KINETIC STUDY OF *MONASCUS PURPUREUS* IN SOLID-STATE FERMENTATION FOR RED PIGMENT PRODUCTION

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ABSTRACT

***(Keywords:** Pigment, Monascus purpureus, oil palm frond, solid-state fermentation, Kinetic study)*

Red pigment produced by *Monascus* species is known as an edible pigment, rich in wide range of biological activities. The aim of this research is to study the kinetic characterization of red pigment production throughout the solid state fermentation. Factors such as nutritional factors (nitrogen source and carbon source) and environmental factors (moisture content, pH, inoculum size) on red pigment were investigated. The results indicated that the highest maximum growth rate (μ_{max}), mean cell (P_{cells}), conversion of substrate into biomass ($Y_{X/S}$), conversion of substrate into red pigments ($Y_{P/S}$) and conversion biomass into red pigments ($Y_{P/X}$) were obtained at day 5 of fermentation period at 2% peptone. Where, the values were 0.1182 day^{-1}, 119.7 g ml^{-1} day^{-1}, 92.53 g g^{-1}, 1.35 g g^{-1} and 0.016 g g^{-1}, respectively. The results also indicated that the conversion of biomass to red pigment is improved with higher peptone concentration. Overall, the finding concluded that the red pigment produced by *Monascus purpureus* FTC 5357 in solid state fermentation produced the highest pigment at 2% peptone, 5g of oil palm fronds, pH 6, 50% of moisture content, at 30°C and 10^8 spores ml^{-1} inoculum size. These findings are important knowledge to be used for optimizing the red pigment production.

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ABSTRAK

Pigmen merah yang dihasilkan oleh spesies *Monascus* dikenali sebagai pigmen yang boleh dimakan, kaya dengan pelbagai aktiviti biologi. Tujuan kajian ini adalah untuk mengkaji pencirian kinetik pengeluaran pigmen merah sepanjang penapaian secara pepejal. Faktor seperti faktor pemakanan (sumber nitrogen dan sumber karbon) dan faktor alam sekitar (kandungan lembapan, pH, saiz inokulum) pada pigmen merah disiasat. Hasilnya menunjukkan bahawa kadar pertumbuhan maksimum tertinggi (μ_{max}), bermakna sel (P_{cells}), penukaran substrat menjadi biomas ($Y_{(X / S)}$), penukaran substrat menjadi pigmen merah ($Y_{(P / S)}$) dan biojisim penukaran ke dalam pigmen merah ($Y_{(P / X)}$) diperoleh pada hari 5 tempoh penapaian pada pepton 2% (sumber nitrogen). Di mana, nilai-nilai itu ialah 0.1182 $hari^{-1}$, 119.7 g ml^{-1} $hari^{-1}$, 92.53 g g^{-1}, 1.35 g g^{-1} 0.016 g g^{-1}, masing-masing. Hasilnya juga menunjukkan bahawa penukaran biomas kepada pigmen merah diperbaiki dengan kepekatan pepton yang lebih tinggi. Secara keseluruhan, penemuan itu menyimpulkan bahawa pigmen merah yang dihasilkan oleh *Monascus purpureus* FTC 5357 dalam penapaian keadaan pepejal menghasilkan pigmen tertinggi pada 2% pepton, 5g pelepah kelapa sawit, pH 6, 50% kandungan lembapan, pada 30^{o}C dan 10^{8} spora ml^{-1} saiz inokulum. Penemuan ini adalah pengetahuan penting yang akan digunakan untuk mengoptimumkan pengeluaran pigmen merah.

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SSF	solid state fermentation
EFB	empty fruit bunch
PPF	palm pressed fiber
OPS	oil palm shell
OPK	oil palm kernel
Sp.	species
OPT	oil palm trunk
OPF	oil palm frond
IMC	initial moisture content

CHAPTER 1

INTRODUCTION

1.1 Background of study

Pigment or colour is the signal that is reflect to the optical sense of human and attributed to the acceptance to the product, for instance food product. Fundamental behavioural response of human being started from eyes, later translated to the mind; which affected by the colour which reflected to the food attractiveness. Requirements of colorants are increasing day by day. People are looking for better choice of colorants. According to the report by Technavio, the global market of natural colorants is predicted to increase during 2018-2020 (www.technavio.com). In line with that, awareness on the hazardous and adverse effects of synthetics colors in food ingredients is increasing among consumers. Preference of natural colorants has been great demand.

Synthetic colors are produced by chemical synthesis and cannot be found naturally. They were originally manufactured from coal tar (Amchova et al., 2015). While, natural colors are derived either from fungi, plants, animals or cyanobacteria (Mortensen, 2006). Natural colors derived from fungi could produce pigments in high yield. The fungi such as *Monascus*, *Paecilomyces*, *Serratia*, *Cordyceps* and *Penicillium* (Malik et al., 2012). Among the fungi, *Monascus* pigment well known as highly safe and naturally occurring edible coloring agents (Babitha, 2009). Generally, in industrial

scale, pigment production has been carried out using submerged fermentation (SmF) (Babitha et al., 2006). However, solid state fermentation (SSF) has emerged as an effective way due to the production, as SSF provides an adequate habitat for fungi. In addition, with SSF, high pigment production can be obtained, and relatively low-cost process attained, when agro-industrial wastes are used as substrate (Subhasree et al., 2011).

In Malaysia, palm oil plantation is one of the largest biomasses contributing sector in the country. Approximately 55.75 million tons of oil palm biomass was recorded, comprising most part of oil palm fiber; empty fruit bunch (EFB), palm pressed fiber (PPF), oil palm shell (OPS), oil palm kernel (OPK), oil palm trunk (OPT) and oil palm frond (OPF) (Shuit et al., 2009; Khalil et al., 2012). The oil palm frond (OPF) is one of the most unpleasant and neglected. These large quantities of OPF produced by oil palm plantation each year make it a very promising source of substrate for *Monascus* sp. fermentation.

Many factors such as inoculum size, substrate material, minerals, nitrogen source, temperature and/or pH influenced the pigment production process as well as fungal growth (Said, 2010; Hailei et al, 2011; Prajapati et al, 2014; Hajjaj et al, 2015; Torres et al, 2016). Process kinetics is related to the reaction rates which affected by the process variables/factors on process fermentation, for instance in SSF. Although there was various research on the pigment production by *Monascus sp*. (Rajeswari et al., 2014; Said, 2010; Srianta et al., 2016; Subhasree et al., 2011). However, there still limited study on the kinetic study of red pigment of *Monascus* in SSF.

For this reason, studying the effects of operating conditions on red pigment production and fungal growth are of crucial importance, primarily because such studies can be used to optimize the performance and cost of the process (Infantes *et al*., 2011).

1.2 Objective

1. To determine the effects of the operational factors such as initial moisture content (IMC), initial pH, supplementation of nitrogen source and inoculums size on the red pigments and biomass production using oil palm frond (OPF) as a substrate.
2. To investigate effect of nutritional factor on the kinetic study of the red pigments and biomass productions.
3. To investigate effect of environmental factors on the kinetic study of the red pigments and biomass productions.

1.3 Research scope

The following are the scopes of this research:

i. Characterization of oil palm frond (OPF) which use as a substrate.
ii. Identification the effect of initial moisture content ranges from 45% to 65% on the biomass and red pigment productions.
iii. Evaluation the effect of percentage of peptone ranges from 1% to 5%.
iv. Investigation the effect of initial pH of substrate ranges from pH 4 to pH 8.
v. Identification the effect of inoculum size ranges from 10^5 to 10^9 spores ml^{-1}.
vi. Investigation the kinetic parameters of the above factors to the biomass and red pigment productions grown on of oil palm frond (OPF) in solid state fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural colorant versus synthetic colorant

Color can enhance the optical and chemical senses of humans which attributes to acceptance of the food product. Attractive food colors usually translated into increased consumption, which is a fundamental behavioral response. There are two types of colorant available in market, natural and synthetic color. Natural colorants are derived from plants, fungi, animals or cyanobacteria (Mortensen, 2006). Synthetic colors are produced by chemical synthesis and cannot be found naturally. They were originally manufactured from coal tar (Amchova et al., 2015).

Most of the synthetic color have been found to be hazardous to human health, only limited kinds of colors are permitted to be used in food industries in many countries. Recent increasing concern on the use of edible coloring agents has banned various synthetic coloring agents, which have a potential of carcinogenicity and/or teratogenicity (Fabre et al., 1993). Thus, there is need a need to develop alternative sources of natural food colorants. Consequently, there is a growing demand for eco-friendly, non-toxic colorants for industrial application (Velmurugan et al., 2011).

There are number of natural colorants, but only a few available in enough quantity of industrial use, as they are directly extracted from plant flowers, fruits, leaves

and roots (Lauro, 1991). Natural coloring agents from microorganism and fungi such as *Monascus*, *Streptomyces*, and *Serratia* (Trias, 1998), are become interest for pigment production. This circumstance has inevitably increased demands for highly safe and naturally occurring edible coloring agents, which is *Monascus* pigment (Francis, 1987).

2.2 *Monascus* sp.

Over 20 species of *Monascus* have been identified since the genus *Monascus* proposed by French scientist van Tieghem in 1884 (Shao *et al*., 2010; Li *et al.*, 2013). The genus *Monascus* belongs to the phylum *Eumycota*, subphylum *Ascomycotina*, and class *Plectomycetes*, order *Eurotiales. Monascus sp.* belongs to the family *Monascaceae* of the phylum Ascomycecota.

The genus *Monascus* generally divided into 9 species: *M. pilosus, M. ruber, M. purpureus, M. floridanus, M.eremophilus, M. pallens, M. sanguineus, M. lunisporas, and M.argentinensis. Monascus sp* has ability to produce secondary metabolites of polyketide structure which are synthesized by the polymerization of acetyl and propionyl subunits in a similar process to fatty acid synthesis (Juzlova *et al*., 1996). The structures of pigments as secondary metabolites are depend on the substrate types, pH, temperature and moisture content (Patcharee et al., 2007).

2.3 *Monascus purpureus*

Monascus purpureus can produce at least six major related pigments (Rajeswari et al., 2014) the colors are: two are orange (rubropunctain and monascoubrin), two are yellow (monascin and ankaflavin) and two are red (rubropunctaminea and

monascorubramine). Among these, the red pigments are of interest, because red is the most popular food color and true red natural pigments suitable for food use are hard to obtain (Chen at al.,1993). Numerous studies report that microorganisms of the genus *Monascus* produce red pigments, which are used as for coloring some foods and increasing the demand for the highly safe pigments (Lin, 2005). *Monascus* helps to lower blood cholesterol, prevent cancer, osteoporosis, stroke, alzheimer's disease and other dementias and muscular degeneration (Cesar et al., 2005).

The morphology and taxonomy of *Monascus purpureus* has been study to understand more about this microorganism. A research has been done by Rasheva et al (1998) claim that the shape of conidia is oval or pyriform aleiroconidia, single or in a chain up to 3-4. Perithecia (flask shape structure opening by a pore) can be observed with globose in shape and diameter 30-35 μm. For ascospore, it has been identifying has oval with diameter 4-5 μm. The morphology of *Monascus purpureus* slightly difference according to the strains. The color of *Monascus purpureus* itself are orange, white edge, and red with flat in shape. The mycelium is long and white abundant.

2.4 Fermentation

Fermentation has been practiced for centuries in food processing and production of traditional fermented food, particularly in the orient and Asian region. Traditional products such as tempe, tapai, red fermented rice, and wine are produced using the fermentation technique. There are two types of fermentation, namely solid-state fermentation (SSF) and submerged fermentation (SmF). Table 2.1 shows the comparison of SSF and SmF.

Table 2. 1: Comparison between SSF and SmF

Solid State Fermentation (SSF)	Submerged Fermentation (SmF)
• No water or almost no water • (12%-70% moisture) added into the solid substrate. • The final fermented product is in wet state substrate. • The growth of microorganism will be inhibited due to the difficulties in mixing the nutrient to all the the solid substrate. • More suitable for fungi.	• Water is present. • The final fermented product is in liquid state. • The growth of microorganism is maximum because it is easy to mix the nutrient to all the solid substrate. • More suitable for bacteria (require high water activity).

Adapted from: Chen (2013); Nigam and Pandey (2009); Wang and Yang (2007)

2.4.1 Solid state fermentation (SSF)

Solid state fermentation (SSF) has been describe as fermentation process which involves solid matrix and is carried out in absence of free water, but it is necessary for the substrate to have the required moisture for SFF to promote growth of the microorganism. SFF is suitable for limited number of microorganisms, primarily for fungi and yeast because of it is contains low moisture content. Hence, it will reduce the contamination by undesirable growth of unwanted bacteria, and produced more concentrated product (Prabhakar et al., 2005).

Solid state fermentation (SFF) has been proven too has high impact on the bioprocess technology due to its promising application in the production of biologically active secondary metabolites (Thomas et al., 2013). SFF has built up credibility and

appear as a promising alternative to submerge fermentation. Advancement in bioprocess, such as bioremediation, bioleaching, biopulping, biobeneficiation, biological delignification, bioconversion of biomass, and production of value-added products such as enzymes, antibiotics, aroma compounds and organic acids are some examples process involved SFF in development (Singhania et al., 2009)

The potential lies in SSF as the microorganism is culture in close vicinity of substrate and obtains the highest substrate concentration by providing the natural habitat of microorganism. As the SFF resemble the natural environment it enhances the microorganism to grow and produce product in high concentration (Babitha et al., 2007; Cheirsilp et al.,2015; Melikoglu, et al.,2013; Thomas et al., 2013; Velmurugan et al., 2011). It appears that this kind of environment is the reason why the microorganism performs well in SFF compare to SmF. According to Cheirsilp et al (2013) SSF has been widely used especially for culture fungi. SSF give more benefits than SmF because it used smaller bioreactor volume, reduced downstream processing costs, higher productivity, simple technique, reduced energy requirement, efficient conversion of product and low wastewater output. While other research shows that SFF give higher enzymatic activities, higher fermentation productivity, higher end concentration of product and lower catabolic repression (Čertík et al., 2013; Mateos Diaz et al., 2006; Melikoglu et al., 2013; Prabhakar et al., 2005; Singhania et al., 2009; Zhang et al.,2015)

Due to the high cost of the current liquid culture-based fermentation technology has restrained the industrial use of *Monascus* pigments. Hence, there is a growing need for developing low cost production of natural pigments or coloring agents, which could replace synthetic pigments. The *Monascus* pigments can be obtained from both solid state and submerge culture. Solid state fermentation (SSF) has emerged as an efficient

alternative for liquid, culture-based fermentation technology. Solid state fermentation is more preferable than submerge fermentation because the substrates used in SSF supply the basic nutrients to the microorganisms and serve as an anchor for the cells (Subhasree et al., 2011). Interestingly, recent studies report that SSF provides a more adequate habitat for fungi, resulting in high pigment production in a relatively low-cost process when agro-industrial wastes are used as substrate.

2.5 Factors that contribute to the red pigment production

2.5.1 Nutritional factors

2.5.1.1 Nitrogen source

Nitrogen source is essential for growth of microorganisms. Nitrogen source has different type which influences growth, sporulation different pigment produced by *Monascus* sp. If the nitrogen substrate is low, supplementation of nitrogen may require stimulating growth and pigmenting production.

The chemistry of the *Monascus* pigment suggests that the orange pigment forms the red pigment. Yellow pigment is unable to react with NH group to produce corresponding amine. It depends on the cultural conditions and C-N ratio (Gunjan et al., 2011). The addition of external nitrogenous compounds showed a positive impact on water-soluble pigment production. Organic nitrogen sources such as monosodium glutamate (MSG), peptone and some amino acids have been reported to stimulate *Monascus* growth and pigment production, especially the production of the red and yellow pigment (Babhita *et al*., 2006).

2.5.1.2 Carbon source

Carbon source provides energy for growth and secondary metabolites. Besides, it also provides carbon making various cell structures, organic chemicals and metabolite. The most commonly used of carbon sources for growth are glucose, maltose and starch. But in the most studies, glucose is superior as carbon source for pigment production (Lin et al., 1992).

According to Juzlova *et al*. (1996) suggest that glucose concentration should kept at less than 20 g L-1 to prevent the Crabtree effect. Crabtree is defined as excess glucose in the culture medium results in ethanol formation under aerobic conditions (Chen et al., 1994). Crabtree effect also involves a shift and metabolism from aerobic to partly anaerobic even though plenty of oxygen may available (Carvalho *et al*., 2003).

The other evidences show that ethanol and sucrose may be superior to glucose for pigment production as they reduce growth rate compared to glucose. Thus, it makes this result in higher biomass specific pigments production (Juzlova *et al*., 1996). The other carbon sources such as lactose, fructose and xylose have been found to be inferior for growth and pigment production (Lin et al, 1992).

2.5.2 Environmental factors

2.5.2.1 pH

Red pigment production dominated at pH range of 5.5 to 6.5, while if pH higher than 6.5 and lower than 5.5 resulted in a decreased of red pigments. The optimum pH for growth is not the same as that for pigment production. The maximum biomass is obtained at pH of 4.5, while the highest levels of red pigment are obtained at a pH of

6.0, a pH level close to the natural pH of the rice samples soaked in distilled water (Kyu Lee *et al*,. 2002). This phenomenon is attributed to inhibited rice reddening due to drop in the pH level during cultivation. They found that the initial pH of the culture determined the pigment yield, regardless of subsequent changes in the pH. It is suggested that it is because of the pH on the nutrient absorption.

2.5.2.2 Inoculum size

Inoculum size determination is another factor that gives effect to red pigment production. Theoretically, if the inoculum size is too small, this can slow down microorganism growth and lead the contamination by undesirable organism however, if inoculum is too large, this can be produced too much biomass and deplete the substrate, which is necessary for production formation. Based on research done by Lee et al. (2002), the red pigment production saturated at a level of about $1\ x10^4$ spore cells (grams of dry solid substrate) and at level lower than this, the value of the red pigment and biomass decreased in proportion to decrease in the inoculum size.

2.5.2.3 Moisture Content

Moisture contents also a key parameter that can control the growth of microorganism and metabolite production (Pandey, 2003). Selection of initial moisture content depends on the types of microorganism and the nature of substrate. For the fungi usually needs lower moisture, 40 to 60% initial moisture content which more enough but depends on selection of substrate. It also depends on several factors mainly related with cost and availability that may involve the screening of several agro-

industrial residues (Pandey *et al*., 2007). Too high a moisture content will reduce substrate porosity and mass transfer of oxygen. But if too low a moisture content, it reduced accessibility of nutrients to the fungus (Babitha *et al*., 2007).

2.6 Oil Palm Frond (OPF)

Now days the development of oil palm industries in Malaysia is considerably growing. Malaysia is the world's second largest palm oil producer. According to MPOB, Oil palm planted area in 2015 reached 5.64 million hectares, an increase of 4.6% as against 5.39 million hectares recorded in the previous year. In the year 2015 Malaysia has generated crude palm oil approximately 19.96 million tons as against 19.67 million tons produced in 2014. With such a large area for palm trees plantation, no wonder the biggest portion of total agricultural waste in Malaysia comes from oil palm fields. The chopped trunks, dead fronds, empty fruit palm bunches (EPFB), shell and fibers are some examples of waste produce in large amount at the plantation. Thus, OPF can be found abundantly as solid agro-waste on oil palm plantation (Goh et al.,2010). Oil palm fronds (OPF) is the largest biomass which makes up to 70% of the total residues generated from palm oil industry. Until now, there is no proper way to decompose OPF, the OPF is direct decaying in the natural environment or buy burning on site with small amount can be decompose. Thus creating another environmental pollutions, and the alternative ways either to utilize or decompose are needed (Tan et al.,2011). With the enhance development of technology, utilization of OPF for the benefits of society has been discovered by many researchers. The biomass can be utilized for production bioethanol (Lim et al.,2012) ,or use as substrate in fermentation technology which give attractive approach and avoiding pollution problems.

Numerous agro-industrial residues such as jackfruit seeds, palm kernel, rice bran, cassava have been used for pigment production. Yet, no effort has been made so far to utilize oil palm fronds (OPF) as a substrate of pigment production. The average density of oil palm fronds is about 700 kg/m3. The weight of each OPF is between 15 and 20 kg depending on the age and condition of the palm tree. The OPF have two major components which are petiole and leaflets. The dry matter weight ratio of petiole to leaflets (including the rachis) is 1.5 (Sulaiman et al., 2010). Chaney (2010) reported that, typically, the main biomass composition is carbon. It comprises between 30% to 60% of the dry matter. After that, typically 30% to 40% is oxygen. Hydrogen is the third main constituent making up between about 5–6%. Nitrogen and sulfur (and chlorine) normally make up less than 1% of dry biomass. The OPF has reasonably high carbon content which is 42.65% and the content of nitrogen and sulfur are low. The H:C and O:C ratios are 0.13 and 1.17, respectively. Since OPF are rich in carbon content, it can be used as potential substrate to produce food grade pigment.

2.7 Kinetic study

Kinetics is concerned with reaction rates in general, 'process kinetics' simply suggests a primary concern with the rates of commercially practiced reactions and, particularly, with the effects of process variables on them. Since fermentation is another type of chemical process, possibilities for applying kinetics techniques for efficient exploitation of biochemical reaction systems should always be sought (Gaden, 2000). 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.

For this reason, studying the effects of operating conditions on red pigment production and fungal growth are of crucial importance, primarily because such studies can be used to optimize the performance and cost of the process (Infantes et al., 2011).

Microbial growth illustrates the growing of chemical components with existence of suitable medium and the culture environment. Growth of the cell mass or cell number can be defined as a doubling of biomass per unit time for filamentous organism such as fungi.

Batch culture is conducted in closed system that contains an initial limited amount of substrate. The inoculated microorganism will pass through several growth phases. During the log phase, cell numbers are increase exponentially at a constant maximum rate. While in exponential phase, nutrients are abundance and the microorganism are growing at maximum specific growth rate, μ_{max} prevailing conditions.

2.7.1 Batch models

Red pigment is a secondary microbial metabolites (Morales et al.,2016). Pigment is formed simultaneously with the growth of cells. In other words, the pigment concentration increases with cell concentration or growth linked product. Formation of growth linked product can be defined as Equation 2.1:

$$\frac{dp}{dt} = q_p x \qquad \textbf{Equation 2. 1}$$

Where, p is the concentration of product, q_p is the specific rate of product formation (mg product/g biomass/h). Product formation can also be expressed in relation to biomass as Equation 2.2:

$$\frac{dp}{dt} = Y_{p/x} \qquad \textbf{Equation 2. 2}$$

Where, $Y_{p/x}$ is the yield of product in terms of biomass (g product/ g biomass). A product formation model is proposed as given in Equation 2.3.

$$q_p = Y_{p/x}.\mu \qquad \textbf{Equation 2. 3}$$

In addition to this, Monod also related the yield coefficient $Y_{X/S}$ (Equation 2.4) to the specific rate of biomass growth (μ) and the specific rate of substrate utilization (q) (Equation 2.5) (Okpokwasili et al., 2005).

$$Y_{X/S} = \frac{dx}{ds} \qquad \textbf{Equation 2. 4}$$

$$\mu = \frac{Y_{X/S}}{X}.\frac{ds}{dt} \cong Y_{X/S}.q \qquad \textbf{Equation 2. 5}$$

In batch fermentation of pigment production, substrate is used for cell growth and maintenance as well as for pigment production. The substrate utilization rate equation can be written as shown in Equation 2.6.

$$\frac{dS}{dt} = -\frac{1}{Y_{xs}}\mu X = -\frac{1}{Y_{xs}}\frac{\mu_m S}{K_S+S}X \qquad \textbf{Equation 2. 6}$$

Where, Y_{xs} is the biomass yield coefficient. The biomass yield coefficient is the efficiency of conversion of substrate to biomass and can be calculated as in Equation 2.7.

$$\text{Biomass} = \frac{Dry\ weight\ of\ biomass\ produced}{Weight\ of\ substrate\ used} \qquad \textbf{Equation 2. 7}$$

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

Figure 3.1 shows the summary of overview process applied in this study. The process involved three steps, which are sample preparation, fermentation process and sample analysis.

