtained from Universiti Malaysia Pahang. The strain is maintained in Potato Dextrose agar (PDA) medium agar plate and incubated at 30°C for 7 days, preserved 4°C [16][17]. After cultivating for 7 days, 1mm x 1mm from PDA are inoculated at the centers of agar plates for 7 days at 30°C and then continuous inoculated in agar slant for another 7 days.

2.2. Inoculum Preparation

One loop of agar slope culture (7-days old) is diluted in distilled water. The spore is scrapped off under aseptic condition to produce homogenous spore suspension as inoculum [16]. The spore suspension is carried out in sterile universal bottles which contains medium inoculated. Then, haemocytometer is used to calculate spore suspension (spores/ml) before it is transferred to fermentation medium.

2.3. Substrate Preparation

The oil palm fronds (OPF) are obtained from local agricultural fields at Felda Lepah Hilir, Gambang, Pahang, Malaysia. The substrate is dried in sunlight before grinding into smaller pieces. OPF is ground to 1mm of size of particle using grinder (Retsch, zm200).

2.4. Solid State Fermentation (SSF)

A 5 gram of substrate is placed in 250ml conical flasks, and then the substrate is autoclaved (Hirayama, HVE-50A) at 121°C for 10 minutes. After cooling, substrate–based medium is inoculated with seed culture of *Monascus purpureus* and incubated (Memmert,Germany, BE600) at 30°C. The parameter involved is nitrogen source (1%-5%) . The pH is adjusted with 1M NaOH to 6.0, 50 % moisture content is fixed while the nitrogen factors are being investigated. Samples were taken every day for 7 days .The fermentation is run for 7 days after inoculation at 30°C to finish the cycle [16]. The samples are made triplicate

2.4. Pigment Extraction

Fermented substrate is dried in oven at 60°C for 24 hours. The fermented substrate is taken for pigment extraction using 60% ethanol and soaked for 3 hours before shaken in incubator shaker (Infors AG CH-4103 Bottmingen) at 200 rpm and 30°C for 1 hour. Then the extracts are allowed to settle down at room temperature and filtered with Whatmann no.1 filter paper [16]. The ethanol extract of unfermented substrate is kept as blank. The pigment production is measured at wavelength of 500 nm (red colour) using spectrometer (Hitachi, U-1800). The absorbance values (OD) are converted into pigment units (AU/g) in (1).

Color value unit (AU/g) =

$$OD \times \frac{Dilution\ volume\ of\ extract\ (ml)}{Weight\ of\ sample\ (g)} \quad (1)$$

2.5. Determination of Biomass

According to methodology adapted from [17], the fungal biomass was estimated by determining the amount of N-acetyl glucosamine released by acid hydrolysis of chitin, present in the mycelia cell wall. Chitin hydrolysis was carried out by using 2ml of H_2SO_4 and autoclaved. The hydrolysate was neutralized to pH 7.0, mixed with acetyl acetone reagent and followed by Ehrlich reagent. The optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine was used as a standard. Where, Abs_{530} is absorbance at 530 nm, V_{Mix} is a total volume of mixture and V_S is sample volume.

Biomass (µg cell dry weight/ g dry matter) =

$$\frac{Abs_{530}}{0.0013} \times \frac{V_{Mix}\ (ml)}{V_S\ (ml)} \times \frac{60\ mL}{0.5\ g} \quad (2)$$

2.6. Determination of Total Reducing Sugar

Total reducing sugars were determined based on method done by [17]. The reaction mixture contained 0.5 ml of crude extract and 0.5 ml dinitrosalicylic acid reagent. The tubes were heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. Glucose served as the calibration standard for total reducing sugar determination.Where, Abs_{560} is absorbance at 560 nm, V_T is total volume in test tube, V_D is volume of distilled water, V_S is sample volume and W_{OPF} is weight of OPF sample.

$$\text{Glucose concentration (g/g)} = \frac{Abs_{560}}{3.7005} \times \frac{V_T\ (ml)}{V_S\ (ml)} \times \frac{V_D(ml)}{W_{OPF}(g)} \quad (3)$$

3. Results and Discussions

3.1. Effect of Nitrogen on Pigment Production, Biomass and Glucose Concentration

Kinetics of growth and product formation in solid state fermentation for nitrogen effect was examined at a fixed pH 6, 50% initial moisture content, $\mathbf{10^8}$ (spores/ml) inoculum size, 5g of OPF, while peptone (nitrogen) used were manipulated from 1% to 5% of peptone concentration. The samples were taken daily and were made in triplicate.