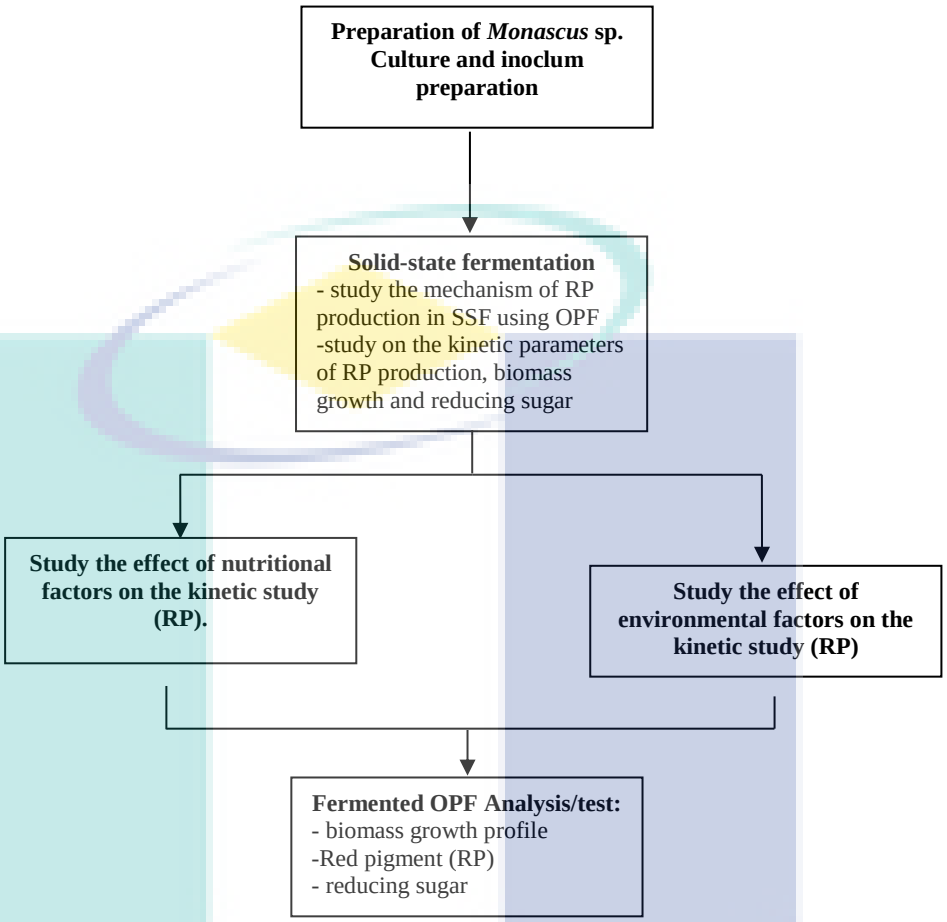

Figure 3. 1: Process flow diagram

3.2 Chemicals

Sodium hydroxide (NaOH), 95% Ethanol, Potato Dextrose Agar, Peptone, Acetyl Acetone, Ehrlich Reagent, Sulphuric Acid, Hydrochloric Acid.

3.3 Procedure

3.3.1 Culture

Monascus purpureus FTC 5336 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 (Dikshit and Tallapragada, 2011; Said, FBM 2010). 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.

3.3.2 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 (Dikshit and Tallapragada, 2011). The spore suspension is carried out in sterile universal bottles which contain medium inoculated. Then, haemocytometer is used to calculate spore suspension (spores/ml) before transferred to fermentation medium.

3.3.3 Substrate

Petiole oil palm fronds (OPF) are obtained from local agricultural fields. The substrate is dried in sunlight before grinding into smaller pieces. OPF is ground to 1mm of size of particle using grinder.

3.3.4 Solid state fermentation (SSF)

A 5 gram of substrate is placed in 250ml conical flasks, and then the substrate is autoclaved at 121°C for 10 minutes. After cooling, substrate–based medium is inoculated with seed culture of *Monascus purpureus* and incubated at 30°C. There are 5 parameters involved, there are nitrogen source (1 % - 5 %), pH (pH 4-pH 8), moisture content (40% - 60%) and inoculum size ($10^5 - 10^9$) spores ml^{-1}. The pH is adjusted with 1 M NaOH to 6.0, 50 % moisture content and 2% of peptone are fixed while the other factors are being investigated. Samples were taken every day for 10 days. The fermentation is run for 10 days after inoculation at 30°C to finish the cycle (Dikshit and Tallapragada, 2011). The samples are made triplicate.

3.4 Assay Method

Three types of assay method were performed to analyze red pigment production, glucose concentration and biomass production (cell dry weight).

3.4.1 Pigment extraction

Fermented substrate is dried in oven at 60°C for 24 hours. 1 grams of fermented substrate is taken for pigment extraction using 10 ml of 95% ethanol and shakes in incubator shaker 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.2 filter paper (Dikshit and Tallapragada, 2011). Ethanol extract of unfermented substrate is kept as blank. The

pigment production is measured at wavelength of 500nm (red color). The absorbance values are converted into pigment units (AU/g) in Equation 3.1.

$$\boldsymbol{Color\ value\ unit = OD \times \frac{Dilution\ volume\ of\ extract\ (ml)}{Weight\ of\ sample\ (g)}} \qquad \textbf{Equation 3. 1}$$

3.4.2 Cell dry weight

0.5 g of dried fermented solids powder is mixed with 2 mL of 60% (vol/vol) sulfuric acid and the mixture is kept at 25℃ for 24 h. Next, the mixture is diluted with distilled water to make 1 N solution of sulphuric acid and autoclaved. Then left the mixture to cool at room temperature and neutralized with 5 N NaOH to pH 7 and the final volume was made up to 60 mL with deionized water. The diluted solution was filtered through a 0.45 μm filter. 1 mL of filtered diluted solution was mixed with 1 mL of acetyl acetone reagent is freshly prepared as it was stable only for 2-3 h at 18℃. The mixture is then held in a boiling water bath for 20 min. after cooling to room temperature, ethanol 95% (6 mL) is added followed by 1 mL of Ehrlich reagent (2.67% (w/v) of p-dimethylaminobenzaldehyde in 1:1 mixture of ethanol and concentrated hydrochloric acid. The mixture is then being incubated in a water bath at 65℃ for 10 minutes. After cooling, the optical density is read at 530 nm against the reagent blank using UV visible spectrophotometer. The blank is prepared with the same method using unfermented OPF (Said F.B.M., 2010). The cell dry weight was calculated as in Equation 3.2.

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

$$\frac{\boldsymbol{Abs_{530}}}{\boldsymbol{0.0669}} \text{ x } \frac{\boldsymbol{total\ volume\ of\ mixture\ (mL)}}{\boldsymbol{sample\ volume\ (mL)}} \text{ x } \frac{\boldsymbol{60\ mL}}{\boldsymbol{0.5\ g}} \text{ x df x } 10^{-3} \text{ x } \frac{\boldsymbol{1}}{\boldsymbol{cf}} \qquad \textbf{Equation 3. 2}$$

CHAPTER 4

RESULTS AND DISSCUSSION

4.1 Introduction

In solid state fermentation, various factors such as nutritional and environmental factors play vital role to the biosynthesis of the fungal growth and the development of by-product. The production of red pigment by *Monascus purpureus* can be affected by several conditions. It can be either from environmental factors or nutritional factors. Factors that falls under nutritional are carbon and nitrogen sources, whereas for environmental factors are pH level, moisture content and inoculum size.

4.3.2 Effect of moisture content on pigment, biomass and glucose concentration

Kinetics of growth and product formation in solid state fermentation for moisture content effect was examined at a fixed 5g OPF, pH 6, 10^8 (spores/ml) inoculum size, 2% peptone (nitrogen), while moisture content used were manipulated from 40% to 60%. The samples were taken-out daily and were made in triplicate.

The optimum moisture content is one of the most crucial factors that lead to determine the yield of red pigments in fermentation. The results are recording in Figure 4.13 illustrates the color value for each moisture content range from 40% to 50% for 7 days. The moisture content affected the production of the red pigment. Lower moisture

levels adversely affected pigment production (Fig 4.1) along with biomass concentration (Fig 4.2). Decreasing the moisture to 40% reduce the pigment production to 2.204 AU/g at day 6.

According to Lee et al., (2002) the mycelium of *Monascus* should permeate inside the substrate and then use it to manufacture pigments. Based on Figure 4.1 and Figure 4.2 the peak production of red pigment and biomass was attained at moisture content of 50%, this is parallel with finding by Lee et al (2002). No further increase in pigment production with increasing moisture content.

At moisture content 60%, the red pigment production decreased drastically. High moisture content causes the particles of substrate move further from each other, make it more difficult to produce pigments. This aggregation of substrate particles resulted in oxygen starvation and poor distribution of mycelia, therefore resulting in poor pigment production (Lee et al.,2002).

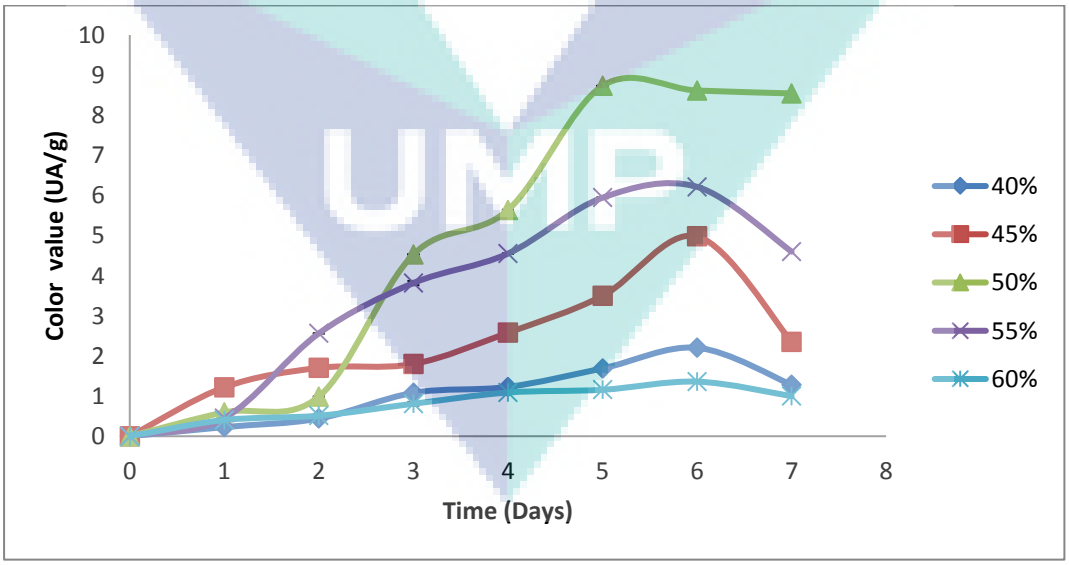

Figure 4. 1 Effect of moisture content on pigment production

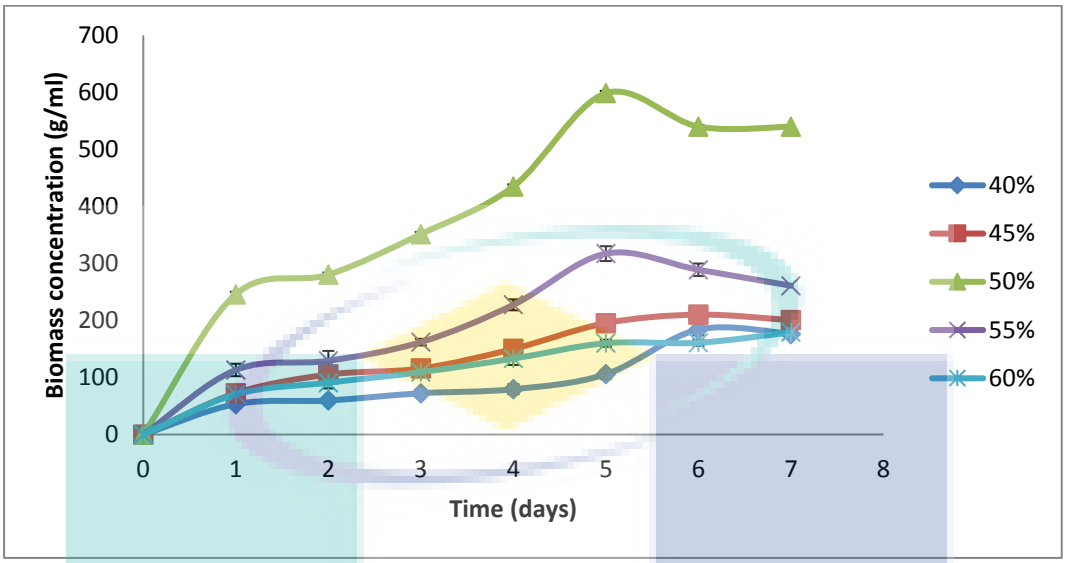

Figure 4. 2 **Effect of moisture content on biomass concentration**

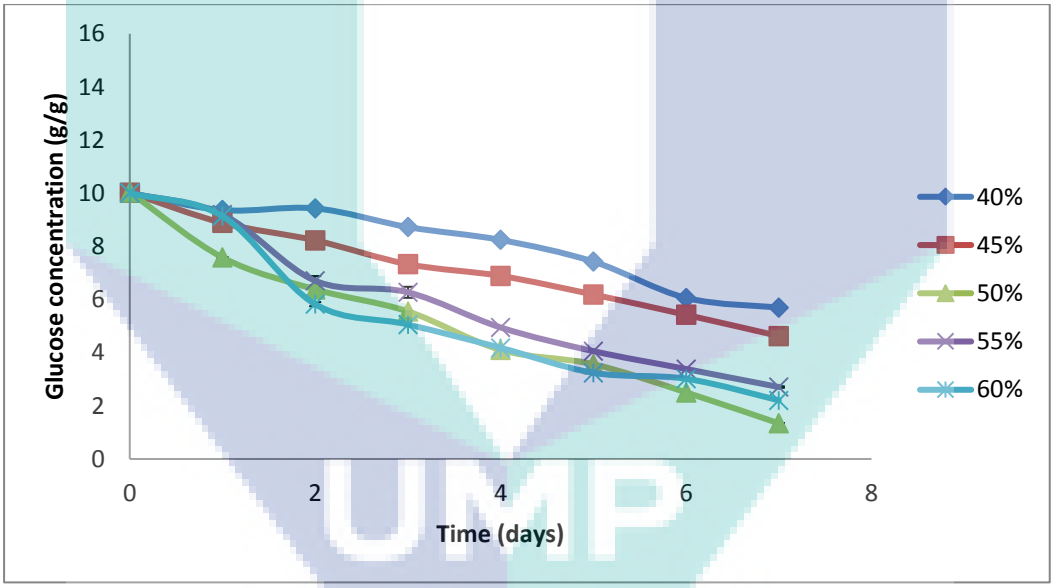

Figure 4. 3 **Effect of moisture content on glucose concentration**

4.2 Nutritional factors

4.2.1 Effect of nitrogen on pigment, 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, 10^8 (spores/ml) inoculum size, 5g of OPF (carbon), while peptone (nitrogen) used were manipulated from 1% to 5% of peptone concentration. The samples were taken-out daily and were made in triplicate.

Figure 4.4 illustrates the colour value for each concentration of peptone range from 1% to 5% for 7 days. The higher red pigment production was obtained with 2% peptone, no further increased 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). While the other concentration showed the slow progress and low pigment production.

Figure 4.4 and 4.5 show the influence of different concentration nitrogen sources on pigment and biomass concentration. Generally, the biomass growths for nitrogen follow 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.

Figure 4.6 shows the reduction of glucose concentration throughout the fermentation process.

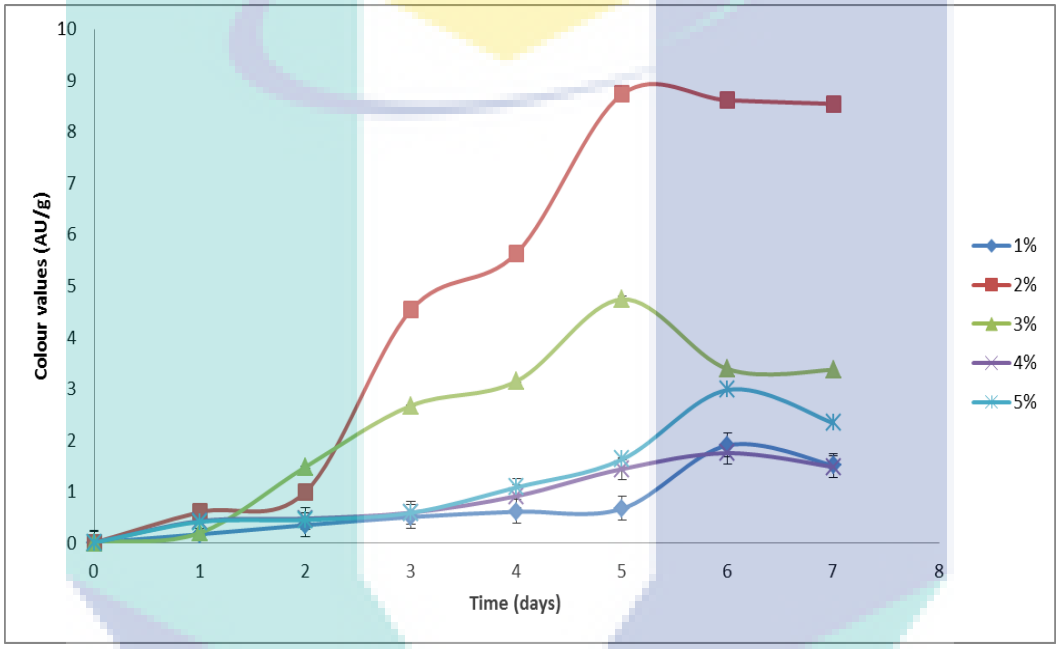

Figure 4. 4 Effect of nitrogen on pigment production

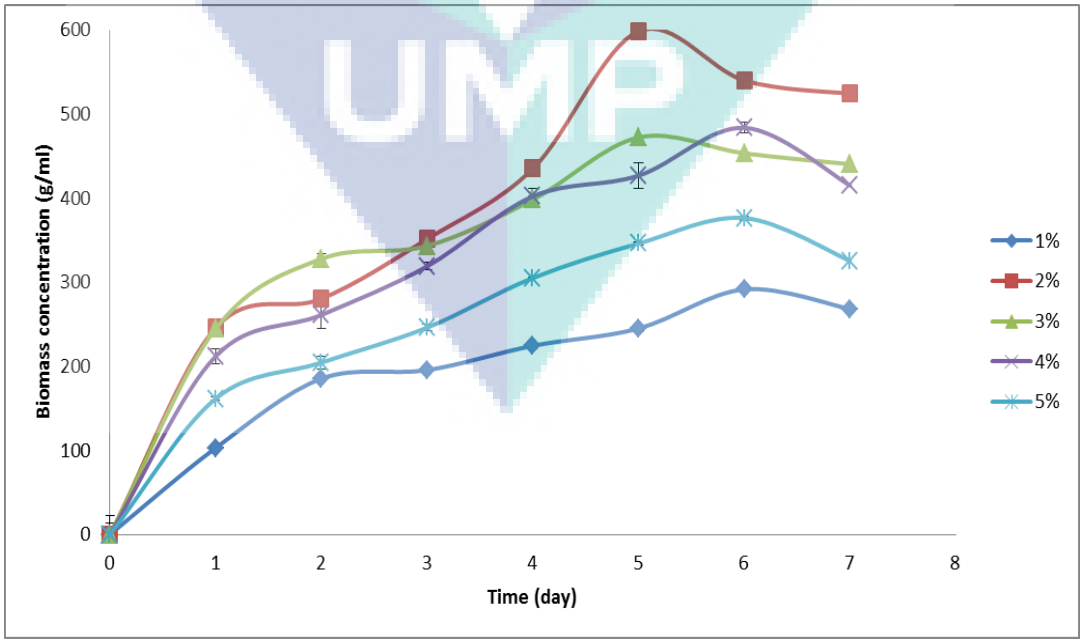

Figure 4. 5 Effect of nitrogen on biomass concentration

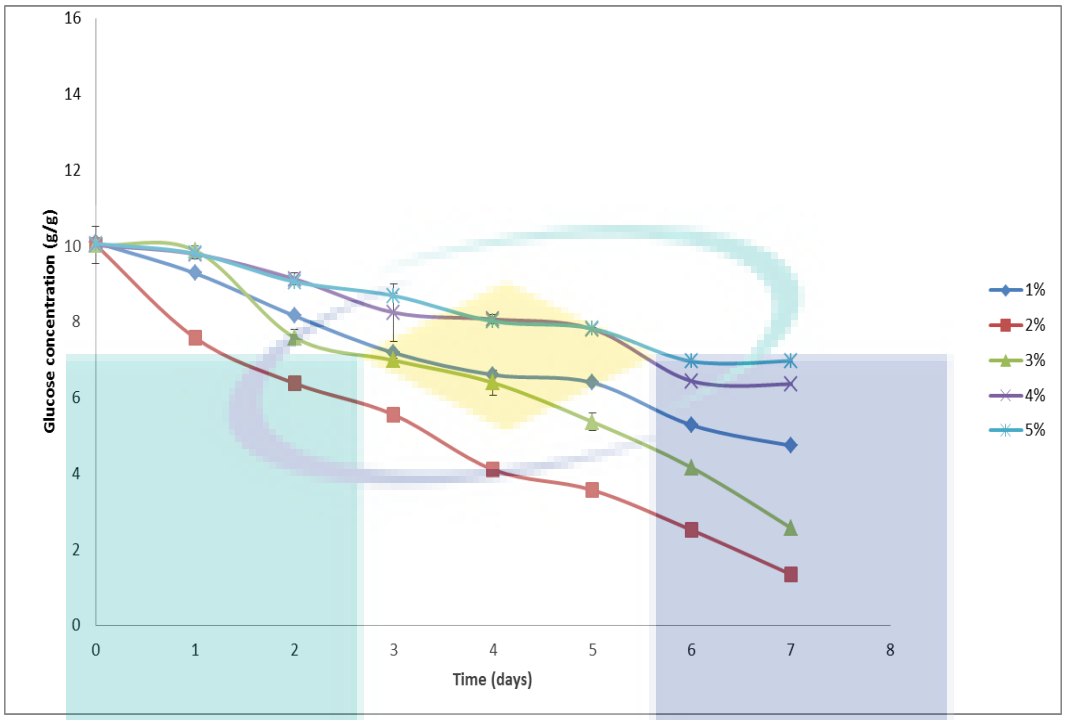

Figure 4. 6 Effect of nitrogen on glucose concentration

Figure 4.6 shows the trend of glucose concentration throughout the fermentation process. The glucose trend was continuously dropped throughout the fermentation period. However, only at 2% and 3% peptones showed a constant declined and were found to be dependent to the fungal growth (Figure 4.5). Where, the maximum biomass obtained at 600 g/ml and 480 g/ml, by 2% and 4%, respectively. The peaked biomass was found on day 5 and 6, for 2% and 4% peptone, respectively. These phenomena demonstrated the consumption of glucose simultaneously from OPF for the growth of fungi. Shortly after the glucose decreased and biomass increased, approaching the exponential phase (Figure 4.5). Higher amount of peptone (4% and 5%), did not accelerate the glucose consumption, instead slow-down the process. It was due to the difficulty of the fungi to assimilate the glucose.

Figure 4.5 shows that biomass concentration for 2% peptone constantly increased throughout the fermentation period. Initial biomass concentration 245.85

g/ml at day 1 was increased to 598.50 g/ml at day 5. While initial glucose concentration 10.03 g/g at day 1 was 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, approaching the exponential phase of the fungi. From day 4 of fermentation it was possible to observe an increased in the production of pigment (Figure 4.4) 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.

Substrate with minimal percentage of peptone (2%-3%) had showed a high value of pigment production however high percentage of peptone will deplete the pigment production. This may due the high concentration of peptone may create high osmotic pressure to the *Monascus* fungi, thus inhibit pigment production. Low concentration of peptone seemed to be effective to promote the production of *Monascus* pigment. According to Shi et al (2015), peptone was more favourable nitrogen source for the growth of *Monascus* and the pigments production when compared to the other two nitrogen sources (ammonium and nitrites) and glucose exhausted more quickly than the other two conditions.

4.2.1.1 Kinetic study of peptone on red pigment

The result from Table 4.1 and Table 4.2 demonstrate the kinetic parameters for various peptone concentrations.

Table 4. 1 Kinetic parameters of at various concentration of peptone

Kinetic parameters	Peptone concentration % (w/w)				
	1%	**2%**	**3%**	**4%**	**5%**
μ_{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 was higher for 2% (119.67 mg $ml^{-1}day^{-1}$), followed by 4% peptone (80.61 mg $ml^{-1}day^{-1}$). While the averages yield of pigment were 1.747 $AU_{500}\, day^{-1}$ and 0.948 $AU_{500}\, day^{-1}$ for 2% and 3% peptone respectively.

The data of specific growth rate (μ_{max}) , mean cell activity (P_{cells}), maximum pigment production and average yield of pigment(P_M) for nitrogen are shown in Table 4.1. 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 4. 2 Conversion factors for nitrogen (peptone)

Kinetic parameters	Nutritional factors: Nitrogen (peptone)				
	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})	6.5x10^3	1.5x10^2	1x 10^2	3.6x10^3	7.9x10^3

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.

4.2.2 Effect of carbon on pigment, biomass and glucose concentration.

Kinetics of growth and product formation in solid state fermentation for carbon effect was examined at a fixed pH 6, 50% initial moisture content, 10^8 (spores/ml) inoculum size, 2% peptone (nitrogen), while oil palm frond (OPF) as carbon source used were manipulated from 3g to 7 g of carbon concentration. The samples were taken-out daily and were made in triplicate.

The effect of various carbon concentration used on the red pigment production was studied by measuring the absorbance at 500nm as illustrated in Figure 4.7. Sample with 5g of OPF was found to give the maximum yield of 8.73 AU/g at day 5. Next to 5g, 4g OPF yield 4.35 AU/g also at day 5. Too high of carbon concentration did not improvise good pigment production, the pigment value at day 6, sample with 7g OPF produced with the least pigment yield of 1.47 AU/g when compared with other carbon concentration. Lag phase changed to log phase faster for 5g OPF (within 5 days),

compared with 7g (within 6-7 days). High concentration of carbon consumes more time on lag phase, indicating that there may be a restricted nutrient that makes less favorable for fungi growth. Exponential phase by 5g of OPF at day 5 (Figure 4.8) showed that hyphae branches are initiated, and then the new hypha extends at a linear rate into uncolonized regions of substrate. Figure 4.8 and Figure 4.9 show the trend of biomass and glucose concentration throughout the fermentation process

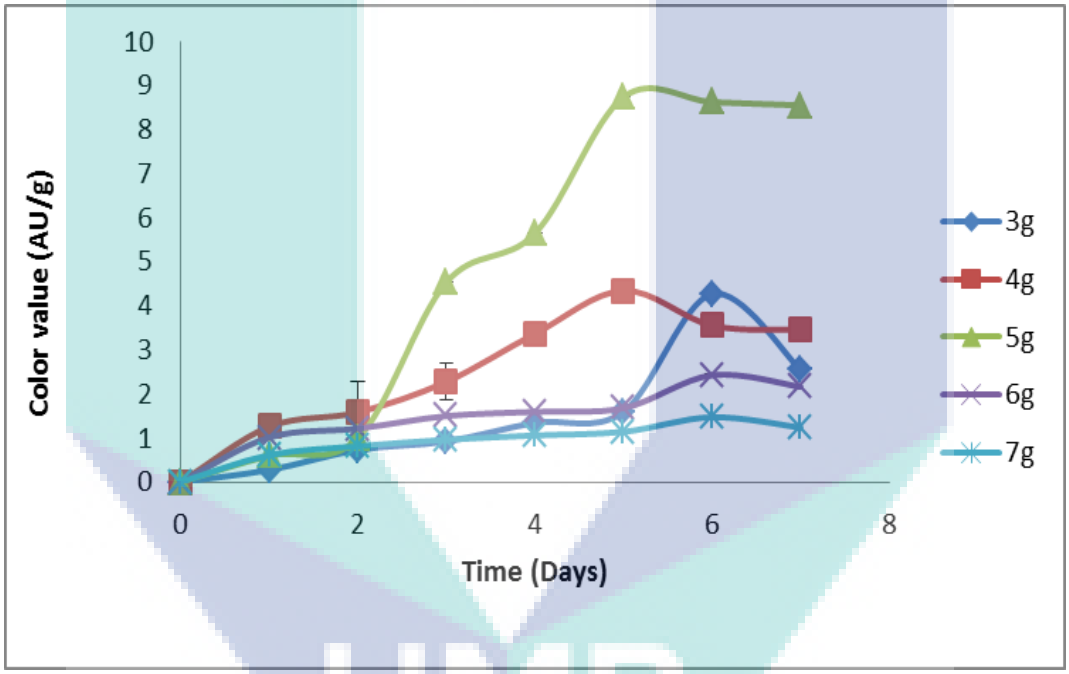

Figure 4. 7 Effect of carbon on pigment production

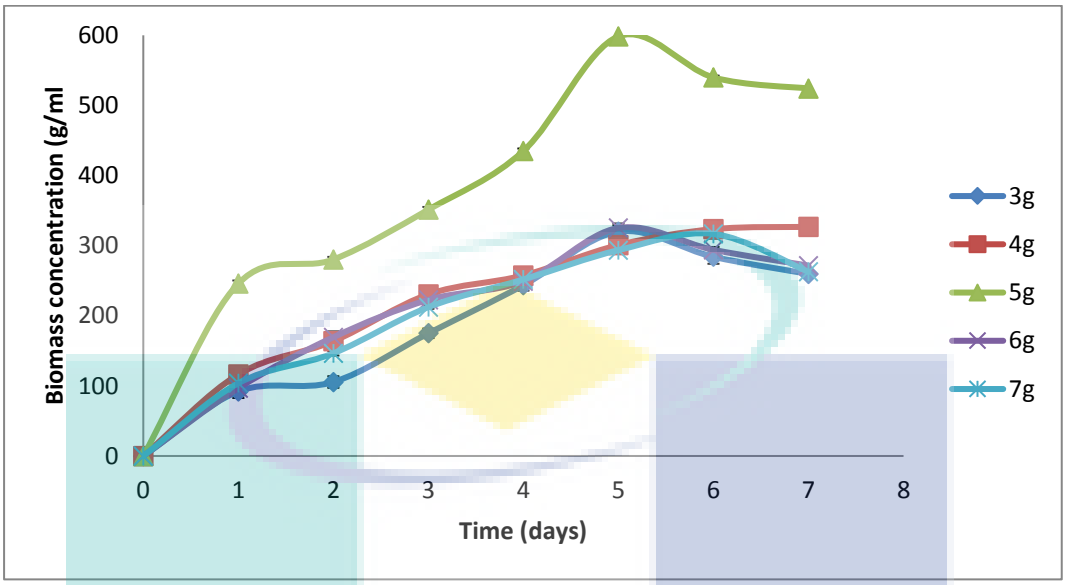

Figure 4. 8 Effect of carbon on biomass concentration

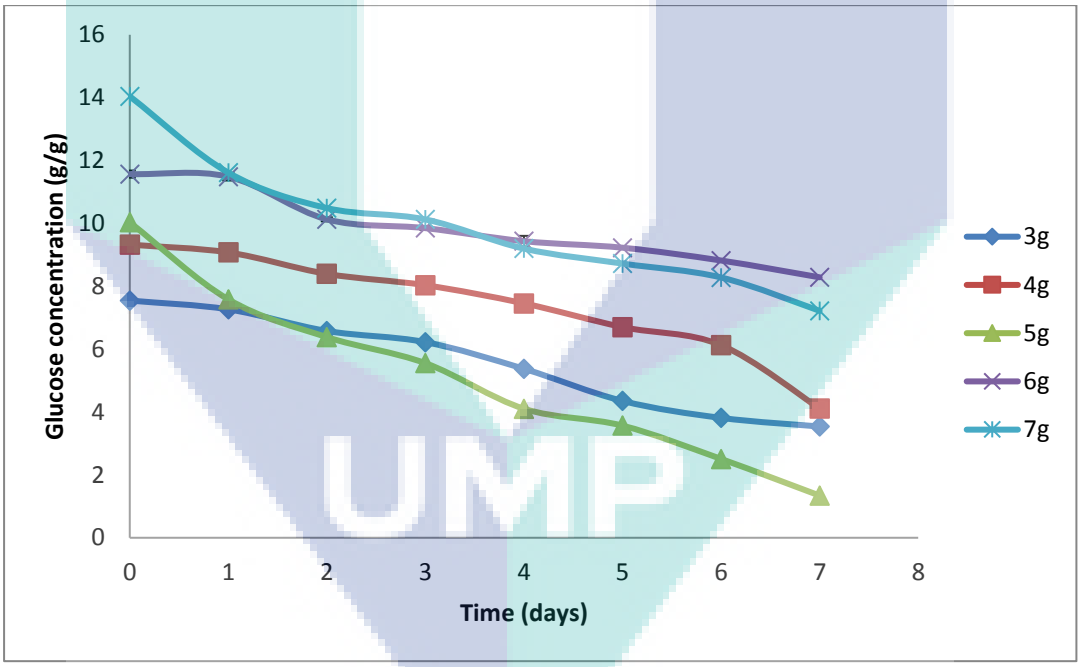

Figure 4. 9 Effect of carbon on glucose concentration

Figure 4.8 illustrates that the biomass of the growing fungus in all samples. Samples with 5g OPF were increased per unit time and reached its maximum biomass of 598.50 g/ml at day 5. This is true as the nutrients are in excess, growth remains

constant during the exponential phase. Biomass trends for all samples followed the growth profile, proved that fungi were adjusting and growing in all samples.

Figure 4.9 shows the initial glucose concentration were varies as the concentration were differ at the beginning. The optimum amount of carbon enhances the production the production of pigment while too high inhibit the growth and less utilization of glucose.

4.2.2.1 Kinetic study of carbon on red pigment

The result from Table 4.3 and Table 4.4 demonstrated the kinetic parameters for various carbon concentrations. Table 4.3 shows the kinetic data in the production of pigments for carbon factors maximum growth rate (μ_{max}), maximum pigment production (AU_{500}) and mean cell (P_{cells}) and pigment (P_M) productivity.

Table 4. 3 Kinetic parameters for carbon

Kinetic parameters	**Nutritional factors: Carbon**				
	3g	**4g**	**5g**	**6g**	**7g**
μ_{max} (day^{-1})	0.190	0.116	0.118	0.113	0.132
P_{cells} (mg $ml^{-1}day^{-1}$)	47.34	53.92	89.98	65.05	52.79
Maximum pigment production (AU_{500})	4.266	4.338	8.732	2.422	1.467
$P_M(AU_{500}\, day^{-1})$	0.711	0.590	1.436	0.334	0.244

Specific growth rates ranged from 0.113 day^{-1} to 0.190 day^{-1}, where the highest rate was observed using 3g OPF and the lowest using 6g OPF. The average cell

productivity (P_{cells}) was higher for 5g (89.98 mg $ml^{-1}day^{-1}$) , followed by 6g (65.05 mg $ml^{-1}day^{-1}$). While the higher values for average yields of pigment (P_M) were 1.436 $AU_{500}\,day^{-1}$ and 0.711 $AU_{500}\,day^{-1}$ for 5g and 3g OPF respectively. Carbon does help in microbial growth based on result of specific growth rate (μ_{max}) but when high carbon concentration was used it does not contribute to the high pigment production. Pigment production for 7g was 1.46 AU/g which is very low value than 5g, 8.73 AU/g.

Table 4.4 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 4. 4 Conversion factors for carbon

Kinetic parameters	**Nutritional factors: Carbon**				
	3g	**4g**	**5g**	**6g**	**7g**
$Y_{X/S}$ (g g^{-1})	76.09	101.2	71.72	97.57	36.96
$Y_{P/S}$ ($AU_{500}\,ml\,g^{-1}$)	1.142	1.108	1.144	0.715	0.255
$Y_{P/X}$(AU_{500} $ml\,g^{-1}$)	0.015	0.011	0.027	0.003	0.004

The highest conversion of substrate into biomass ($Y_{X/S}$) was 4g OPF. While for conversion of substrate into red pigments ($Y_{P/S}$) and the highest conversion biomass into red pigments ($Y_{P/X}$) was 5g OPF.

4.3 Environmental factors

4.3.1 Effect of pH on pigment, biomass and glucose concentration.

Kinetics of growth and product formation in solid state fermentation for pH effect was examined at a fixed 5g OPF, 50% initial moisture content, 10^8 (spores/ml) inoculum size, 2% peptone (nitrogen), while pH used were manipulated from pH 4 to pH 7. The samples were take-out daily and were made in triplicate.

Figure 4.10 illustrates the colour value for each concentration of pH range from pH 4 to pH 8 for 7 days. The higher red pigment production was obtained with pH 6, no further increased in pigment production with increasing pH concentration. Based in result, pH 6 produced the maximum red pigment obtained was at day 5 (8.733 CVU/g), while with pH 7, the maximum pigment obtained was at day 6 with lesser value, 6.712 AU/g. This is supported by research done by B. Kang et al., (2013), at pH 6 a relatively higher concentration of extracellular red pigments due to the red pigment derivates was an ionizable compounds. Too low of pH concentration did not improvise good pigment production, the pigment value at day 6 for pH 4 was 1.306 AU/g. According to Shi et al (2015), when compared pH 2.5 and pH 4, pH 4 produced more pigments and glucose depleted rapidly indicating that red pigments production start to dominant at this pH. However, higher red pigments production was observed at pH 6.5 with absorbance value of 510nm.

Data showed that lower pH concentration did not high effect on growth and pigment productions. *Monascus purpureus* survives at a wide range pH from pH 4 to pH 8. Figure 4.11 displays the effect of pH on biomass, while Figure 4.12 illustrates effect of pH on glucose.

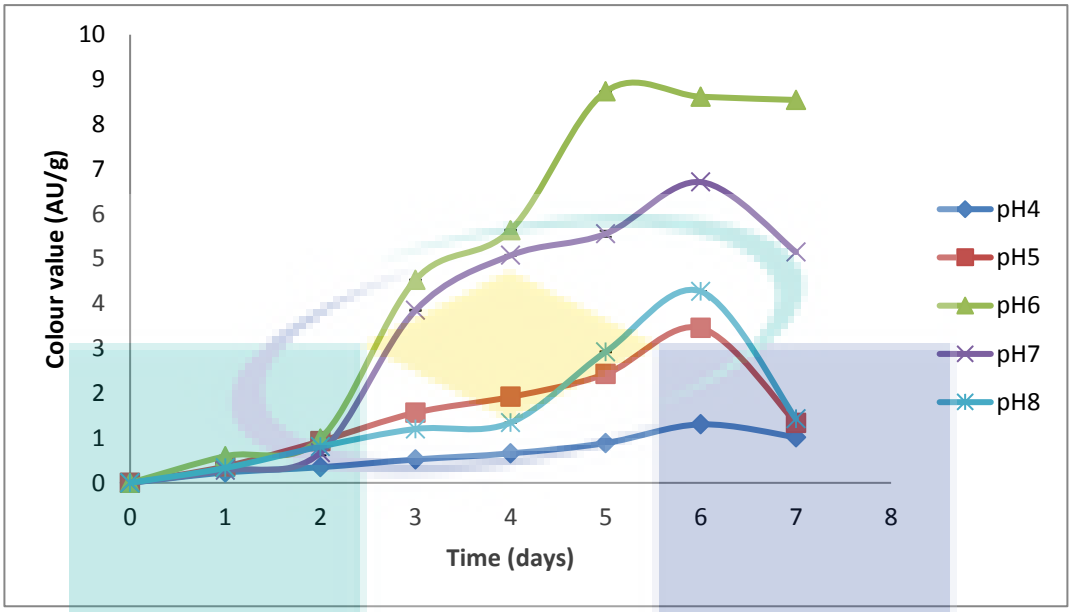

Figure 4. 10 Effect of pH on pigment production

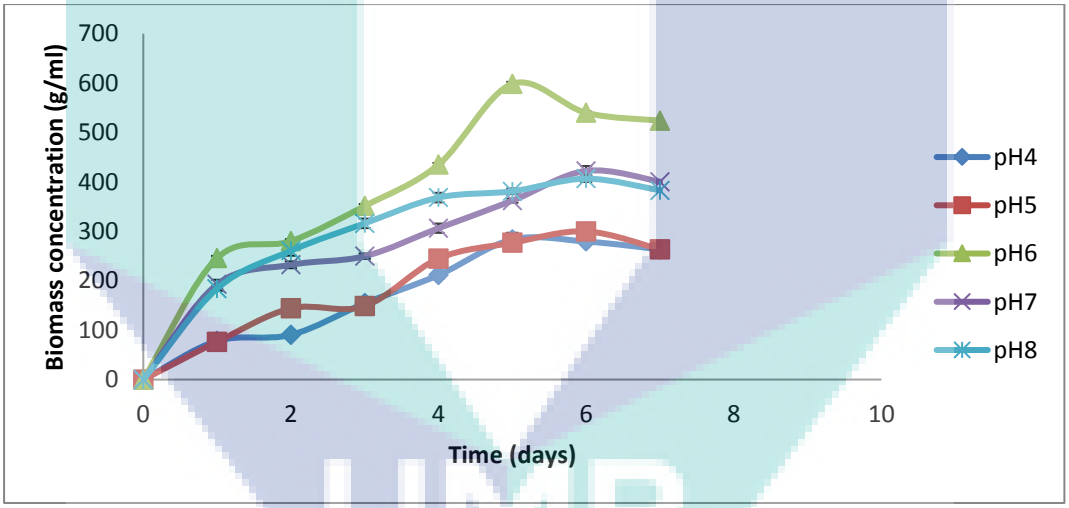

Figure 4. 11 Effect of pH on biomass concentration

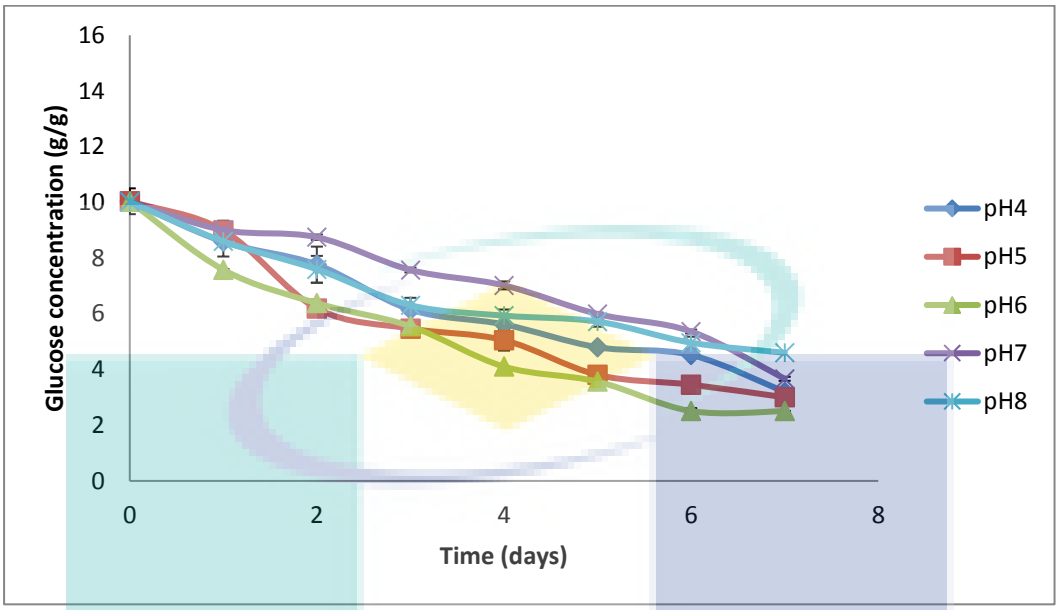

Figure 4. 12 Effect of pH on glucose concentration

Initial stage for all pH showed a slow increase process and started more rapid at day 3 in terms of pigments production. While biomass concentrations for all pH were constantly increased from day 1, the glucose consumptions were constantly decreased. Pigments concentration and biomass exhibited similar trends for pH 6, they rose rapidly to a high value in first 4 days, and then slowly down reaching the peak value at day 5.

4.3.2 Effect of inoculum size on pigment, biomass and glucose concentration.

Kinetics of growth and product formation in solid state fermentation for inoculum size effect was examined at a fixed pH 6, 50% initial moisture content, 5g of OPF (carbon), 2% peptone (nitrogen), while inoculum size used were manipulated from 10^6 to 10^{10}(spores/ml) inoculum size. The samples were taken-out daily and were made in triplicate. Figure 4.13 illustrates the colour value for each inoculum size range from 10^6 to 10^{10}(spores/ml) for 7 days. The highest red pigment production was obtained with 10^8(spores/ml) inoculum size at day 5. The pigment production showed reduction

with increasing inoculum size. Low inoculum size produces lower pigment but not much different if compared with high inoculum size. Data showed that increasing the inoculum size did not affect much on the growth and pigment production much, but it lengthens the lag phase. 10^8(spores/ml) inoculum size change lag to log phase faster (within 5 days), while the other inoculum sizes show slow progress and low pigment production.

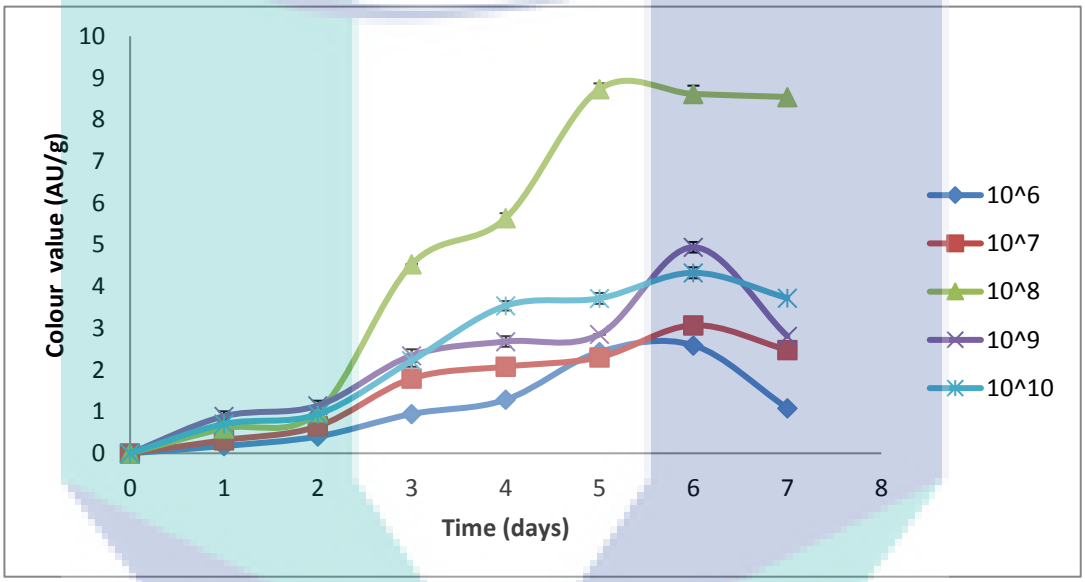

Figure 4. 13 Effect of inoculum size on pigment production

Figure 4.14 and Figure 4.15 show the changes of biomass and glucose concentration throughout the fermentation process. From Figure 4.14 shows that biomass concentration for $\mathbf{10^8}$(spores/ml) constantly increased throughout the fermentation period. Initial biomass concentration 245.85 g/ml at day 1 was increased to 598.50 g/ml at day 5. While initial glucose concentration 10.03 g/g at day 1 was decreased to 2.51 g/g at day 5, demonstrating the consumption of the fungi to grow and produced pigment. Comparing the biomass concentration for low and high inoculum size were not much different, indicating that there may be a restricted nutrient that makes less

favorable for fungi growth if inoculum is too low or high. According to Lee at el., (2000) if the inoculum size is too small, this can slow growth of the fungi and may lead to contamination. If the inoculum is too large, the fungi will produce too much biomass and depleted the substrates, that are necessary for pigment production.