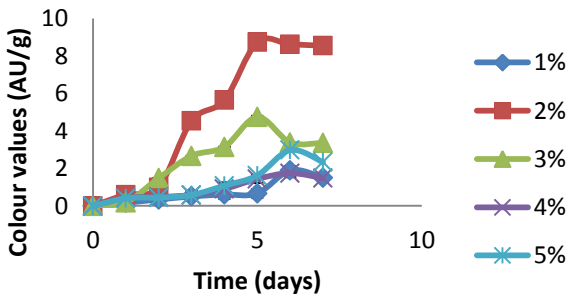

Fig. 1: Effect of nitrogen on pigment production

Figure 1 illustrated the colour value for each concentration of peptone ranging from 1% to 5% for 7 days. The higher red pigment production was obtained with 2% peptone, there is no further increase in pigment production with increasing peptone concentration from 2 to 5%, With 2% peptone, the maximum red pigment obtained was at day 5 (8.733 CVU/g), while with 5% peptone, the maximum pigment obtained was at day 6 with lesser value, 2.976

AU/g. Too low of peptone concentration did not improvise good pigment production, the pigment value at day 6 for 1% of peptone was 1.896 AU/g. Data showed that increasing the peptone concentration did not affect the growth and pigment production much, but it lengthens the lag phase. When comparing 2% and 5% peptone, 2% change lag to log phase faster (within 5 days) than 5% of peptone (within 6 days).Meanwhile, the other concentration showed the slow progress and low pigment production.

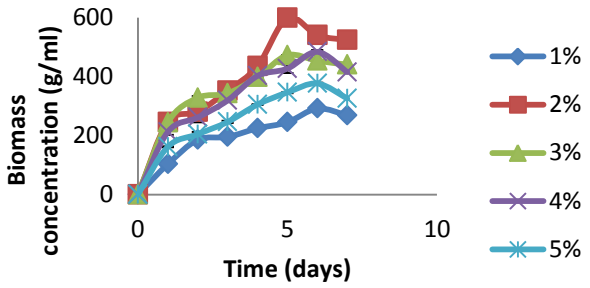

Fig. 2: Effect of nitrogen on biomass concentration

Figure 1 and Figure 2 showed the influence of different concentration nitrogen sources on pigment and biomass concentration. Generally, the biomass growths for nitrogen follows the expected profile of a lag phase (day 0 to day 2), an exponential phase (day 3 to day 5), and the deceleration growth phase (day 6 to day 7). The stationary phase starts at the end of the declaration phase. Fermentation cycle was completed at day 7. Red pigment was produced simultaneously with microbial growth, thus classified this microbial product as growth associated product formation.

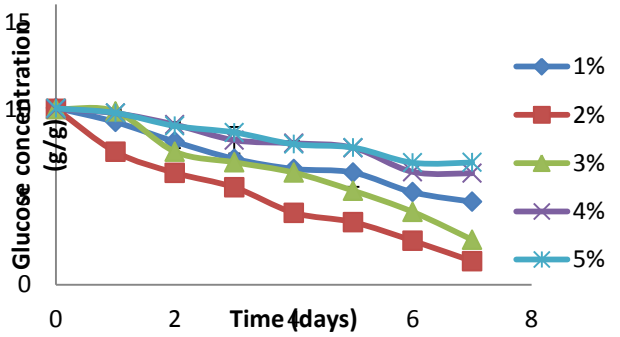

Fig. 3: Effect of nitrogen on glucose concentration

Figure 2 and Figure 3 showed the changes of biomass and glucose concentration throughout the fermentation process. Figure 2 shows that biomass concentration for 2% peptone constantly increased throughout the fermentation period. Initial biomass concentration 245.85 g/ml at day 1 increased to 598.50 g/ml at day 5, while initial glucose concentration 10.03 g/g at day 1 decreased to 2.51 g/g at day 5, demonstrating the consumption of the fungi to grow and produced pigment. Shortly after the glucose decrease and biomass increased, the fungi growth is approaching the exponential phase. From day 4 of fermentation, it was possible to observe an increased in the production of pigment (Figure 3.1) which was reached its maximum after day 5 of fermentation, period in which the reduction in biomass and pigment production and depletion of the substrate of the substrate occurred.