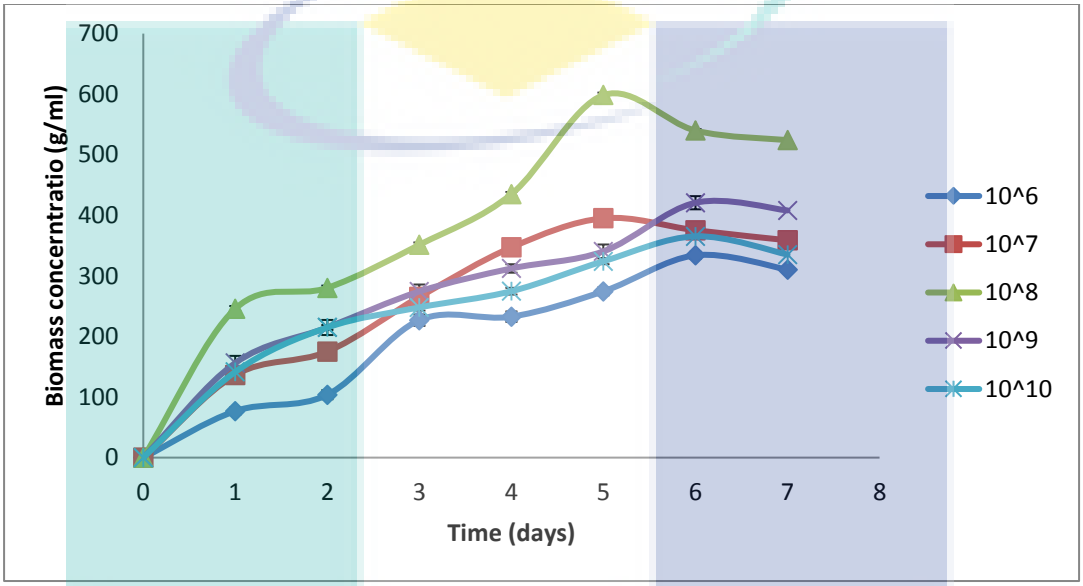

Figure 4. 14 Effect of inoculum size on biomass concentration

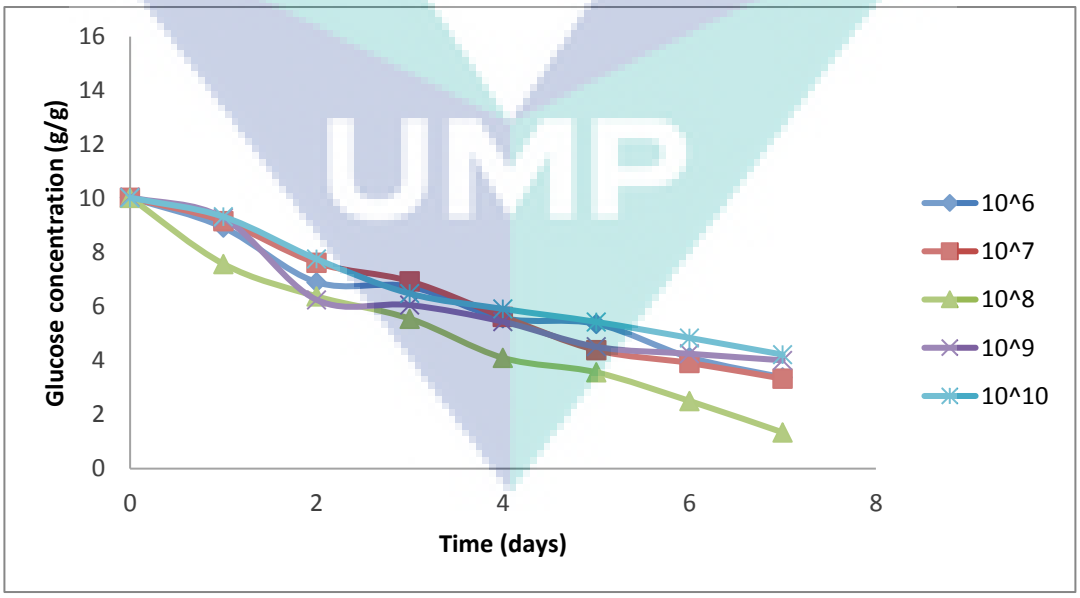

Figure 4. 15 Effect of inoculum size on glucose concentration

4.4 Summary

All the parameter tested supported the growth of fungi and pigment production. The Monascus pigment production appeared to be growth associated as reported by Said (2010). The carbon source that contained 5g of substrate is preferable for fermentation. Substrate that was supplied with 2% peptone proved best for growth and pigment development. The most suitable pH for pigment growth was pH6. 50% proved to best initial moisture content.

CHAPTER 5

CONCLUSION

5.1 Conclusion

This work investigated the kinetic study of red pigment production in solid state fermentation. Sample with 5g OPF, 2% of peptone pH 6, 50% initial moisture content, and 10^8 (spores/ml) inoculum size proved to be most effective for pigment production in nutritional and environmental factors.

For all conditions, either nutritional or environmental, the red pigment production was found to be growth associated product. Based on kinetic value, the best for each parameter for specific growth rate, the average cell productivity (P_{cells}), and average yields of pigment (P_M) has been discussed in Chapter 4.

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ATTACHMENT (PUBLICATIONS)

OPTIMIZATION OF RED PIGMENT PRODUCTION BY *MONASCUS PURPUREUS* FTC 5356 USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT: Factors such as environmental conditions and nutrients are significant for successful growth and reproduction of microorganisms. Manipulations of the factors are the most effective way to stimulate the growth of the microorganism, which can be used to optimize the yield of a product. In this study, Central Composite Design (CCD) of Response Surface Methodology (RSM) was used to optimize the production of red pigment by *Monascus purpureus* FTC 5356 using the petioles of oil palm fronds (OPF) as a substrate in solid state fermentation (SSF). The data was analyzed using Design Expert Software. The optimum combination predicted via RSM was confirmed through experimental work. The interactions between three variables such as initial moisture content (%), initial pH value (pH), and peptone concentration (%) were studied and modelled. The statistical analysis of the results showed that the optimal conditions for red pigment production 47 AU/g with the biomass of 425.1 mg/g was at 55% initial moisture content, 3% of peptone, and at pH 3. The RSM results showed that the initial pH value had a significant effect on red pigment production (P-value <0.05). The validation of these results was also conducted by fermentation with predicted conditions and it was found that there was a discrepancy of 0.39% between the values of the experimental result and those of the predicted values.

ABSTRAK: Keadaan persekitaran dan nutrien merupakan faktor-faktor penting dalam pertumbuhan mikroorganisma. Manipulasi faktor-faktor tersebut adalah kaedah terbaik bagi meningkatkan pertumbuhan mikroorganisma dan mengoptimumkan penghasilan produk. Kajian ini mengguna pakai Rekaan Gabungan Pusat (CCD) melalui Kaedah Tindak balas Permukaan (RSM) bagi penghasilan pigmen merah optimum oleh *Monascus purpureus* FTC 5356 menggunakan batang pelepah kelapa sawit (OPF) sebagai perumah dalam proses penapaian pepejal (SSF). Data telah dianalisis menggunakan perisian Design Expert. Gabungan parameter optimum seperti cadangan RSM telah disahkan secara eksperimen. Interaksi antara tiga pemboleh ubah seperti kandungan lembapan awal (%), nilai pH awal (pH), dan kepekatan pepton (%) telah dikaji dan dimodelkan. Analisis statistik menunjukkan penghasilan optimal pigmen merah adalah pada 47 AU/g dengan biomas sebanyak 425.1 mg/g, pada 55% lembapan awal, 3% pepton dan pada pH 3. Hasil keputusan RSM menunjukkan pH awal memberikan kesan signifikan kepada penghasilan pigmen merah (nilai P <0.05). Pengesahan analisis juga telah dijalankan melalui proses penapaian dan hasil ujikaji mendapati 0.39% lebih tinggi daripada nilai jangkaan.

KEYWORDS: *response surface methodology; red pigment; oil palm frond; Monascus pigment*

1. INTRODUCTION

In recent years, colorants have been extensively used in the food industry. However, to overcome the unlimited usage of synthetic pigment, which is found to be hazardous and toxic to human health, the development of alternate sources for the production of natural pigment has been focused on. Nowadays, productions of pigment from microbial origin have attracted more attention from the food industry. Particular focus has been given to *Monascus* sp., which is a nontoxic fungi that has been widely used as a natural colorant and food additive in East Asia. *Monascus* pigment can produce three groups of pigment: orange, red, and yellow. Among these pigments, the red pigment is gaining high market demand for its use [1].

It is important to study the effect on the red pigments produced by *Monascus* sp. under different culture conditions, for the safe and successful application in food and pharmaceutical industries [2]. Previous study was done on the usage of petioles and leaflets of oil palm frond (OPF) as a substrate [3]. The finding revealed that 100% petiole rendered the best results. Thus, the goal of this study was to optimize the most significant of the multivariable factors for substrates made solely of petiole, in order to influence red pigment production. Factors observed include initial moisture content, peptone concentration, and initial pH value.

The traditional 'one factor at a time' (OFAT) approach used for optimizing a multifactor system is not only effort and time consuming, but also often misses in representing the interaction effect between different factors [4]. However, OFAT could be used as a preliminary experiment to set the range of the factor efficiently, making the results more reasonable and credible [5]. Therefore, the traditional approach of OFAT still can be applied. The range of factors obtained in OFAT can be used by adopting a statistical approach, such as response surface methodology (RSM), to solve the complexity involved in red pigment production. Recently, many types of statistical experimental design methods have been discovered for optimization [6-8]. Among them, RSM is the most suitable technique to reduce the number of experimental trials needed. It is also used to evaluate the most significant single factors and to effectively seek the optimum conditions for the multivariable system [8]. Several studies have applied RSM for optimization of red pigment [9-11]. However, to the best of our knowledge, no research has been reported on the application of RSM for optimization the red pigment production using *Monascus purpureus* FTC 5356 on petioles of oil palm fronds (OPF). Therefore, in order to determine the significant optimization factors in red pigment production, response surface methodology was applied in the present study.

2. MATERIALS AND METHODS

2.1 Microorganism

The strain used in this study was *Monascus purpureus* FTC 5356 obtained from Malaysian Agricultural Research and Development Institute, Serdang, Malaysia. The stock culture was maintained on potato dextrose agar (PDA) media and incubated at 28-30 ^{o}C for 7 days, preserved at 4 ^{o}C and sub-cultured once every 4 weeks [12].

2.2 Inoculum Preparation

Monascus purpureus FTC 5356 was grown on PDA slants at 30 ^{o}C for 7 days. The spores were then scrapped off and suspended in 5 ml sterile distilled under aseptic conditions at room temperature. The suspension was adjusted to 10^8 spores/ml with sterile distilled

water. The spore numbers were counted using a Neubauer hemacytometer (Cole-Parmer 79001-00). The adjusted spore suspension (10% v/w) was used for further solid state fermentation [12].

2.3 Substrate Preparation

Fresh oil palm fronds (OPF) were obtained from the Federal Land Development Authority (FELDA) Bukit Goh, Kuantan, Pahang. The leaflets and petioles were separated from the OPF. The petioles were then cut into small pieces approximately 3-4 cm in length, washed, and dried at 60 ^{o}C for 3 days. The dried petiole was shredded and ground using a commercial grinder (Retsch ZM-200, Germany) to a particle size smaller than 1 mm by passing through 1 mm sieve screens using a vibrator sieve shaker (Retsch, Germany).

2.4 Solid State Fermentation

The experimental work was done based on the experimental design being set by Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA), (Table 1 and Table 2). The best range of each factor was selected by applying the One Factor at A Time (OFAT) method as in the preliminary experiment (data not shown). All experiments have been carried out in replicates and the whole flasks were discarded. Each substrate was inoculated and incubated in the dark at 30 ^{o}C for 8 days.

Table 1: Independent variables, responses and the levels in the experimental design.

No	Designation	Factors	-1	0	+1
1	X_1	Initial moisture content (%)	40	55	70
2	X_2	Peptone concentration (%)	2	35	5
3	X_3	Initial pH value	6	8	10
		Response			
4	Y_1	Red pigment production (AU/g)			
5	Y_2	Biomass (mg/g)			
6	Y_3	Glucose concentration (µg/g)			

Table 2: The central composite design matrix developed for three independent variables

Run	X_1	X_2	X_3
1	0	0	-1
2	-1	-1	-1
3	+1	-1	-1
4	-1	+1	-1
5	+1	+1	-1
6	0	0	0
7	-1	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	+1	0	0
12	0	+1	0
13	0	-1	0
14	+1	-1	+1
15	+1	+1	+1
16	0	0	+1
17	-1	+1	+1
18	-1	-1	+1

2.5 Pigment Extraction and Determination

The harvested fermented solid was dried at 60 ^{o}C for 24 hours in an oven (Memmert UFB-500). The dried fermented solid was extracted with 95% ethanol in a ratio of 1:10 w/v for 1 hour at 200 rpm, in an incubator shaker (Infors AG-CH-4103 Bottmingen). The extract was then allowed to stand for 15 min, and filtered through Whatman No.1 filter paper. Ethanol extracts of unfermented substrates were used as blanks. Analysis of pigment concentration was done using a UV-Vis spectrophotometer (Hitachi U-1800). The wavelength used was 500 nm. Pigment yield was expressed as absorbance units (AU) per gram of dried solids [12-14].

2.6 Reducing Sugar Determination

Reducing sugar was measured using a dinitrosalicylic acid (DNS) method [12, 15]. The reducing sugar was measured at 575 nm by UV-Vis spectrophotometer (Hitachi U-1800).

2.7 Biomass (Cell Dry Weight)

Total fungal biomass was determined by measuring the N-acetylglucosamine released by acid hydrolysis of the chitin present in the fungal cell walls. The acid hydrolysis of the sample was carried out by mixing 0.5 g of dry fermented OPF powder with 2 ml of 60% (vol/vol) sulfuric acid, H_2SO_4 and the mixture was incubated at 25 ^{o}C in a fume hood for 24 h [16]. Then the mixture was diluted with distilled water to make a 1 N solution of sulfuric acid that was then autoclaved at 121 ^{o}C for 1 h. The mixture was allowed to cool at room temperature and neutralized with 5 N NaOH to pH 7 and the final volume was brought up to 60 ml with deionized water. Later, the filtered acid hydrolysis sample (1 ml) was mixed with 1 ml of acetyl acetone reagent (2% (vol/vol) of acetyl acetone in 1 N sodium bicarbonate (Na_2CO_3) before being placed in a boiling water bath for 20 min [12]. After cooling, 6 ml of ethanol (95%) was added, followed by 1 ml of Ehrlich reagent (2.67% (w/v) of *p*-dimethylaminobenzaldehyde (Merck) in 1:1 mixture of ethanol and concentrated hydrochloric acid) [17]. The mixture was incubated in a water bath at 65 ^{o}C for 10 min. After cooling, the optical density was read at 530 nm against the reagent blank, N-acetylglucosamine (Sigma-Aldrich) as the external using a UV-visible spectrophotometer [12, 18].

2.8 Experimental Design

The red pigment production was developed and optimized using response surface methodology (RSM) provided by Design Expert Software (Version 7.1.6, 2008, Minneapolis MN, USA). A standard RSM design tool known as Central Composite Design (CCD) was applied to study the significant production factor of red pigment. The three identified independent variables were the initial moisture content (40-70%), peptone concentration (2-5%), and initial pH (pH 6-8). The critical ranges of selected factors were determined by preliminary experiment using OFAT and screening by factorial design (data not shown). During the screening process of petiole used as a substrate, the initial moisture content (IMC), initial pH, interaction of peptone, and pH were found to be significant. Thus, three factors were chosen for optimization. Screening was done to eliminate the insignificant factor. Table 1 lists the ranges and levels of the three independent variables with actual and coded levels of each factor. The lower and upper levels were coded as -1 and +1; the middle level was coded as 0. A total of 18 runs with 4 central points were generated. The center points are usually repeated 4-6 times to determine the experimental error (pure error) and the reproducibility of the result. Three responses, red pigment (AU/g), biomass (mg/g) and glucose concentration (µg/g), were measured. The experiments were run in triplicate. The

complete design matrix corresponding to the CCD design in terms of real and coded independent variables is displayed in Table 2.

2.9 Validation Experiment

The validation experiment was performed by conducting the experiment with the suggested optimal conditions of higher pigment.

3. RESULTS AND DISCUSSION

The statistical significance of the model equation was evaluated by the F-test analysis of variance (ANOVA). The ANOVA statistics for responses Y_1, Y_2, and Y_3 were summarized in Table 3, 4, and 5, respectively. Multiple regression analyses of the response surface design were developed as in Equations 1, 2, and 3. In order to determine the optimal level of each variable for maximum production of red pigment and biomass, a 3D surface plot was designed as a function of two factors at a time, holding all other factors at a fixed level. This design was helpful for understanding both the main and the interaction of the two factors. The response values for the variables can be predicted from these plots.

Table 3: ANOVA analysis for red pigment production (Y_1)

Source	**Sum of squares**	**DF**	**Mean square**	**F-value**	**Prob>F**	
Model	4915.48	9	546.16	52.33	<0.0001	Signi-ficant
X_1- Initial moisture content	9 x 10^3	1	9 x10^3	8.6 x 10^4	0.9773	
X_2- Peptone concentration	26.9	1	26.9	2.58	0.1471	
X_3- Initial pH value	126.74	1	126.74	12.14	0.0083	
X_1X_2	16.24	1	16.24	1.56	0.2475	
X_1X_3	0.18	1	0.18	0.017	0.8988	
X_2X_3	0.13	1	0.13	0.012	0.9156	
X_1^2	44.47	1	44.47	4.26	0.0729	
X_2^2	444.04	1	444.04	42.54	0.0002	
X_3^2	1195.10	1	1195.10	114.51	<0.0001	
Residual	83.50	8	10.44			
Lack of fit	76.15	5	15.23	6.22	0.0817	Not signi-ficant
Pure error	7.35	3	2.45			
R^2	0.9833					
Adeq precision	18.345					

Table 4: ANOVA analysis for biomass response (Y_2)

Source	Sum of squares	DF	Mean square	F-value	Prob>F	
Model	1.28×10^5	9	14169.99	7.63	0.0044	Signi-ficant
X_1- Initial moisture content	124.61	1	124.61	0.067	0.8022	
X_2- Peptone concentration	108.24	1	108.24	0.058	0.8153	
X_3- Initial pH value	2982.53	1	2982.53	1.61	0.2408	
X_1X_2	296.46	1	296.46	0.16	0.7	
X_1X_3	5.95	1	5.95	3.2×10^3	0.9563	
X_2X_3	0.66	1	0.66	3.6×10^4	0.9854	
X_1^2	4302	1	4302	2.32	0.1666	
X_2^2	9802.12	1	9802.12	5.28	0.0507	
X_3^2	25466.58	1	25466.58	13.71	0.0060	
Residual	14864.29	8	1858.04			
Lack of fit	13890.42	5	2778.08	8.56	0.0536	Not signi-ficant
Pure error	973.87	3	324.62			
R^2	0.8956					
Adeq precision	7.105					

Table 5: ANOVA analysis for glucose concentration response (Y_3)

Source	Sum of squares	DF	Mean square	F-value	Prob>F	
Model	90779.97	9	10086.66	91.77	<0.0001	Signi-ficant
X_1- Initial moisture content	1.51	1	1.51	0.014	0.9095	
X_2- Peptone concentration	442.89	1	442.89	4.03	0.0796	
X_3- Initial pH value	3997.20	1	3997.20	3637	0.0003	
X_1X_2	1074.62	1	1074.62	9.78	0.0141	
X_1X_3	7.57	1	7.57	0.069	0.7997	
X_2X_3	25.56	1	25.56	0.23	0.6425	
X_1^2	1516.91	1	1516.91	13.80	0.0059	
X_2^2	9162.68	1	9162.68	83.37	<0.0001	
X_3^2	17555.18	1	17555.18	159.73	<0.0001	
Residual	879.26	8	109.91			
Lack of fit	235.72	5	47.14	0.22	0.9319	Not signi-ficant
Pure error	643.54	3	214.51			
R^2	0.9904					
Adeq precision	25.360					

3.1 Optimization of Red Pigment Production

The second order polynomial equation model for prediction of the optimal point between the response variable (red pigment production) and the independent variables was expressed in Eqn. 1:

$$Y_1(\text{red pigment}) = 45.66 - 0.03X_1 + 1.64X_2 + 3.56X_3 - 1.42X_1X_2 + 0.15X_1X_3 + 0.13X_2X_3 - 405X_1^2 - 12.80X_2^2 - 21X_3^2 \quad (1)$$

where Y_1 is the response for red pigment production, X_1 is the code for initial moisture content, X_2 is for peptone concentration, X_3 is for initial pH value.

Based on the ANOVA Table, as presented in Table 3, the quadratic model indicated that this model could be accepted to navigate the design space. The Model F- value of the response Y_1 with the value 52.33 implies that the model was significant at 95% confidence level. The P-value was used as a tool to check the significance of each coefficient, which in turn designates the pattern of interaction between the factors. The smaller the P-value, the larger the significance of the coefficient was. As in Table 3, the P-values for the Y_1 was <0.0001, which was less than 0.05. Therefore, it can be concluded that the model terms were statistically significant. In addition, the main model terms indicated that the significant factor was the initial pH (X_3), while the significant quadratic terms were peptone concentration (X_2^2) and initial pH (X_3^2). The lack of fit test with P-value (0.0817), which was not significant (p-value> 0.05 is not significant), supported the hypothesis that the model was satisfactorily fitted to the experiment data. The 'not significant' term of lack of fit is most-desired as a significant of lack of fit indicates the presence of the contribution in the regressor-response relationship that is not accounted for by the model [19]. The correlation coefficient (R^2) is a tool to identify the 'goodness of fit' between the experimental and the predicted values. Based on Table 3, the R^2 for Y_1 (0.9833) was found to be close to 1, which indicated the presence of a good relation between experimental and predicted values for red pigment (Y_1). The adequate precision for Y_1 is 18.345. These large values of adequate precision demonstrated that these quadratic models were significant for the process. The evaluation of residuals was analyzed to validate the adequacy of the model. A normal probability plot of the residuals for Y_1 is displayed in Fig. 1. Based on the figure, it clearly shows that the residuals distribution was nearly a straight line. Thus, it can be concluded that the errors were distributed evenly.

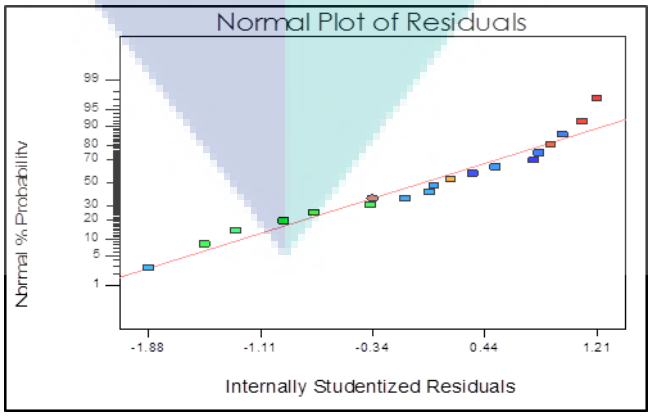

Fig. 1: Normal plot of residuals for red pigment production (Y_1).

Figures 2a, 2b, and 2c show the 3D surface plots of the relationship between the main factors X_1X_2 (initial moisture content and peptone concentration), X_1X_3 (initial moisture content and initial pH), and X_2X_3 (peptone concentration and initial pH), respectively. In Fig. 2a, the increment of initial moisture content from low level 40% to 55% leads to the increase in red pigment to a maximum level. However, a further increase in the initial moisture content (>55%) did not further increase the pigment. This result clearly shows that the red pigment decreased above and below the 55% initial moisture content. The poor yield of red pigment at high moisture content (>55%) was possibly due to the agglomeration of substrate, consequently reducing oxygen supply for the growth of *Monascus*. While, the decrease in red pigment at low moisture content was because of the insufficient nutrient supply due to the low nutrient salt dissolution [19].

A similar trend of effect on the response was observed for the initial moisture content and the initial pH. An increase of the initial moisture content and initial pH, up to the optimum point, maximized the red pigment production and a further increase of the factors decreased the red pigment, as shown in Fig. 2b. This reaction process was in agreement with Orozco and Kilikian [20] in which changing the pH value of the medium from pH 5.5 to pH 8, caused the drastic excretion of the red pigment. In addition, they also claimed that the best condition for red pigment production was at alkaline medium (pH 8.0 or pH 8.5). Between these two pH values, pH 8 had been chosen to be the best condition due to the maximum yield of red pigment production.

The interaction effect of the peptone concentration with initial pH as shown in Fig. 2c clearly suggested the best combination for production of red pigment. An increase in the peptone concentration with initial pH, optimized the red pigment gradually. However, at higher peptone concentration (> 3.5%) and higher initial pH (> pH 8), the pattern is reversed. The decrease in yield may due to excessive nutrients provided in the medium that became toxic and inhibited the red pigment production. Therefore, the optimum conditions for maximum red pigment production were obtained at the initial moisture content of 55%, peptone concentration of 3.5%, and initial pH value of 8. The maximum red pigment achieved was 47.9 AU/g.

3.2 Optimization of Biomass Production

Based on the experimental results and regression analysis, a quadratic polynomial equation was developed to determine the relationship between the biomass of *Monascus purpureus* and the factors. The model of coded units can be stated as in Eqn. 2:

$$\boldsymbol{Y_2(\text{biomass}) = 382.75 + 3.53X_1 + 3.29X_2 + 17.27X_3 - 6.09X_1X_2 - 0.86X_1X_3 - 0.29X_2X_3 - 39.85X_1^2 - 60.15X_2^2 - 96.95X_3^2} \quad (2)$$

where Y_2 is the response for biomass production, X_1 is the code for initial moisture content, X_2 is for peptone concentration, and X_3 is for initial pH value.

From the analysis of variance (ANOVA) as presented in Table 4, the model for biomass was highly significant (P-value, 0.0044) and the R^2 (0.8956) was relatively good, as evidenced by the significance of the model. There was no significance of a single factor or interaction between factors that influenced the biomass production, however, the quadratic terms of initial pH value was found to be significant. Furthermore, the lack of fit was not significant with P-value of 0.0536 (>0.05), indicating that the experimental data obtained fitted well with the model.

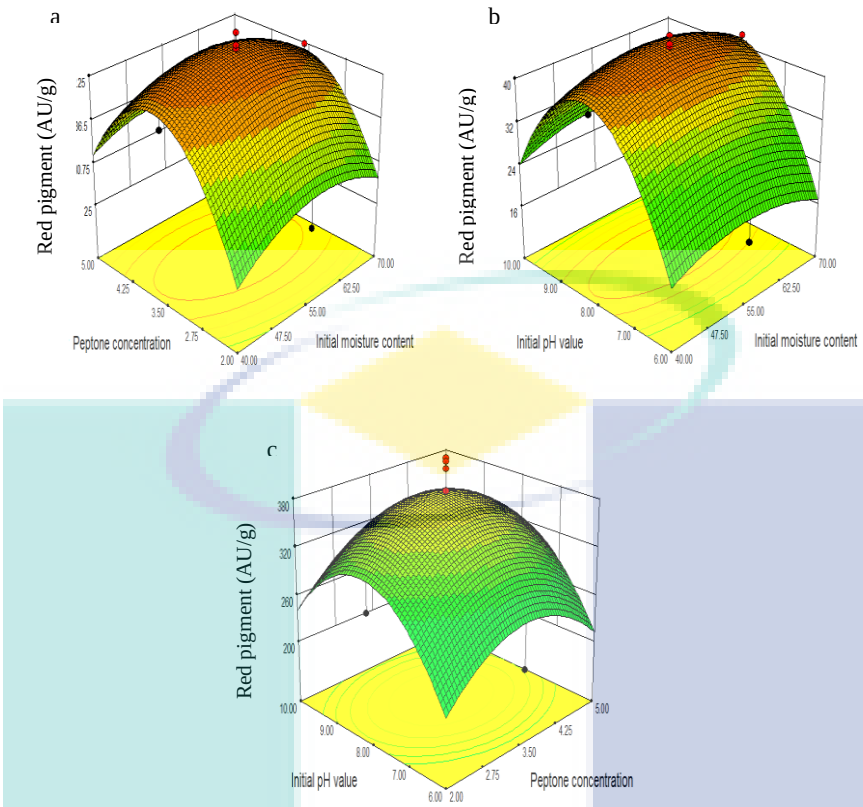

Fig. 2: Response surface curve showing combined effect between the main factors: (a) initial moisture content (X_1) and peptone concentration (X_2), (b) initial moisture content (X_1) and initial pH value (X_3), (c) peptone concentration (X_2)and initial pH value (X_3).

The residual analysis was carried out for the confirmation of the adequacy of the model. This was done by observing the normal probability plot of the residual in Fig. 3 where the residuals were on a straight line, suggesting that the errors were distributed evenly.

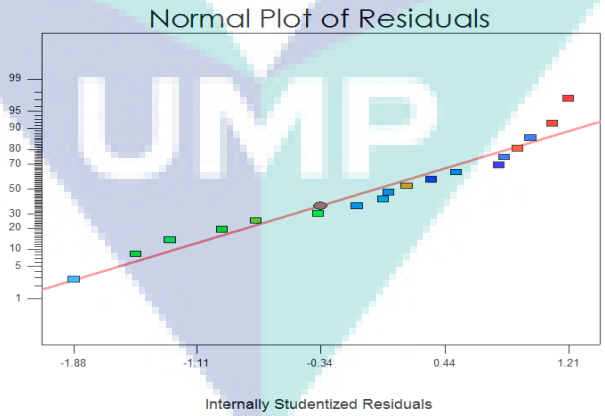

Fig. 3: Normal plot of residuals for biomass response (Y_2).

Figures 4a, 4b, and 4c show the 3D surface plots of biomass responses after combining the effect of the main factors. The effect of the initial moisture content and peptone concentration on the biomass is shown in Fig. 4a. An increase of initial moisture content with peptone concentration up to the optimum point increased the fungal biomass to a maximum level and a further increase in the initial moisture content and peptone

concentration did not further increase the trend. This finding was supported by Krishna [21], who stated that the low initial moisture content could reduce nutrient diffusion consequently affecting the growth of the *Monascus.* However, if the initial moisture content is too high, water will occupy the voids where airflow is required for fungal growth.

Increased factors of initial moisture content and initial pH up to the optimum point, maximized the biomass production (Fig. 4b). From the 3D plot, it was obviously shown that *Monascus* was grown successfully at pH 8 indicating that the biomass achieved the maximum yield. However, the fungal biomass production started to decrease with a further increase of initial pH (> pH 8) of substrate.

The interaction effect of the peptone concentration with initial pH in Fig. 4c clearly suggested the best combination for the production of fungal biomass. An increase in the peptone concentration and initial pH optimized the biomass gradually but at higher peptone concentration and initial pH, the pattern is reversed. It was studied that nitrogen is the major element of cell membranes and nucleic acid, therefore supplying nitrogen sources to the medium may facilitate the growth of the fungus. However, if the nitrogen concentration is too high (> 3.5%), it might inhibit the fungal growth. Therefore, the optimum biomass was observed at the initial moisture content of 55%, peptone concentration of 3.5%, and initial pH of substrate of pH 8. The maximum biomass achieved was 430.8 mg cell dry weight/g dry matter.

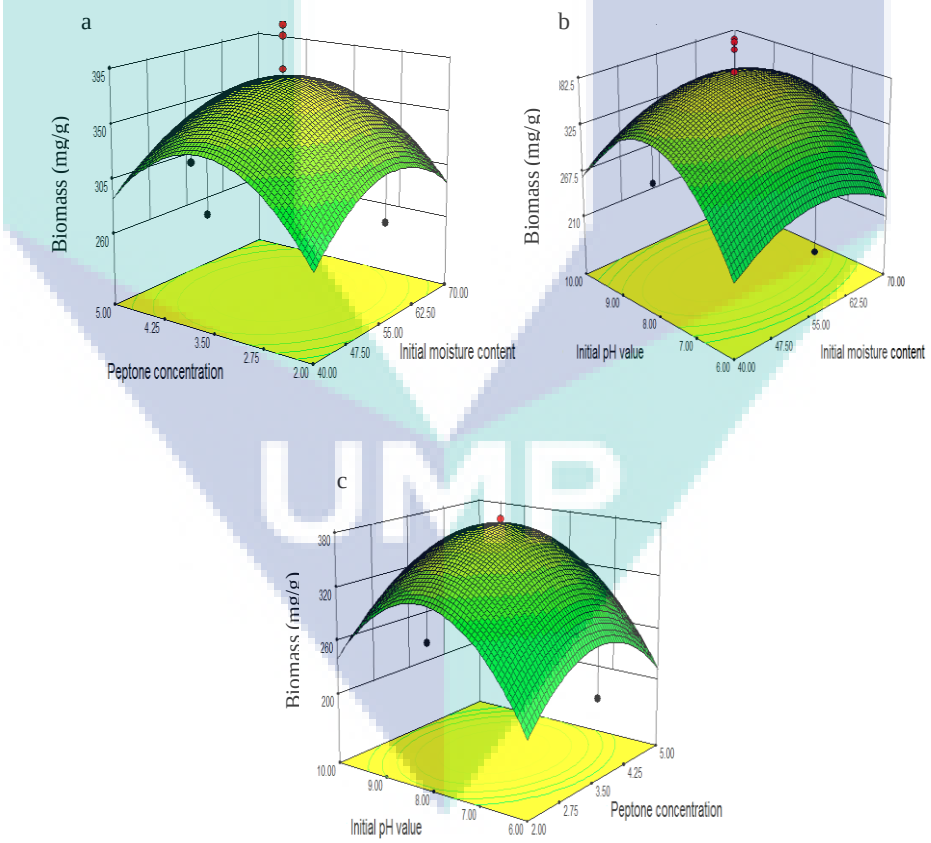

Fig. 4: Response surface curve showing combined effect between the main factors:
(a) initial moisture content (X_1) and peptone concentration (X_2),
(b) initial moisture content (X_1) and initial pH value (X_3),
(c) peptone concentration (X_2) and initial pH value (X_3).

3.3 Glucose Concentration

On the glucose consumption, a second order polynomial can be obtained by the Design Expert. Multiple regression equations (in term of coded factors) were represented in Eqn. 3:

$$Y_1(\text{glucose concentration}) = 130.65 - 0.39X_1 - 6.66X_2 - 19.99X_3 + 11.59X_1X_2 - 0.97X_1X_3 - 1.79X_2X_3 + 23.66X_1^2 + 58.15X_2^2 + 80.49X_3^2 \quad (3)$$

where Y_3 is the response for glucose concentration, X_1 is the code for initial moisture content, X_2 is for peptone concentration, X_3 is for initial pH value.

The ANOVA Table implies that the model was significant with the F-value of 91.77 (Table 5). The P-value (<0.0001) was less than 0.05, which indicated the model terms were highly significant. In addition, the main model terms indicated that the significant factor was initial pH value (X_3) and the interaction terms were found to exist between initial moisture content (X_1) with peptone concentration (X_2). While, the significant quadratic terms were initial moisture content (X_1^2), peptone concentration (X_2^2), and initial pH value (X_3^2). The lack of fit value of 0.22 confirmed that the lack of fit was not significant, relative to the pure error when p-value was 0.9319 and > 0.05. The insignificant lack of fit demonstrates the good predictability of the model. In addition, the value of R^2 was 0.9914, indicating that the model was fitted and explains 99.14% of the variability in glucose concentration. The high values of adequate precision with the value of 25.360 demonstrated that these quadratic models were significant for the process.

Figure 5 displays the normal plot of residuals of response Y_3 glucose concentration. It was obviously shown that the points cluster around the diagonal line which indicated the good fit of the model.

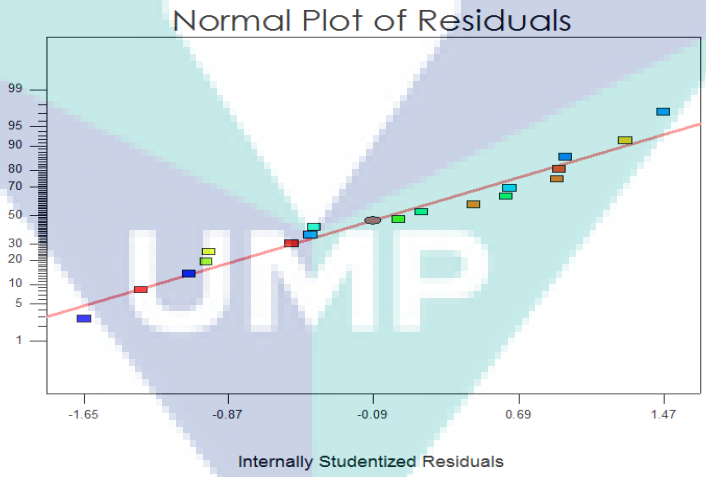

Fig. 5: Normal plot of residuals for glucose concentration response (Y_3).

Figures 6a, 6b, and 6c show the 3D surface plots of glucose concentration response after combining the effect between the main factors. From the figure, it was observed that the glucose was decreased when the initial moisture content, peptone concentration and initial pH value were 55%, 3.5% and pH 8, respectively. The 3D surface plots of glucose concentration were totally different with the previous figures (Figures 2a, 2b, 2c, 4a, 4b, and 4c). The glucose concentration decreased when the fungal biomass and red pigment production achieved the maximum yield. This phenomenon suggested that the rapid consumption of glucose by *Monascus* caused the depletion of glucose, consequently

resulting in an insufficient glucose supply that reached its supply limitation [22]. The lowest final glucose concentration of 114.73 µg/ g was obtained.

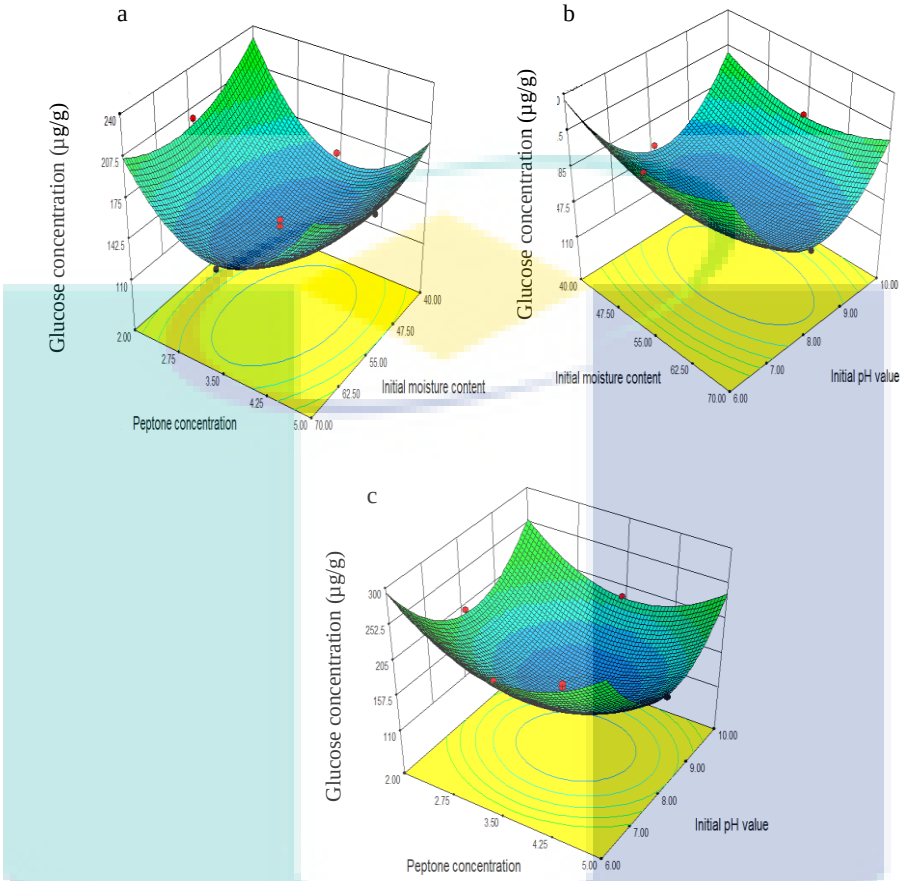

Fig. 6: Response surface curve showing combined effect between the main factors: (a) initial moisture content (X_1) and peptone concentration (X_2), (d) initial moisture content (X_1) and initial pH value (X_3), (e) peptone concentration (X_2)and initial pH value (X_3).

3.4 Validation

In order to confirm the optimization of red pigment production by *Monascus purpureus* FTC 5356, an experiment was performed under the predicted optimal conditions. This experiment was conducted in triplicate. Under these suggested conditions, the predicted optimal values of the variables were 56% initial moisture content, 3.5% peptone, and pH 8.2. The prediction of the total red pigment was 45.85 AU/g and the actual value obtained through the triplicate experiments was 46.03 AU/g, as shown in Table 6. The percentage error calculated based on the Eqn. 4 was 0.39%. Therefore, the experimental results agreed well with the model predicted values.

$$Percentage\ error = \frac{(\text{Experimental value} - \text{predicted value})}{\text{Experimental value}} x\ 100\% \quad (4)$$

Table 6: Optimum factors of RSM on red pigment

Factor	Value	Predicted (AU/g)	Actual (AU/g)	Percentage error (%)
Initial moisture content (%)	56	45.85	46.03	0.39
Peptone (%)	3.5			
pH	8.2			

4. CONCLUSION

This study shows that response surface methodology is a fast and error-free approach for optimization of media composition to obtain the best performance of red pigment production and biomass. Besides, an interaction study among all the components was an additional advantage of employing RSM. Results obtained from response surface methodology critically point out the importance of initial pH value of the substrate for red pigment production as well for the fungal biomass.

ACKNOWLEDGEMENT

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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.

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.

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

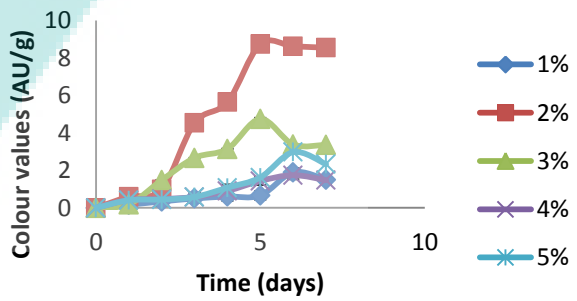

Fig. 1: Effect of nitrogen on pigment production

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.

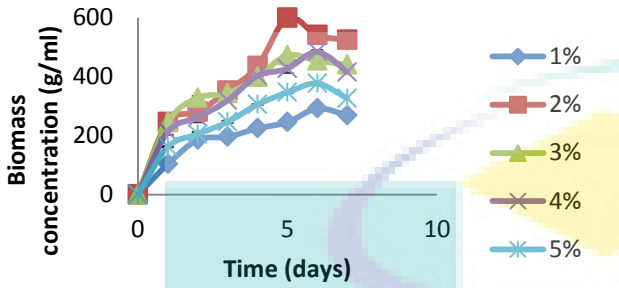

Fig. 2: Effect of nitrogen on biomass concentration

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.

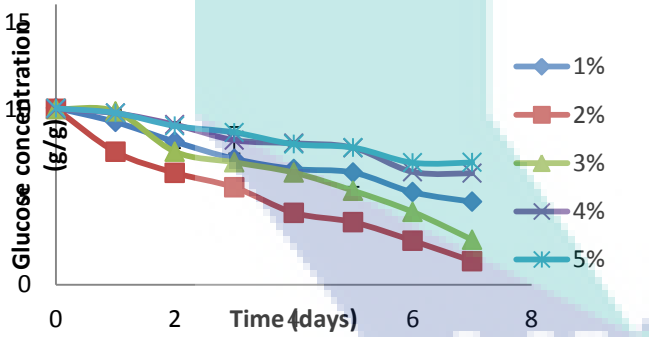

Fig. 3: Effect of nitrogen on glucose concentration

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*.

Acknowledgement

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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 & C Red No.2, E123), erythrosine (FD & C Red No. 3, E127) and tartrazine (FD & 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. Pigments are made from various sources including extraction from microorganisms, plants and animals [5]. 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. Approximately 80 million tonnes of oil palm biomass were produced in 2011 [22]. 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 30ºC 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 60ºC 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 121ºC for 15 minutes, which is known as autohydrolysis process [29]. The treated OPF was later washed with distilled water and oven-dried at 45ºC 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 30ºC. 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 60ºC 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

The concentration of crude fermentable sugar was analyzed by dinitrosalicylic acid (DNS) method [32] 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 121ºC. 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 Figure 1 and 2. In terms of structure, Figure (1a and 2a) 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 Figure 1b and 2b. 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 Lim and the colleagues [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.

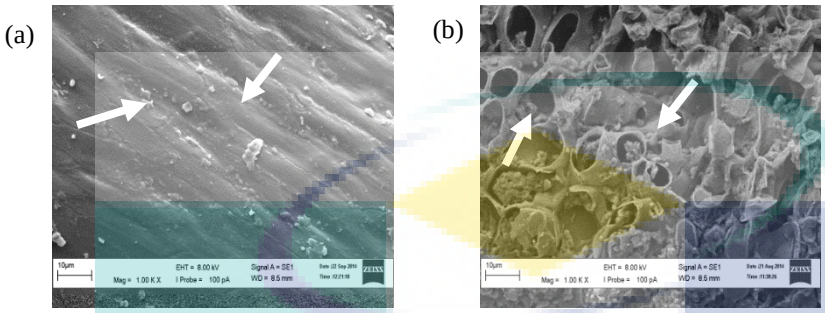

Fig. 1. SEM micrograph of the OPF leaflet under 1000x magnification. (a) Non-treated; (b) Treated

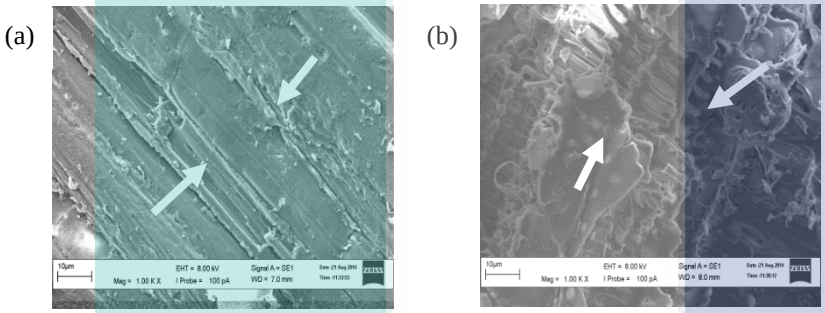

Fig. 2. SEM micrograph of the OPF petiole under 1000x magnification. (a) Non-treated; (b) Treated

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) (Figures 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 (Figure 1b and 2b). 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.

3.2. Effect of initial moisture content (IMC) on the 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 Figure 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) (Figure 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 (Figure 3).

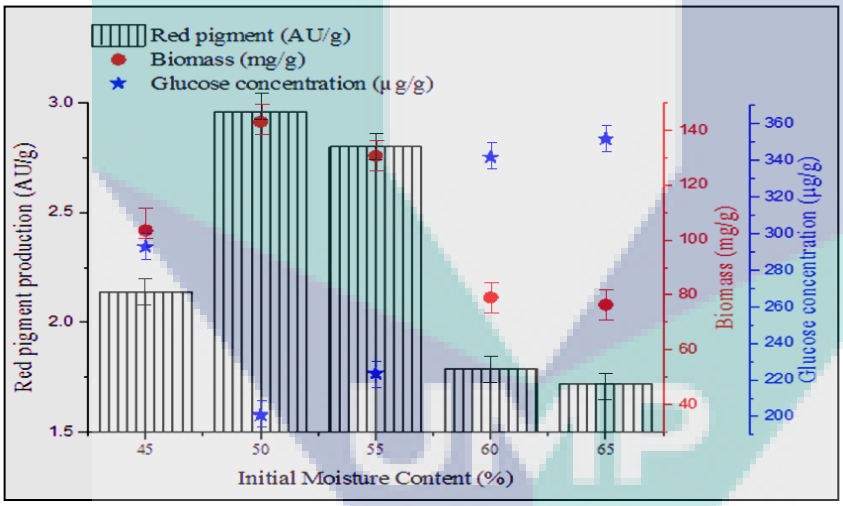

Fig. 3. Effect of initial moisture content on the red pigment production

3.3. Effect of nitrogen source on the 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 (Figure 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].