Figure 2 shows the growth trends of fungi for 5% peptone, it was slowly increased, demonstrating the difficulty of peptone assimilation by the fungi. However, the presence of glucose was detected at the beginning of fermentation (10.06 g/g at time day 0), was consumed throughout this fermentation has decreased to (6.95 g/g at day 6) suggesting the production pigment by *Monascus* but does not gave a high production. The pigment and biomass was observed to reach maximum reading at day 6 and decreased after this period.

Substrate with minimal percentage of peptone (2%-3%) had showed a high value of pigment production. However a high percentage of peptone will deplete the pigment production. This may be due to the high concentration of peptone which may create high osmotic pressure to the *Monascus* fungi, thus inhibiting pigment production. Low concentration of peptone seemed to be effective to promote the production of *Monascus* pigment.

3.2. Kinetic study on red pigment

The result from Table 1 demonstrated the kinetic parameters for various peptone concentrations. Table 1 shows the kinetic data in the production of pigments for nitrogen factors maximum growth rate (μ_{max}), maximum pigment production (AU_{500}) and mean cell (P_{cells}) and pigment productivity (P_M).

Table 1: Kinetic parameters for various peptone concentrations

Kinetic parameters	Peptone concentration (%)				
	1%	2%	3%	4%	5%
$\mu_{max}(day^{-1})$	0.059	0.118	0.054	0.099	0.099
P_{cells} (mg $ml^{-1}\,day^{-1}$)	48.58	119.7	75.54	80.61	62.72
Maximum pigment production (AU_{500})	1.896	8.733	4.739	1.743	2.975
$P_M(AU_{500}\,day^{-1})$	0.316	1.746	0.947	0.291	0.496

Specific growth rates ranged from 0.059 to 0.118 day^{-1}, where the highest rate was observed using 2% peptone and the lowest using 3% peptone. The average cell productivity (P_{cells}) was higher for 2% (119.67 mg $ml^{-1}day^{-1}$), followed by 4% peptone (80.61 mg $ml^{-1}day^{-1}$). While the average yields of pigment (P_M) were 1.747 $AU_{500}\,day^{-1}$ and 0.948 $AU_{500}\,day^{-1}$ for 2% and 3% peptone respectively.

The maximum specific growth rate for nitrogen was when substrate supplemented with 2% of peptone. Nitrogen does help in microbial growth based on result of specific growth rate (μ_{max}) but when high peptone concentration was used it does not contribute to the pigment production. Pigment production for 5% peptone was 2.98 AU/g which is lower than 2% peptone, 8.73 AU/g.

Table 2 shows the factors of conversion of substrate into biomass ($Y_{X/S}$), substrate into red pigments ($Y_{P/S}$) and biomass into red pigments ($Y_{P/X}$).

Table 2: Conversion factors for peptone

Kinetic parameters	Peptone concentration (%)				
	1%	2%	3%	4%	5%
$Y_{X/S}$ ($g\,g^{-1}$)	60.56	92.53	70.08	218.8	168.2
$Y_{P/S}$ (AU_{500}ml g^{-1})	0.394	1.350	1.017	0.486	0.959
$Y_{P/X}$ (AU_{500}ml g^{-1})	6.5 x 10^3	1.5 x 10^2	1.0 x 10^2	3.6 x 10^3	7.9 x 10^3

The highest conversion of substrate into biomass ($Y_{X/S}$) and conversion of substrate into red pigments ($Y_{P/S}$) was 4% peptone. While the highest conversion biomass into red pigments ($Y_{P/X}$) was 2% peptone.

4. Conclusion

Based on the kinetic value of pigment productivity, when substrates were supplemented with 2% of peptone, the conditions had stimulated high pigment production with 8.733 CVU/g with a low biomass to red pigment conversion ($Y_{P/X}$). Meanwhile, there is no further increase of the kinetic values with the increasing of peptone concentration from 2% to 5%, except on $Y_{P/X}$ the values was high with higher concentration. Higher and lower values peptone does not give significant values for a high pigment production. Future studies could use 2% of peptone in order to be applied it in the production of pigments by *Monascusp purpureus*.

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