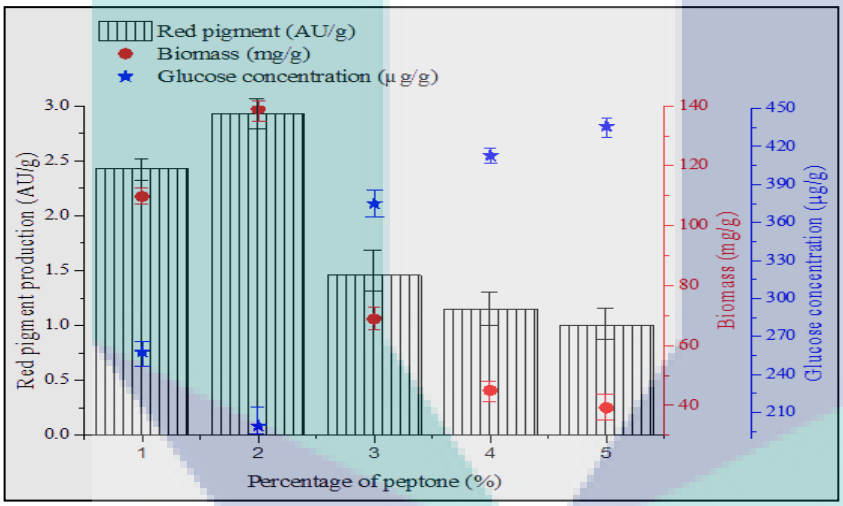

Fig. 4. Effect of peptone concentration on the red pigment production

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. Figure 6 demonstrates the effect of various percentages of petiole to leaflet ratio on the red pigment production. The Figure 6 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 (Figure 6). 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.

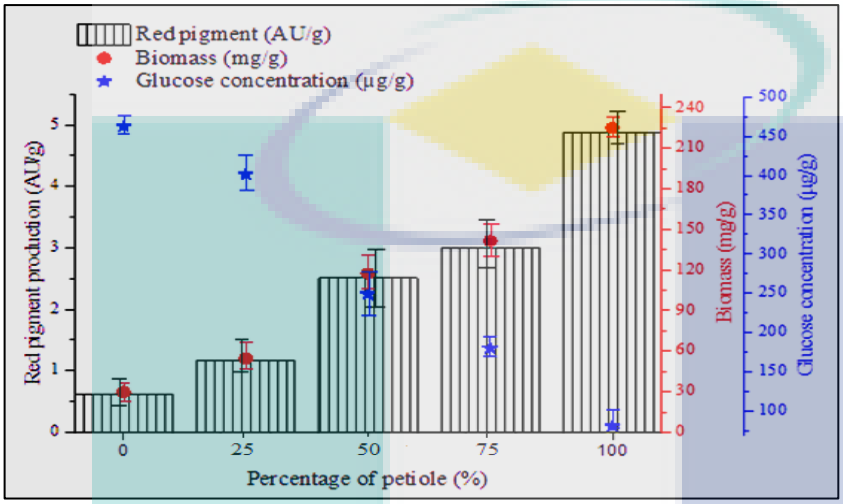

Fig. 5. Effect of percentage petiole to leaflet on the red pigment production.

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 (Figure 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 (Figure 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.

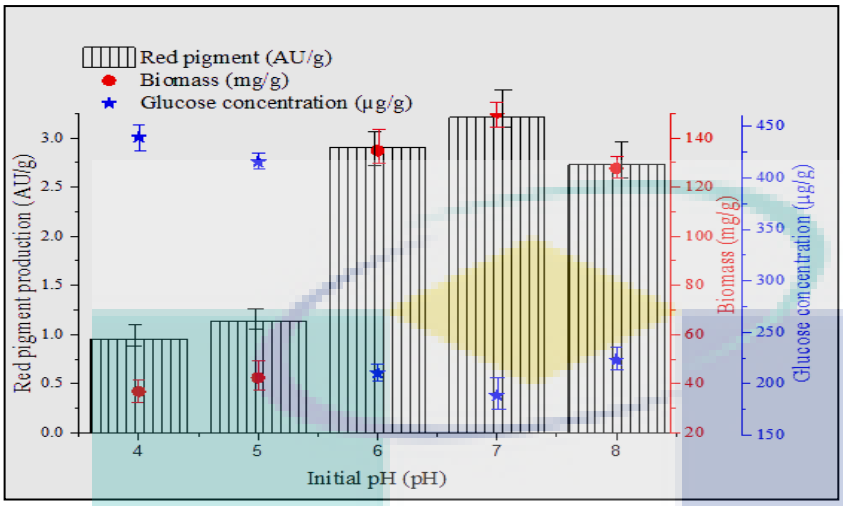

Fig. 6. Effect of pH on the red pigment production

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) (Figure 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 (Figure 7).

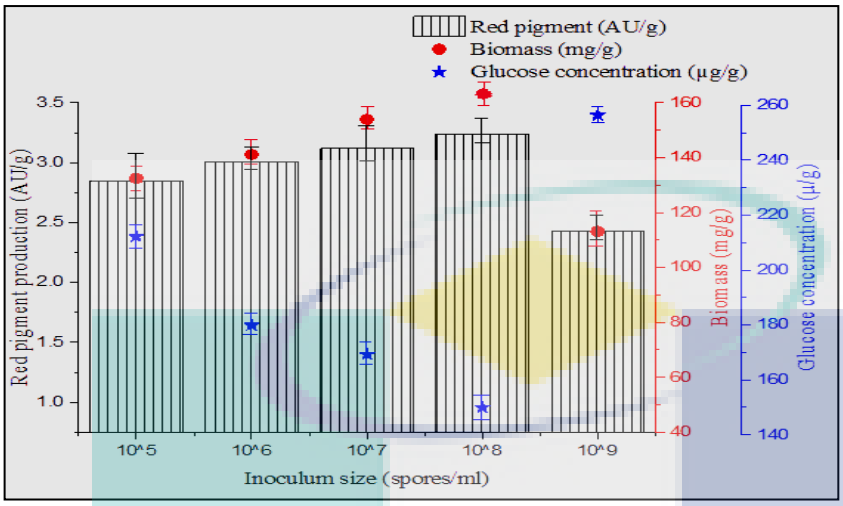

Fig. 7. Effect of inoculum size on the red pigment production.

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.

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Nomenclatures

w/w	Weight per weight
%	percentage
g	gram
mg	milligram
kg	Kilogram
w/v	Weight per volume
nm	Nanometer
C:N	Carbon to nitrogen
µg/g	Microgram per gram

Abbreviations

OPF	oil palm frond
OFAT	one-factor-at-a-time
IMC	initial moisture content
AU	Absorbance unit
EFB	empty fruit bunch
PPF	palm pressed fibre
PDA	potato dextrose agar
FELDA	Federal Land Development Authority
DNS	dinitrosalicylic acid
SEM	scanning electron microscope
SSF	solid-state fermentation

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Evaluation of Bio-red Pigment Extraction from *Monascus purpureus* FTC5357

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Abstract. A suitable extraction technique helps to increase the extraction yield and stimulate higher quality of pigments. Therefore, investigating the effect of different extraction solvents on red pigment produced via solid-state fermentation (SSF) by *Monascus purpureus* FTC 5357 are essential. In this study, oil palm frond (OPF) was used as a substrate for the fermentation process. The fermentation was conducted at 30 °C for eight days. Variation of solvents (95% ethanol, 60% ethanol and distilled water), pH and time of extractions were applied on the fermented product. The extracted pigment was then analysed using spectrophotometer at 500 nm, for red pigment. Combination of pH 6 and 60% ethanol at 16 h pronounced to be the best conditions to extract the pigment, with an absorbance value of 207 AU/g.d. The advantage of the ethanol as a solvent extraction is cheap and non-toxic. Later, the extracted pigment is safe to be used in food applications.

1 Introduction

Customarily, the manufactured food will be imposed with colorants to amplify its commercial values [1]. According to Martins et al. (2016), pleasing colours might affect the product acceptance. There are two categories of food colorant such as natural food colorant and synthetic food colorant. Recently, the awareness on the application of the former to the food product has increased due to the harmful effect caused by the latter [2-4].

Natural pigments are coloured compounds extracted from living organisms; such as from plant [5], animal [6, 7] and fungus [8-10] and most of the available natural pigment was extracted from plant [11-13]. Despite the popularity of pigment extracted from plant, pigment produced by microorganisms hold a promising potential to meet present day challenges. *Monascus* species is known be able to produce an edible pigment and it is highly safe [14, 15]. Furthermore, *Monascus* pigments not only improve the marketability of the product but also have varied biological activities such as anti-inflammatory [16, 17], anti-tumor [18, 19], anti-oxidant [20-22] and regulation of cholesterol levels characteristics [23, 24].

Generally, pigment production in industrial scale has been carried out using submerged fermentation (SmF) [25]. However, solid state fermentation (SSF) has emerged as an effective way due to the high production yield [26]. In addition, by SSF process, a relative low-cost process can be achieved, especially when agro-industrial wastes are used as substrate [27].

In Malaysia, there are more than 4.98 million hectares of oil palm plantations [28]. The main problem in the oil palm tree cultivation and its related industries is its substantial amount of biomass

wastes. Oil palm frond (OPF) is one of the biomass waste generated. However, the utilization of OPF is limited. Previous study were done on the usage of petiole and leaflet of OPF as a substrate by SSF to produce red pigment [29-31]. Yet, the challenging occurred on how to extract the red pigment from the fermented OPF via SSF, since the extraction is one of the most expensive steps in the production of natural colorants [32].

Solid liquid extraction (SLE) is the most common technique for the removal of pigment from fermented substrate by SSF [33-36]. A suitable extraction technique helps in increasing the extraction yield, besides prevent the degradation of the extracted pigments [37].

A number of researchers have proposed various extraction methods, however, most of the methods are on plant material [5, 38-41]. Due to the limitation of previous study in extracting the natural pigment from fungi via SSF, thus, this study aims to investigate the performance of red pigment extracted from *Monascus purpureus* FTC 5357. Extraction process is generally affected by several factors such as temperature, time and solvent type [32, 42, 43]. Hence, the above mentioned factors are identified on the efficiency to extract the red pigment produced by different extraction solvents under various conditions.

2 Methodology

2.1 Culture and Solid State Fermentation

Monascus purpureus FTC 5357 was purchased from culture collection Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Petiole oil palm fronds (OPFs) were obtained from agricultural fields, Felda Lepar Hilir, Gambang, Pahang, Malaysia. The fresh OPFs were cut into smaller pieces and dried in an oven for 1 day, ground and sieve to get 1 mm particle size using a sieve shaker (Retsch AS 200 Basic, Germany) [30]. Later, the OPFs powder were autoclaved with distilled water in a 1:18 ratio (w/v) at 121 °C, for 15 min and cooled at room temperature [44, 45]. The pre-treated OPFs were filtered and washed with distilled water, before being oven dried at 60 °C for 24 h [30]. The pre-treated OPFs were mixed with distilled water to get approximately 75% initial moisture content, adjusted to pH 8 and 4% (w/w) of peptone. The medium was autoclaved at 121 °C for 20 min. Then, the sterilized OPFs were inoculated with 1.0×10^7 spores/mL and incubated at 30 °C, for 8 days.

2.3 Extraction Methods

Fermented OPFs were dried in an oven at 60 °C for 24 h. In order to determine the performance of different extraction conditions, three different solvent mixtures were evaluated which are distilled water, 60% ethanol (v/v) and 95% ethanol (v/v).

A 0.5 g of dried fermented OPFs were placed in 250 mL Erlenmeyer flasks and mixed with different solvents in a ratio of 1:160 (g/ml). The solvent extraction was performed in an incubator shaker at 180 rpm, 30 °C for 1 h. After that, another experiment was repeated with different pH (i.e. pH 2, 4, 6 and 8) using the best solvent obtained in the previous experiment in a ratio of 1:160 (g/ml) at 180 rpm, 30 °C for 2 h. The pH of the solvent was adjusted using hydrochloric acid (HCl) and sodium hydroxide (NaOH). Later, with the optimum condition found earlier, six individuals Erlenmeyer flasks were exposed to six different extraction times (i.e. 4, 8, 12, 16, 20 and 24 h).

2.4 Pigment Assay

In all cases, at the end of extraction period, the mixtures were allowed to stand for 15 min at room temperature and filtered through Whatman no.1 filter paper [46-48]. The supernatants were analyzed by a spectrophotometer at a wavelength of 500 nm, for red pigment, taking into consideration the dilution factor of the sample [49]. The results were expressed as absorbance units per g of dried solid (AU/g.d).

3 Results and Discussion

3.1 The effect of the extraction solvent

The solvent selection is very important to determine the affiliation of the solvent composition to the particles to be extracted [50]. Extraction by different types of solvent mixture were investigated on the dried fermented OPF, separately. Figure 1 shows that all the solvents tested were able to extract the red pigment. Among the solvents tested, 60% ethanol shows the best extraction with an absorbance value of 219.2 AU/g.d, followed by 95% ethanol (41.6 AU/g.d) and distilled water with an absorbance value of 12.8 AU/g.d. The absorbance value of 95% ethanol and distilled water are lower than 60% ethanol by 81% and 94%, respectively, due to the polarity of solvent. Where, the ethanol is able to react with both polar and non-polar compounds due to its unique structure molecule. The hydroxyl (OH) group with the high electronegativity of oxygen allow the hydrogen bonding to take place known as polar compound, while the ethyl (C_2H_5) group acted as non-polar compound. At lower ethanol concentration (60%), the polarity of the solvent was quite higher when compared to the 95%. Where, at 60% ethanol the hydrogen bond (OH) in water was mixed together in ethanol. Too high polar solvent (i.e. distilled water) did not promote to better pigment extraction because it consists of only OH group.

Karacabey & Mazza (2008) and Carvalho et al. (2007) reported that moderate polar compounds were suited to be extracted with 50-70% ethanol concentration. The efficiency of the extraction is based on the selectivity of the solvent to the compound that need to be extracted. The result obtained is compatible with previous research which reported that water has the lowest yield of *Monascus* pigment due to high polarity of distilled water [51]. Ethanol 60% appeared to be the best solvent for red pigment extraction due to the close on the polarity of the red pigment produced by *Monascus* and the solvent [52, 53]. Thus 60% ethanol was used for the next experiment.

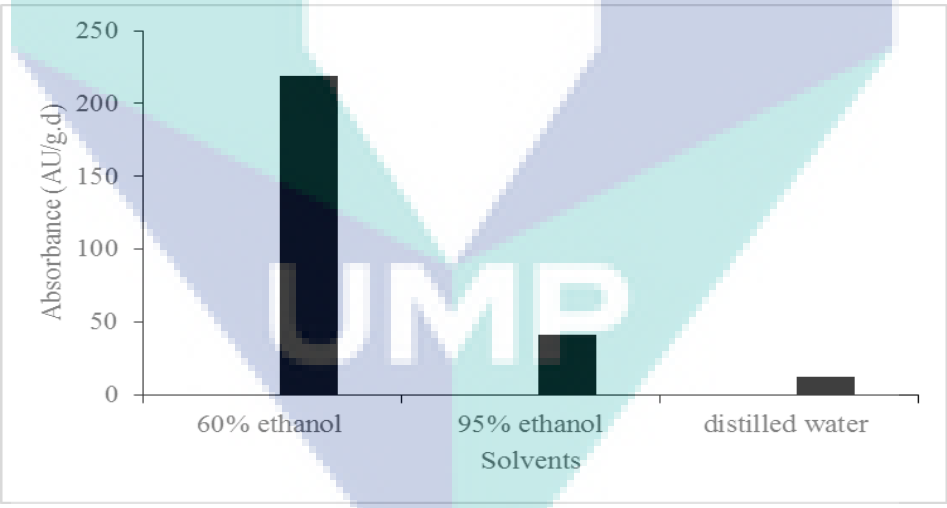

Figure 1. Absorbance of red pigments extracted using different solvents.

3.2 Effect of pH on extraction

The pH value plays a crucial role in the extraction process. Next a series of experiments at different pH value were studied. *Monascus* pigment was extracted at different pH of ethanol (60%) ranging from pH 2 to 8. The wavelength of 500 nm denoted as red pigment as agreed by many researchers [36, 45, 47, 54-56]. On the other hand, the wavelength of 400-420 nm, indicated yellow pigment [57, 58]. Figure 2 shows that pH 2 (170 AU/g.d) and pH 6 (172 AU/g.d) produced high red pigment compared to pH 4 (143 AU/g.d) and pH 8 (135 AU/g.d). But, pH 2 produced higher yellow pigment compared to the red pigment. While, at pH 6, the absorbance value for yellow and red pigments were almost

comparable.

It was observed that, the pH solvent for extraction was comparable with the pH medium for *Monascus* to growth. It has been reported that when the pigment produced at lower pH (pH<6), there was predominance of yellow pigment and at higher pH (pH≥6), there was predominance of red pigment [46, 47, 59, 60]. Feng et al. (2012) and Orozco et al. (2008) reported that the pH ranged from 5.5 to 8.0 are shown to stimulate *Monascus* growth and the red pigment production. As shown in Figure 2, the best conditions to extract the red pigment occurred at pH 6 and yellow pigment at pH 2, using 60% of ethanol. Thus, pH 6 was used for the next experiment.

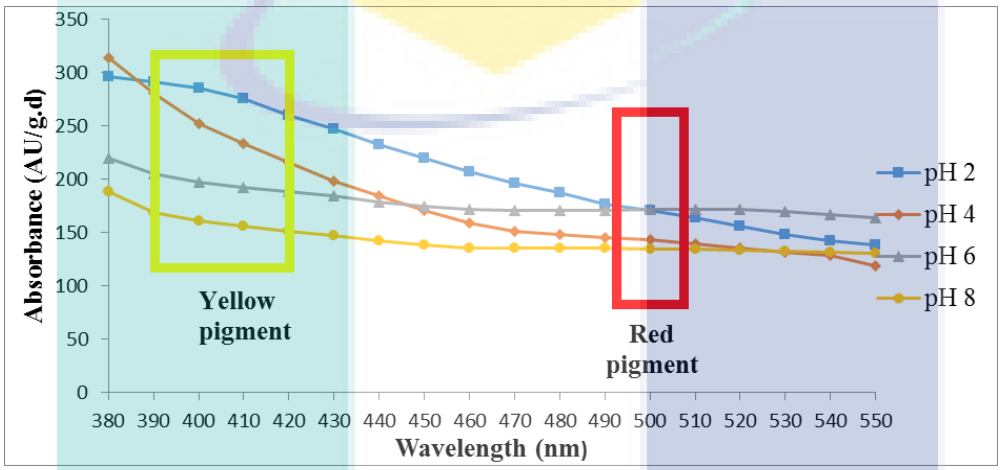

Figure 2. Effect of pH on extraction.

3.3 Effect of time on extraction

Monascus pigment was soaked in the best pH (pH 6) using 60% of ethanol solution as discussed in previous section, at different soaking time from 4 h to 24 h. As shown in Figure 3, the trend was increased as the soaking time extended to 16 h, with the absorbance values of 207 AU/g.d. At 16 h of extraction, an absorbance value was increased up to 2.3–fold when compared to the 4 h extraction. No further increased of the pigment value when the extraction time increased to more than 16 h. The result indicated that the longer the exposure of solute to the solvent, the greater the pigment can be extracted from the solid substance (fermented OPF). At longer soaking time, the contact time between the fermented OPF to the solvent is greater, allowing the phase equilibrium to be established [50]. Hence, the reaction complete, as a result more pigment is extracted from the fermented OPF. Similar findings were reported by Henriques et. al. (2007), Kumar et. al (2017) and Sinha et al., (2012), where the pigments extracted from marine microalga, *Bougainvillea glabra* and *Butea monosperma*, respectively, improved at longer time.

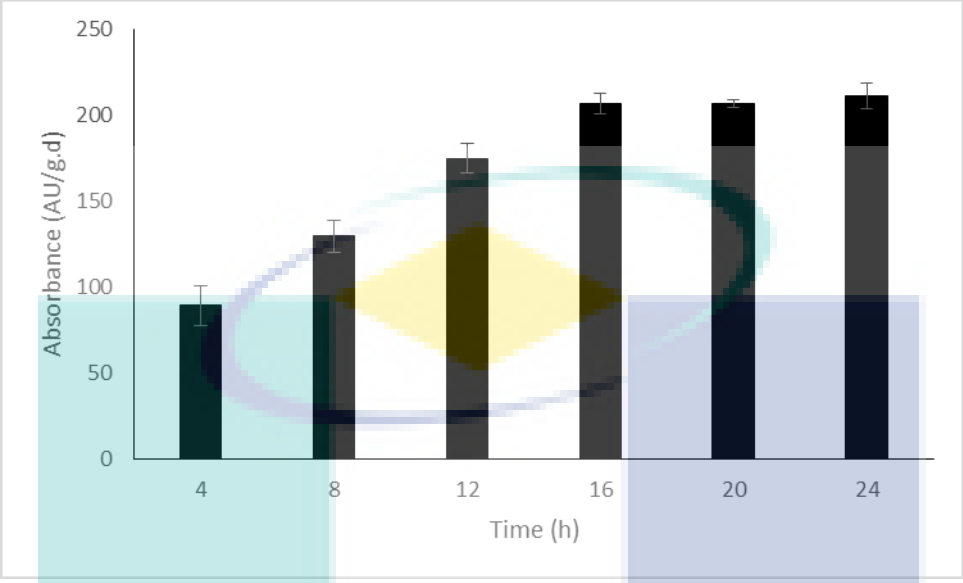

Figure 3. Effect of time on extraction.

4 Conclusion

The key point in pigment extraction is the selectivity of the solvent. Ethanol was used as extraction solvent due to its characteristic such as non-toxic and volatile, which could be significant point to be used in food industry. It was confirmed that red *Monascus* pigment yield can be improved: (i) by using 60% ethanol, (ii) applying pH 6 of ethanol and (iii) extraction time of 16 h. In order to analyze more about extraction method in extracting the red pigment by *Monascus purpureus* on OPF, an optimization of parameters in speed of extraction and extraction temperature need to be investigated in the future. This will in turn provide more important information in order to apply in industrial applications.

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Optimization of Lovastatin in Solid-State Fermentation using Oil Palm Frond

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Abstract. Lovastatin plays a role in lowering the cholesterol level in the human blood, especially the bad cholesterol or low density lipoproteins (LDL). Concurrently, lovastatin increase the good cholesterol or high density lipoproteins (HDL), to prevent the formation of plaque inside the blood vessels. The objective of this research was to experimentally optimize the lovastatin compound produced by *Monascus purpureus* FTC5357 under solid state fermentation (SSF) using oil palm frond (OPF). In order to identify the optimal condition to produce lovastatin, four parameters which were pH, initial moisture content, peptone and potassium, were optimized using Box–Behnken design. Based on the ANOVA analysis performed, initial moisture content, potassium and peptone contributed significantly to the lovastatin production. Meanwhile, pH had the least impact to the lovastatin production. Peptone pronounced to be the most contributed factor, as the lovastatin production increased with the increasing of peptone in the substrate. Under optimized condition (pH 5.50, moisture content at 60%, 3.40 g of potassium, and 3.30 g of peptone) maximum lovastatin yield was 45.84 µg/g. The lovastatin produced through SSF using OPF as a substrate by *Monascus purpureus* FTC 5357 has a great potential to be utilized as a source of lovastatin in future.

1 Introduction

Hypercholesterolemia is the accumulation of cholesterol in blood plasma that causes atherosclerosis (blockage of the artery), leading to the coronary heart disease and heart attack [1]. According to the report of the World Health Organization (WHO), cholesterol problem is estimated to cause 2.6 million deaths annually, which simultaneously increases the risks of heart disease and stroke [2]. Further, hypercholesterolemia stimulates the chances of diabetes development, obesity and certain types of cancers [3].

3–hydro–3–methyl glutaryl coenzyme–A (HMG–CoA) is the key enzyme which inhibits the rate limiting step in cholesterol biosynthesis. Structurally similar to the substrate HMGA–CoA, such as lovastatin can compete the key enzyme and used as a drug for hypercholesterolemia treatment because of their proven efficiency and safety profile [1-4]. Lovastatin belongs to a group of fungal secondary metabolites known as statins. Lovastatin and pravastatin are natural statins; simvastatin is semi-synthetic while atorvastatin and fluvastatin are synthetic statins. Natural statins can be produced through microbial fermentation [5].

Lovastatin can be naturally produced by several fungal species, such as *Aspergillus* species; *Aspergillus niger, Aspergillus terreus, Aspergillus flavus, Aspergillus flavipes, Aspergillus parasiticus* and some *Monascus* species including *Monascus ruber, Monascus paxi, Monascus anka,* and *Monascus*

purpureus [2, 6]. Compared to the other *Aspergillus* species, *Monascus* species possess several advantages, such as non–pathogenic and traditionally acted as fermentation food in Asian countries [2, 7].

Both solid-state fermentation (SSF) and submerged fermentation (SMF) can be applied to produce lovastatin [2]. Compared to SMF, SSF is considered to be the best approach for lovastatin production as it offers numerous advantages such as high concentration of the end-product, less waste water, low sterility and low product repression effect [1, 2, 6, 7]. In addition, production of lovastatin by fermentation decreases the production cost compared to the costs of chemical synthesis [4].

The microbial process especially through SSF process may utilize the agro industrial product such as long-grain rice, sorghum grain, barley, wheat bran, sago, corn, bagasse, barley, soybean meal, gram bran and fruits waste [1, 8]. However, these substrate materials are normally expensive and are need to compete with the human being and the livestock [9]. Thus, there is a need to find alternative substrate for the microbial process. Recently, large quantity of agro-industrial biomass such as from palm oil industry is produced globally especially in the tropical countries, including Malaysia. Oil palm frond (OPF) has made up approximately 70% of total biomass of palm oil industry [10]. Global production of OPF is found to be around 250 million metric tonnes (MMT) in weight which can accommodate a sustainable production of 34.6 MMT of structural carbohydrates [11].

Hence, this study was conducted to study the optimal condition to produce lovastatin from *Monascus purpureus* FTC5357 using OPF in SSF. In order to identify the optimal condition to produce lovastatin, four parameters which were pH, initial moisture content, peptone and potassium, were studied. The Box–Behnken design approach was applied to achieve the objective of the study.

2 Methodology

2.1 Substrate preparation

The fresh OPF was collected from a local palm oil plantation-Lepar, Pahang, Malaysia. The OPF was cut into smaller pieces, washed thoroughly with tap water and dried in oven at 60 °C for 24 h. Then, the dried OPF were grinded to powder form using grinder (Retsch ZM-200, Germany). The OPF powder were sieved using 1 mm mash hole using a sieve shaker (Retsch AS 200 Basic, Germany). Later, the OPF powder was autoclaved at 121 °C for 20 min with distilled water in a ratio of 1:18 (w/v). The treated OPF were filtered and washed with distilled water, before being oven dried at 60 °C for 24 h before used [12].

2.2 Culture and Inoculum preparation

Monascus purpureus FTC5357 used was collected from Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. *Monascus purpureus* FTC5357 was periodically prepared by maintained on Potato Dextrose Agar (PDA), incubated at 30 °C for 7 days and sub-cultured when needed [13] and then stored at 4 ° [2]. The spore were scraped off from agar slant in distilled water under aseptic condition at room temperature. The spore suspension were calculated using Neubauer hemacytometer (Cole-Parmer 79001-00) and adjusted to 10^4 spores per ml for use as inoculum throughout the study [1, 2, 12, 14].

2.3 Solid State Fermentation (SSF) by Box- Behnken's approach

The experimental work was conducted based on the design generated by Box–Behnken of response surface methodology (RSM) using Design Expert 7.0 software. The ranges of the parameters were determined based on the earlier study by Jahromi et al. (2012). The level of parameters for Box-Behnken design was shown in Table 1.

A 5 g of OPF powder was placed into a 250 ml conical flask with desired initial moisture content. The pH, potassium and peptone were adjusted and added to the flask, according to the generated table (Table 2). Next, the medium was autoclaved at 121 °C for 20 min. On cooling, 1 ml of *Monascus*

purpereus FTC 5357 spores were inoculated to the autoclaved medium and incubated at 30 °C for 8 days [12, 15].

Table 1. Level of parameters for Box–Behnken's response surface design.

Parameter	Term	Lower limit (-)	Upper limit (+)
pH	A	4	7
Initial Moisture Content (%)	B	40	60
Potassium (g)	C	0.5	3.5
Peptone (g)	D	0.5	3.5

2.4 Lovastatin extraction and determination

The fermented substrates were dried in an oven at 60 °C for 1 day. A gram of dried fermented OPF was taken for lovastatin extraction and adjusted to pH 3.0 by using hydrochloric acid (HCl). Next, an equal volume of ethyl acetate was added and agitated at 200 rpm at ambient temperature for 15 min using incubator shaker (Infors AG CH-4103 Bottmingen) [6]. The extraction liquid was then filtered through Whatman filter paper [3]. The lovastatin concentration was measured using spectrometer (Hitachi, U-1800) at wavelength of 245 nm [3]. The data collected was subjected to Analysis of Variance (ANOVA). The Response Surface Methodology plots (RSM plots) and contour graph were drawn by using Design Expert software. The means and the standard errors (Mean ± S.E) for each treatment were performed.

3 Result and Discussion

3.1 Design of experiment (DOE) for lovastatin production

RSM plays a very critical role in efficiently exploring the optimal values of explanatory variables. The three dimensional response surface and the contour plot obtained from the RSM, are useful in understanding the main and the interaction effects of the parameters. There can be used to describe and examine the regression equations in a visualized way to reflect the effects of experimental variables on the required response [16].

In this study, the effect of SSF parameters including initial moisture content, pH, amount of peptone and potassium sulphate, on lovastatin production from *Monascus purpureus* FTC3557 were studied. Box-behnken design suggested a set of experiments based on combination of the four parameters (Table 2). The results of lovastatin production was given as an input to the software for further analysis and predict.

From Table 2, run 25 has the lowest amount of lovastatin, whereas run 15 has the highest amount, which were 7.288 µg/g and 46.487 µg/g, respectively. The differences of both conditions were the former was with 40% moisture content, 2 g of potassium and 2 g of peptone, while the latter was with 50% moisture content 3.5 g of potassium and 3.5 g of peptone. From the results obtained, it showed that the higher the amount of peptone used, the higher the amount of lovastatin obtained. This is consistent with Miyake et al. (2006).

Table 2. Optimal design for optimization of lovastatin production.

Run	pH (pH)	Initial Moisture Content (%)	Potassium (g)	Peptone (g)	Response (µg/g)
1	5.50	50.00	2.00	2.00	35.578
2	4.00	50.00	3.50	2.00	21.487
3	5.50	50.00	2.00	2.00	33.630
4	4.00	40.00	2.00	2.00	8.652

5	5.50	50.00	2.00	2.00	33.435
6	5.50	60.00	3.50	2.00	45.794
7	5.50	50.00	0.50	3.50	39.431
8	7.00	50.00	2.00	0.50	15.253
9	4.00	60.00	2.00	2.00	19.496
10	5.50	50.00	0.50	0.50	25.838
11	4.00	50.00	2.00	3.50	23.500
12	5.50	40.00	2.00	0.50	26.119
13	5.50	60.00	2.00	3.50	41.725
14	5.50	40.00	3.50	2.00	29.149
15	5.50	50.00	3.50	3.50	46.487
16	5.50	60.00	0.50	2.00	34.885
17	7.00	50.00	3.50	2.00	9.669
18	4.00	50.00	0.50	2.00	11.704
19	7.00	60.00	2.00	2.00	28.846
20	7.00	40.00	2.00	2.00	10.448
21	5.50	50.00	3.50	0.50	29.668
22	7.00	50.00	2.00	3.50	23.457
23	5.50	40.00	2.00	3.50	33.067
24	5.50	40.00	0.50	2.00	28.500
25	4.00	50.00	2.00	0.50	7.288
26	7.00	50.00	0.50	2.00	10.751
27	5.50	60.00	2.00	0.50	34.171

3.2 Statistical Analysis of lovastatin production

Table 3 shows the Analysis of variance (ANOVA) table of lovastatin production obtained from Box–Behnken experimental design. The ANOVA analysis shows (Table 3) that the developed regression model is highly significant (F-value >16.37), with a p-value of <0.0001. In this study, parameters B, C, D and A^2 are significant model parameters as the p-values were less than 0.05, which implies the regression model is significant. The "lack of fit" F-value of the model was 12.07 with the p-value of 0.0789, implied that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is good as this shows that the suggested model equation fits well with the experimental results. The goodness of fit was evaluated by the R^2 value. The R^2 value found near to 1 indicates that the experimental data are closed to the fitted regression line, where R^2 was 0.9503. The value suggests that the model could predict 95.03% of the variability in the response.

The developed mathematical model for the prediction of lovastatin production can be expressed by equation (1) where the response is the lovastatin production in µg/g. The model suggested as pH (A), initial moisture content (B), amount of potassium (C) and amount of peptone (D). The positive (+) sign in model equation indicates the synergic effects and the negative (-) sign represents antagonistic effects on lovastatin production.

$$\text{Lovastatin Production } (\mu g/g) = -218.0789 + (88.756)\,A - (0.755)\,B + (0.280)\,C + (5.890)\,D + (0.126)\,AB - (1.207)\,AC - (0.890)\,AD + (0.171)\,BC + (0.010)\,BD + (0.358)\,CD - (8.228)\,A^2 + (2.751 \times 10^{-5})\,B^2 - (0.294)\,C^2 + (0.409)\,D^2 \quad (1)$$

Figure 1 shows a graphical representation of the predicted (mathematically calculated) and actual (experimental) plot of model for lovastatin production. The predicted values of lovastatin are quite similar to the experimental values (Figure 1). It demonstrated that the regression model has strong correlation between the model prediction and its experimental results. Thus, the developed regression model is reliable and can be used to predict the lovastatin production [18].

The normality assumptions can be ensured by the construction of normal probability plot of the experimental residual. Figure 2 shows the normal probability plot for the lovastatin. The normal probability shows a straight line, indicated there is no noticeable issue with the normality [18].

Table 3. Analysis of variance (ANOVA) for lovastatin production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3291.690	14	235.121	16.374	< 0.0001*
A	3.306	1	3.306	0.230	0.6400
B	396.551	1	396.551	27.616	0.0002*
C	80.846	1	80.846	5.630	0.0352*
D	400.543	1	400.543	27.894	0.0002*
AB	14.266	1	14.266	0.994	0.3386
AC	29.516	1	29.516	2.056	0.1772
AD	16.035	1	16.035	1.117	0.3114
BC	26.316	1	26.316	1.833	0.2008
BD	0.092	1	0.092	0.006	0.9376
CD	2.600	1	2.600	0.181	0.6780
A^2	1827.861	1	1827.861	127.295	< 0.0001*
B^2	0.404	1	0.404	0.028	0.8697
C^2	2.331	1	2.331	0.162	0.6941
D^2	4.504	1	4.504	0.314	0.5857

*significant

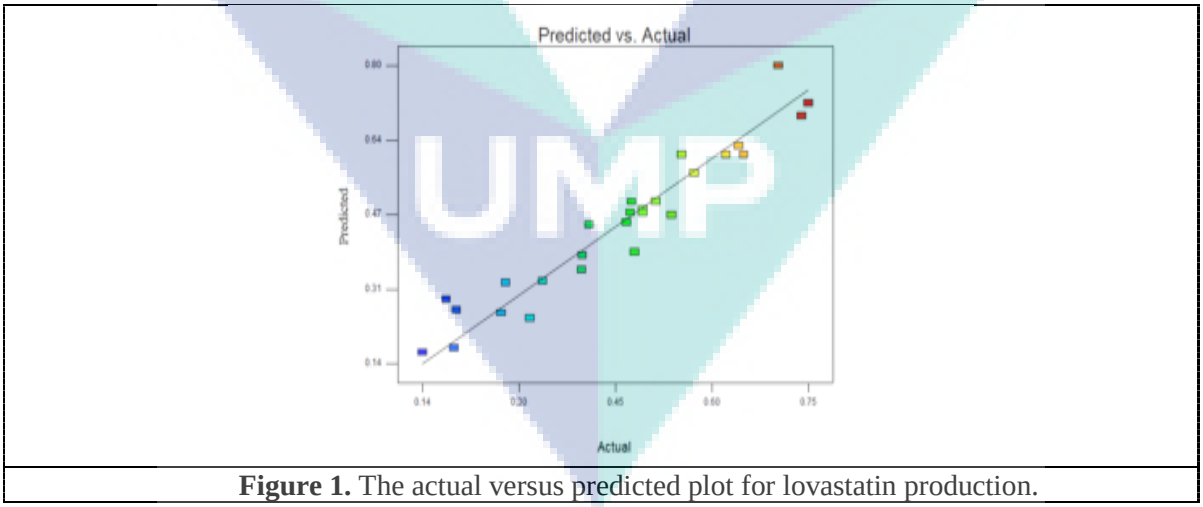

Figure 1. The actual versus predicted plot for lovastatin production.

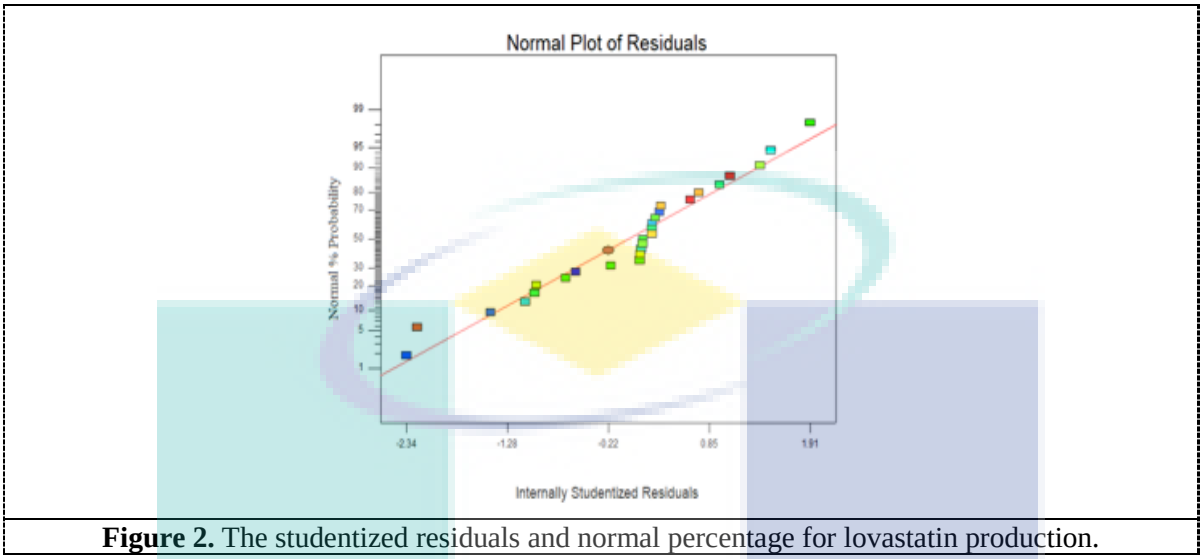

Figure 2. The studentized residuals and normal percentage for lovastatin production.

3.3 Response surface analysis of parameters on lovastatin production

3.3.1 Interaction of pH with other parameters. The three dimensional response surface plots for lovastatin production on pH and the interaction with other parameters are shown in Figure 3. The interactive relationship between AB (pH and moisture content), AC (pH and potassium) and AD (pH and peptone) on the amount of lovastatin produced, are shown in Figure 3(a), 3(b) and 3(c), respectively. The lovastatin productions were maximum at 41 µg/g, 40 µg/g, and 36 µg/g, with respect to interactive parameters of AD, AB, and AC, respectively. At the same condition of pH 5.5, peptone had the most significant effect to the lovastatin, which affected to the better lovastatin production, compared to the other two parameters (initial moisture content and potassium). No further increment of lovastatin at pH beyond the optimum value (pH 5.5). This is because, pH is strongly influences the transport of various components across the cell membrane which in turn supports the cell growth and product formation [4]. Lower or higher pH than the optimum pH during SSF, it will cause the denaturation and inactivation of *Monascus purpereus* FTC 5357 leading to lower lovastatin production [4]. Similar finding had also been reported by Kumar et al., (2000), where the lovastatin production by *Aspergillus terreus* DRCC 122 in the batch process, was optimum at pH range 5–6.5. On the other hand, the optimum pH for lovastatin production by *Monascus purpureus* MTCC 369 was pH 6 [14] and pH 5 by *Aspergillus flavipes* [1]. The above information, suggested that the optimum pH for lovastatin production in SSF is in the ranges of pH 5–6.5, depending on the types of substrate and microorganism used.

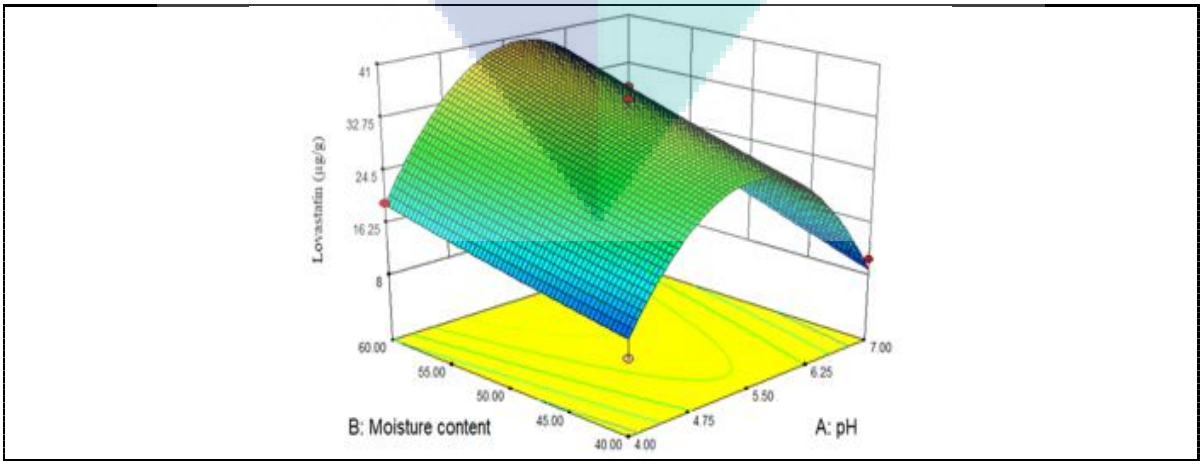

Figure 3(a). The 3D Response Surface of pH and Moisture Content on Lovastatin Activity.

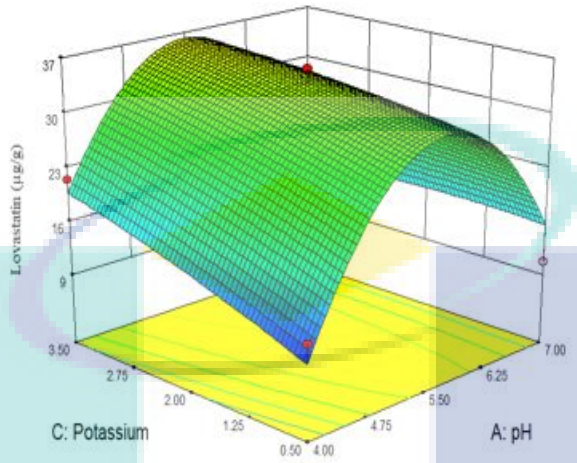

Figure 3(b). The 3D Response Surface of pH and Potassium on Lovastatin Activity.

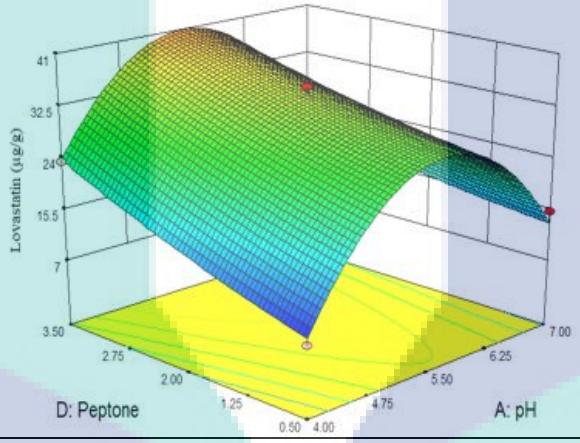

Figure 3(c). The 3D Response Surface of pH and Peptone on Lovastatin Activity.

3.3.2 Interaction between parameters at pH 5.5. The three dimensional response surface plots for lovastatin production at pH 5.5 and the interactive relationship between BC (moisture content and potassium), BD (moisture content and peptone) and CD (potassium and peptone), are shown in Figure 4(a), (b) and (c), respectively. The lovastatin productions were maximum at 44 µg/g, 47 µg/g, and 43 µg/g, with respect to interactive parameters of BC, BD, and CD, respectively. Interaction parameter of BD shows the most significant effect leading to the highest lovastatin production, compared to the interaction parameters of BC and CD.

Figure 4(a) and 4(b) show that increase of moisture content from 40% to 60% facilitate to the production of lovastatin. Initial moisture content of the medium is a key parameter affecting SSF, which control the diffusion of nutrients in the reaction system, maintain the stability and the functional of biological molecules such as proteins, carbohydrates, and nucleotides [19]. *Monascus purpureus* FTC5357 were utilized the substrate at 60% initial moisture content (optimum) and favourable for high production of lovastatin. Furthermore, moisture content basically influence the physical properties of the substrate [7], where the porosity of the substrate is directly decrease as the moisture content increase beyond the optimum value. Resulted in low diffusion of gas exchange. As a result, the lovastatin production started to decline. This is due to the aggregation of substrate particles, reduction of aeration and leading to the anaerobic conditions [3, 9]. Conversely, lower moisture content reduce the metabolic activity and may account for lower lovastatin production [2, 3, 6]. Similar result was obtained by Latha

et al. (2012) whom conducted optimization of lovastatin using coconut oil cake by *Aspergillus fischeri*. They reported that the highest lovastatin yield at 60% moisture content. The other researchers claimed that the optimal initial moisture content of *Monascus ruber, Monascus purpureus* 9901 and *A. terreus* were set in the range of 50–55 %, in SSF [2, 7, 9].

Figure 4(b) and 4(c) clearly show that lovastatin production increased with the increase of peptone. Slow-acting organic nitrogen source, such as peptone, was more favour, due to the long-term biosynthesis of lovastatin [2]. It was because organic nitrogen source possess complex nutrients compared to the inorganic nitrogen source [2]. Selection of carbon and nitrogen in the fermentation medium are key parameters to the lovastatin production, as they acted as precursors and cofactors for the formation of biomass and lovastatin product [2, 4, 7]. Higher concentration of peptone contributed to the better growth of *Monascus purpureus* FTC5357 by shortening the growth period and rapid entry to the stable phase, and consequently synthesize the product [7]. However, extreme level of peptone will repressed the production. There have been several reports of the production of lovastatin using SSF from organic nitrogen source [2, 7, 20]. However, the optimum value of organic nitrogen to give the maximum lovastatin yield, are different. These results might be directly due to the proportion variance of the carbon nature and the nitrogen sources [4].

Figure 4(a) and 4(c) show that potassium ions contribute to the increase of lovastatin. However, if the potassium amount is higher than the optimum value (3.40 g), it reduced the production. It was due to the interactions with lower moisture content (Figure 4 a) and lower peptone (Figure 4 c), which were affected much to the fungal growth, consequently lowering the lovastatin production. Although potassium is an essential growth nutrient and affected to the lovastatin production [21], though peptone and moisture content are more important to the lovastatin production. This behaviour was also observed by Jahromi et al. (2012), where the lovastatin yield increased using OPF in *A. terreus* ATCC 74135 by added the minerals.

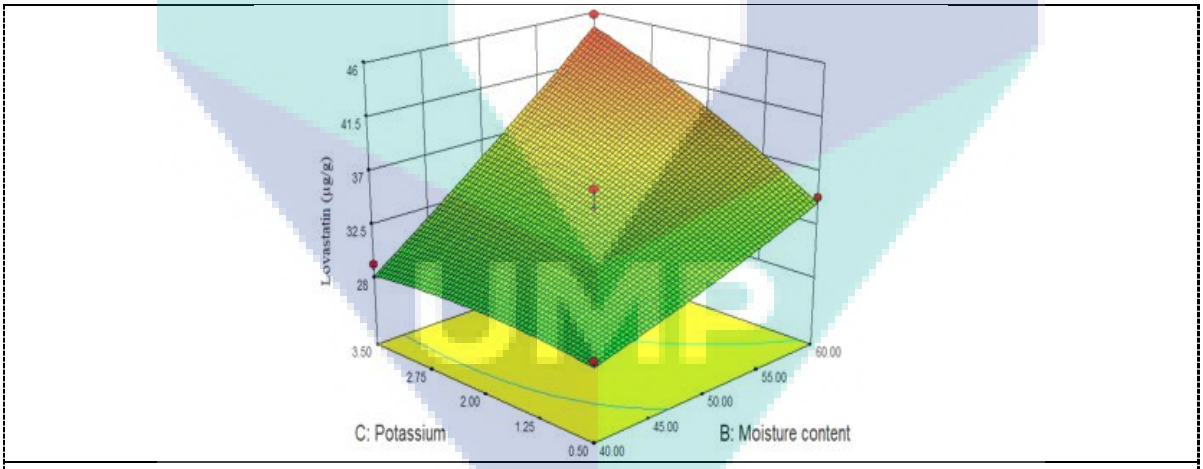

Figure 4(a). The 3D Response Surface of Moisture Content and Potassium on Lovastatin Activity.

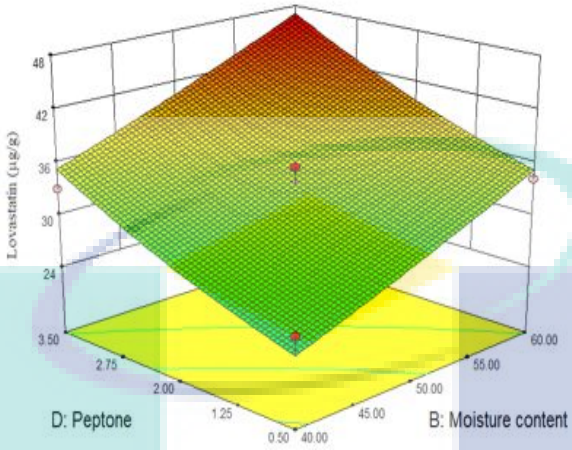

Figure 4(b). The 3D Response Surface of Moisture Content and Peptone on Lovastatin Activity.

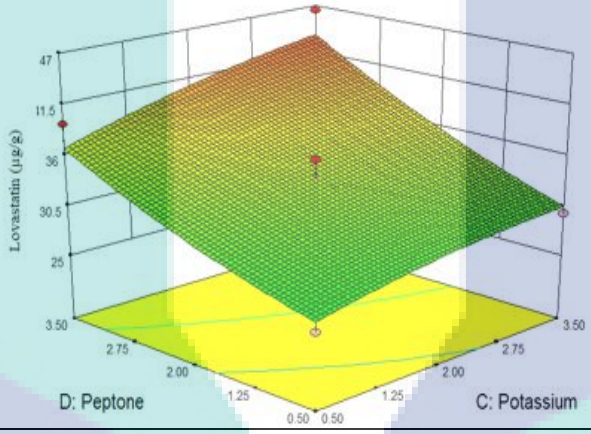

Figure 4(c). The 3D Response Surface of Potassium and Peptone on Lovastatin Activity.

To validate the optimal condition for lovastatin production by *Monascus purpereus* FTC5357, an experiment was conducted under the predicted optimal conditions. Under the suggested conditions, the predicted optimal values of parameters were at 60% of moisture content, pH 5.5, 3.30 g of peptone and 3.40 g of potassium. The predicted amount of lovastatin obtained was 49.63 µg/g. While, the actual value obtained through triplicate analysis was 45.84 µg/g. The percentage error obtained was less than 10%, and it was considered acceptable conditions to optimize the lovastatin by SSF using *Monascus purpereus* FTC5357.

4 Conclusion

Response surface methodology was proven suitable to be applied for the optimization of SSF using OPF as substrate. Box-Behnken experimental design which developed the ANOVA analysis showed that the second-order polynomial model was valid and adequate to study the effects of parameters on lovastatin production. Based on the ANOVA analysis, peptone was the most significant parameter, followed by moisture content, potassium and pH values. In short, it can be observed that the higher the pH, the higher the production of lovastatin, until optimal pH of 5.5. The production efficiency will drop beyond the optimal point. The optimal conditions for lovastatin production via SSF were at pH 5.5, 3.30 g peptone, 3.40 g potassium and 60% moisture content. The maximum lovastatin produced was 49.626 µg/g. Validation experiment was done to confirm the adequacy of the model by producing lovastatin under the optimal condition. The experimental value was 45.8376 µg/g of lovastatin, confirming the validity

of the model with percentage error of 8.63%. Based on the results of the experimental study, the usage of *Monascus purpureus* FTC5357 in SSF which acts as a natural source to produce desired amount of lovastatin. The lovastatin produced through SSF by *Monascus purpureus* FTC5357 using OPF had indicated that it has a great potential to be utilized as the source of lovastatin in future. The results in this study may expand our understanding on the application of SSF using agricultural waste as substrate.

Acknowledgements

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Effect of factors on the red pigment production in the stirred drum bioreactor: Fractional factorial design approach

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Abstract. Public demands on natural colouring over the synthetic colouring are growing due to consumer health concern towards the effect of synthetic colouring. The colorants production by employing fermentation process shows certain advantages. This research has emphasized on the capability of stirred drum bioreactor on performing red pigment in solid-state fermentation (SSF) of *Monascus purpureus* FTC 5357 by using oil palm frond (OPF). A Fractional factorial experimental design 2^4 (FFD) was applied in order to evaluate the effect of initial moisture content (IMC), inoculum size, percentage of peptone, pH, aeration rate, loading capacity and agitation programme on the red pigment production. In the FFD experimental studies, the highest pigment production was obtained at 75% (v/w) initial moisture content, 10^8 spores/mL of inoculum size, initial pH of 4, aeration rate of 1.21 vvm, loading capacity of 35% (v/v), agitation programme of 6 cycles/day, with the yield of 71.86 AU/g. The initial moisture content had the most pronounced affects to the red pigment production, followed by the aeration rate of stirred drum bioreactor, peptone concentration and inoculum size. The loading capacity of the bioreactor, agitation programme and the initial pH had the lesser effect to the red pigment production. The result indicated that FFD was a useful tool to improve the red pigment production in SSF using stirred drum bioreactor by considering all the factors involved.

INTRODUCTION

Generally, most of the food manufacturer imposed to use colorant to enhance the desirability of the food product. Public demands on natural colouring over the synthetic colouring are aggressively growing due to the consumer health concern towards the effect of synthetic colouring [1]. In 2014, the natural food colorant market has reached around the value of US$1.14 billion [2,3], and it was expected to rapidly increased, and continue growing by 10% to 15% annually [4].

Due to this, natural food colorants are largely being extracted from plant sources [5]. However, different concerns are about to rise due to the large-scale production of natural food colorants, especially on the sustainability of the resources. Therefore, the colorants production by utilizing fermentation process shows certain advantages over the other sources [6]. The production of natural colorants (pigments) using microorganisms gained a significant attention due to certain advantage such as able to be grown rapidly under highly controlled conditions compared to the other available sources, which were results in a high productivity [7,8,9]. One of the potential microorganisms for large scale pigment production is from *Monascus* sp., as its competency to produce an intense red pigment as well as other beneficiary metabolic by-products [10]. Regardless of the high pigment yields in solid-state fermentation compared to the submerged cultivation [11], the mechanical aspects such as the bioreactor design for solid-state fermentation are vastly unexplored. Hence, this study was emphasized on *Monascus purpureus* in the solid state fermentation (SSF) using oil palm frond (OPF) in stirred drum bioreactor for red pigment production.

A fractional factorial experimental design 2^4 (FFD) was applied in order to evaluate the effect of seven factors such as initial moisture content (IMC), inoculum size, percentage of peptone, initial pH, aeration rate, loading

capacity and agitation programme to the red pigment production. The fermentation process was conducted in a 2.3 L stirred drum bioreactor.

MATERIALS AND METHODS

Microorganism, inoculum preparation and substrate preparation

Monascus purpureus FTC 5357 culture was maintained on Potato Dextrose Agar (PDA) and incubated in at 30ºC for 8 days [12]. Fully sporulated agar slant culture was prepared prior to inoculum preparation. Sterile distilled water was added to the slant culture, followed by gentle scrapping on the slant surface to harvest the spore. The spore concentration was measured and adjusted accordingly to the desired concentration for fermentation.

The fresh oil palm fronds (OPF) were obtained from a local palm oil plantation in Federal Land Development Authority (FELDA) Bukit Goh, Kuantan, Pahang, Malaysia. The fresh OPF was cut into smaller pieces, cleaned and prepared as mentioned by Hamid and Said (2018) [13].

Experimental design and cultivation condition

A specifically fabricated 2.3 L stirred drum bioreactor from the Faculty of Chemical and Natural Resources Engineering (FKKSA) laboratory, UMP, Malaysia, was used [14]. The experimental work was done based on the experimental design, using fractional factorial design (FFD) 2^4, being set by Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA), (Table 1 and Table 2). The best range of each factor was selected by applying the One Factor at A Time (OFAT) method as in the preliminary experiment (data not shown). All experiments have been carried out in replicates and each run has been harvested on day 8 after inoculation.

An empty bioreactor drum and the treated OPF having specified initial moisture content, peptone, and adjusted pH, as stated in Table 2, were separately autoclaved. After being cooled to room temperature, the substrates were inoculated with specified inoculum volume as in Table 2, evenly mixed and aseptically transferred to the bioreactor. The cultures were cultivated for 8 days at room temperature.

TABLE 1. Independent variables, responses and the levels in the experimental design

Symbol	Factors	-1	1
A	Initial moisture content (% v/w)	55	75
B	Inoculum size (spores/mL)	10^8	10^{12}
C	Peptone concentration (% w/w)	4	6
D	Initial pH (pH)	6	8
E	Aeration rate (vvm)	0.87	1.21
F	Loading capacity (% v/v)	25	35
G	Agitation programme (cycles/day)	6	10
	Responses		
Y_1	Red pigment production (AU/g)		
Y_2	Biomass (mg/g)		

TABLE 2. The fractional factorial design of the independent variables

Run	A	B	C	D	E	F	G
1	55	10^8	4	6	0.87	25	6
2	75	10^8	4	6	1.21	25	10
3	55	10^{12}	4	6	1.21	35	6
4	75	10^{12}	4	6	0.87	35	10
5	55	10^8	6	6	1.21	35	10
6	75	10^8	6	6	0.87	35	6
7	55	10^{12}	6	6	0.87	25	10
8	75	10^{12}	6	6	1.21	25	6
9	55	10^8	4	8	0.87	35	10
10	75	10^8	4	8	1.21	35	6
11	55	10^{12}	4	8	1.21	25	10
12	75	10^{12}	4	8	0.87	25	6
13	55	10^8	6	8	1.21	25	6
14	75	10^8	6	8	0.87	25	10
15	55	10^{12}	6	8	0.87	35	6
16	75	10^{12}	6	8	1.21	35	10

Analytical methods

Red pigments were determined using a UV-VIS spectrophotometer (Hitachi U-1800) [13]. The yield was expressed as absorbance units (AU) per gram of dried solids [15]. Total fungal biomass was determined using spectroscopy method, by measuring the N-acetylglucosamine released by acid hydrolysis of the chitin in the fungal cell walls [13,16].

Statistical analysis

A Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA) software was used for the experimental design of fractional factorial design. Statistical parameters were estimated using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

A fractional factorial design (FFD) was conducted to screen out the insignificant factors in order to gain effective red pigment production in stirred drum bioreactor. Factorial design is efficient to evaluate the effect of factors over a wide range of conditions with a minimum number of experiments.

The selections of significant model factors were done using design expert software. Figs. 1 and 2 show the pareto charts of red pigment and biomass productions, which represented the estimated effects of the factors and their interactions on the responses variables. The pareto charts show 2 different t-limit; Bonferroni corrected t-limit (or Bonferroni limit) and standard t-limit (or t-value limit) (Figs. 1 and 2). Effects that are above Bonferroni limit are considered almost certainly significant, while the t-value limit indicated the possibly significant effects. While, effects that are below t-value limit are not likely to be significant, at a confidence level of 95%. On the other hand, the factors in Figs. 1 and 2 also characterized into two categories; factors with positive and negative effects. Factors with positive effect are directly correlated to the responses' value (red pigment and biomass), and factors with negative effect are inversely correlated. For instance, initial moisture content (A) (Fig. 1) showed the high positive effect, which indicated that the red pigment was greater in higher initial moisture content. Contrarily, for negative effect, higher peptone (C) would contributed to low red pigment production (Fig. 1). This can be explained due to the excessive nutrient provided in the substrate medium make the medium become toxic and inhibit the growth of the *Monascus* sp. [17].

Fig. 1 clearly exposed that the most statistically significant factors to the red pigment were initial moisture content (A), followed by aeration rate (E), peptone concentration (C), initial moisture-inoculum size (AB) and initial moisture-aeration rate (AE). While, inoculum size (B) was the least significant effect to the red pigment.

While, in Fig. 2, factor initial moisture content (A) also showed the most significant factor to the biomass production. Followed by peptone concentration(C), aeration rate (E) and initial moisture content-inoculum size (AB), at 95 % of confidence level. Initial moisture content (A) and aeration rate (E) showed positive effects to the biomass production, while and peptone concentration (C) and initial moisture content-inoculum size (AB) show negative effects (Fig. 2). The effectiveness of initial moisture content (A) was in agreement with several reports in literature stating that the moisture content of the substrate significantly affect the biomass growth of the *Monascus* sp. [15,18].

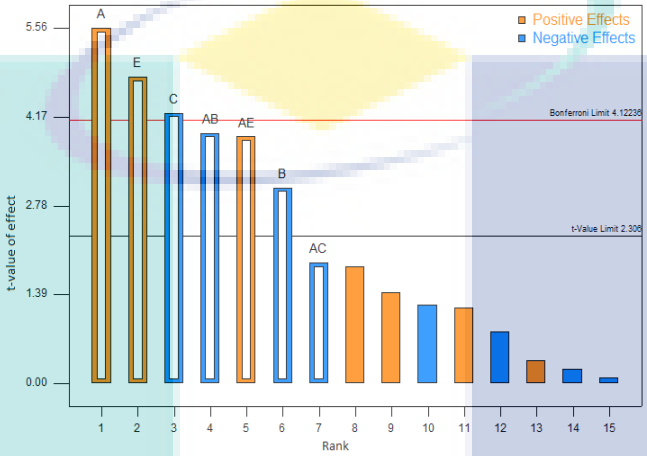

FIGURE 1. Pareto chart for red pigment production

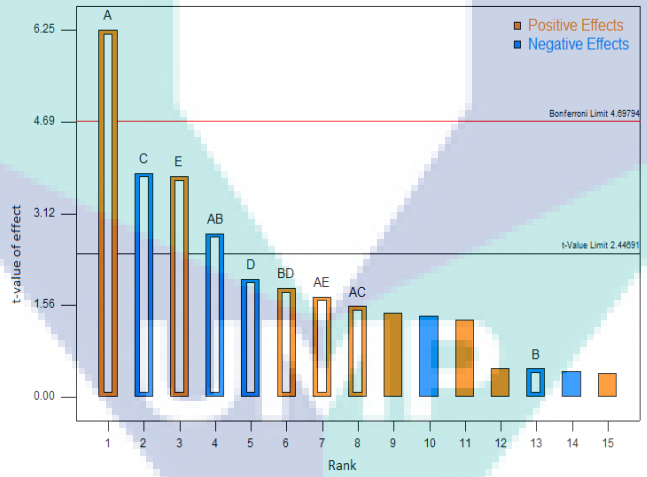

FIGURE 2 Pareto Chart of biomass production

Analysis of variance (ANOVA) for red pigment and biomass productions

The significance of the factors shown in Figs. 1 and 2 were verified by the analysis of variance (ANOVA) in Table 3 and 4, respectively. The quadratic regression model of ANOVA showed that the model was significant to the red pigment with low p-value (0.0004) (Table 3). It also demonstrated that the model has a high correlation with the experimental data. In addition, Table 3 also reveals that the most significant factors to the red pigment was initial moisture content (A), followed by aeration rate (E), peptone concentration (C), initial moisture-inoculum (AB), initial moisture-aeration (AE), and inoculum (B); where all the factors showed the p-value of <0.05. The results were consistent with the pareto chart discussed earlier (Figure 1). The R-squared of the quadratic response surface model of the red pigment obtained was 0.933, which found to be close to 1, indicated the good relation of the predicted and the experimental data of the red pigment. This model also showed a reasonable agreement between the adjusted (0.875) and predicted (0.734) R-squared values. The adequate

precision for the red pigment was 12.3, indicated that the quadratic models obtained were significant for the process [13].

Whereas, in Table 4, the quadratic regression model of the biomass production was significant at 95% confidence level, with relatively low p-value (0.0065). The most significant factors to the biomass was comparable to the red pigment, which was initial moisture content (A), followed by peptone concentration (C), aeration rate (E) and initial moisture-inoculum (AB). Further, the R-squared of the quadratic response surface model of biomass production was 0.934, close to 1. The R-squared obtained showed relatively good relation of both predicted and the experimental data of the biomass production.

TABLE 3. Analysis of variance (ANOVA) for red pigment production

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	4576.75	7	653.82	16.02	0.0004*
A	1261.13	1	1261.13	30.91	0.0005*
B	382.59	1	382.59	9.38	0.0155*
C	731.16	1	731.16	17.92	0.0029*
E	937.89	1	937.89	22.98	0.0014*
AB	625.00	1	625.00	15.32	0.0045*
AC	144.72	1	144.72	3.55	0.0964
AE	607.87	1	607.87	14.90	0.0048*
Residual	326.45	8	40.81		
Cor Total	4903.20	15			
R^2	0.933				
Adj R^2	0.875				
Pred R^2	0.734				
Adeq Precision	12.298				

*significant ($p<0.05$),
Notes: A-initial moisture content, B-inoculum size, C-peptone, E-aeration rate

TABLE 4 Analysis of variance (ANOVA) for biomass production

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	208810.55	9	23201.17	9.41	0.0065*
A	96209.63	1	96209.63	39.02	0.0008*
B	564.54	1	564.54	0.23	0.6492
C	35758.81	1	35758.81	14.50	0.0089*
D	9887.02	1	9887.02	4.01	0.0921
E	34638.79	1	34638.79	14.05	0.0095*
AB	19063.32	1	19063.32	7.73	0.032*
AC	5849.19	1	5849.19	2.37	0.1744
AE	7120.83	1	7120.83	2.89	0.1401
BD	8392.39	1	8392.39	3.40	0.1146
Residual	14793.32	6	2465.55		
Cor Total	223603.87	15			
R^2	0.934				
Adj R^2	0.835				
Pred R^2	0.530				
Adeq Precision	11.129				

*significant ($p<0.05$),
Notes: A-initial moisture content, B-inoculum size, C-peptone, D-pH, E-aeration rate

Interaction factors on the red pigment and biomass production

Figs. 3 and 4 denote the interaction factors of initial moisture content and aeration rate (AE) and initial moisture content and inoculum size (AB), to the red pigment, respectively. In Fig. 3, the pigment production was increased as the AE increased. For instance, the higher aeration rates, the higher red pigment production. The red pigment production was in favor at higher initial moisture content and higher aeration rate. The increased of initial moisture content proportionally increased the solubility of nutrients in the substrate. Hence, promoted to the fungal

growth, consequently increased the production of the red pigment. In addition, an adequate aeration in the bioreactor facilitates to the oxygen transport process [18], besides stimulated to the transformation of accumulated heat in the substrate bed in the bioreactor [14,19]. These interaction (AE) suggested the synergic effects between initial moisture content and the humidified air supplied throughout the fermentation in the bioreactor, to the red pigment production.

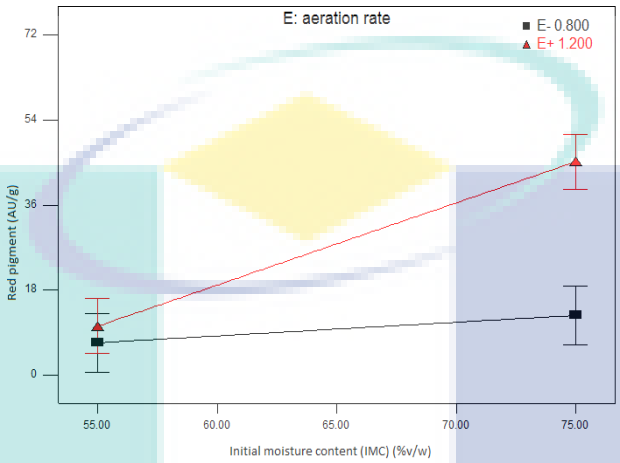

FIGURE 3. Interaction of initial moisture content and aeration rate (AE) on red pigment production. Other factors were fixed at 10^9 spores/ml, 5% (w/w) peptone, pH 7, loading capacity 30% (v/v), agitation programme 8 (cycles/day)

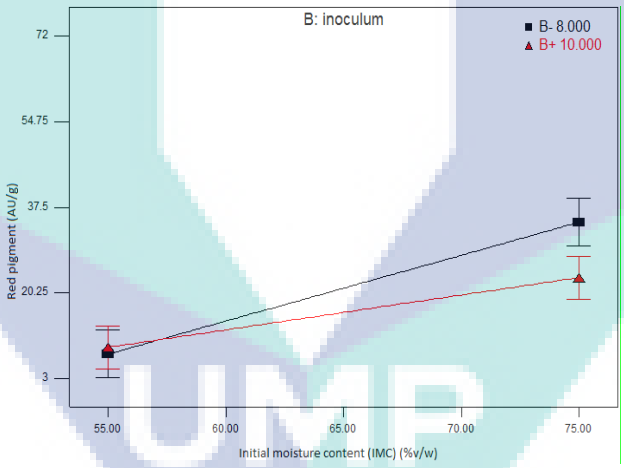

FIGURE 4. Interaction of initial moisture content and inoculum size (AB) on red pigment production. Other factors were fixed at 5% (w/w) peptone, pH 7, loading capacity 30% (v/v), agitation programme 8 (cycles/day), aeration rate 1 (vvm)

In Fig. 4, at low initial moisture content, the response showed relatively in low pigment production to both high and low inoculum size. However, at higher moisture content, the response favored to the lower inoculum size, although generally there was slight increment of response at higher inoculum size. Various possible explanations can be accounted for this occurrence. From the mechanical aspect of fermentation, the high water content would likely to facilitate the heat regulation process in the drum bioreactor. While, on the other aspects, at higher inoculum size, the overpopulation of the fungal might be occurred in the bioreactor. The overpopulation of the *Monascus* sp. in the bioreactor may resulted to the nutrient exhaustion and oxygen depletion [18], thus caused in the unsustainable biological system in the bioreactor. This phenomenon would lead to the disruption of the pigment producing performance in the bioreactor.

Fig. 5 shows the interaction factors of initial moisture content and inoculum size (AB), to the biomass production. At low initial moisture content, the biomass production slightly favored to the high inoculum size. However, at higher moisture content, the biomass was favored to the lower inoculum size (Fig. 5). This result is highly corresponded to the earlier discussion in Fig. 4.

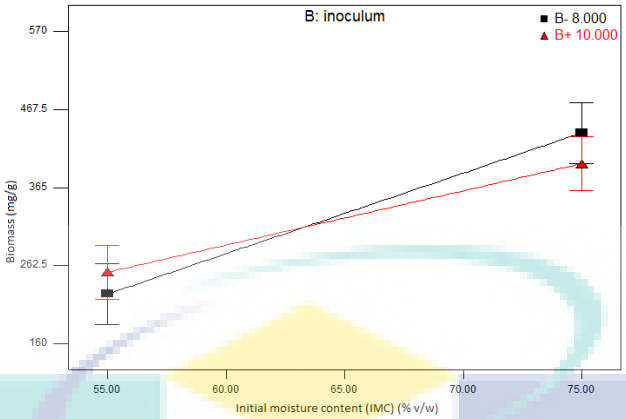

FIGURE 5. Interaction of initial moisture content (A) and inoculum size (B) on biomass production. Other factors were fixed at 5% (w/w) peptone, pH 7, loading capacity 30% (v/v), agitation programme 8 (cycles/day), aeration rate 1 (vvm)

CONCLUSION

The study showed that in the stirred drum bioreactor, the initial moisture content had the most pronounced affects to the red pigment and biomass productions. Followed by the aeration rate and the peptone concentration. The inoculum size, loading capacity of the bioreactor, agitation programme and the initial pH had the lesser effect to the red pigment and biomass production. The result indicated that FFD was a useful tool to screen the significant factors that contributed to the red pigment production to further optimized for larger scale production in stirred drum bioreactor.

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