THE OPTIMIZATION OF RED PIGMENT BY *MONASCUS PURPUREUS* FTC 5356 IN SOLID STATE FERMENTATION

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ABSTRACT

Monascus red pigment had been found to have capability to give natural color that meet demand in the industries. The objective of this research is to identify the optimize condition of red pigment production in solid state fermentation by *Monascus Purpureus* FTC 5356 using local agricultural products as a substrate. The substrates used in this research were banana, corn, papaya, coconut meat, white rice, pumpkin and guava. In determining the best substrates, the initial moisture content was set up at 50%, pH 6 for 10 days at 30°C. Among the local agricultural products, it was identified that banana is the best with 0.3390AU/g.day of red pigment production. The optimum condition for the pigment production in parameter study was 50% of initial moisture content and addition of 1.8% of nitrogen source to the substrate. The optimum growth for pigment production was 7 days. The further experiment will be conducted using Central Composite Design (CCD) of Response Surface Methodology (RSM) by setting parameter of nitrogen source concentration and moisture content of the substrate in producing high pigment production.

ABSTRAK

Pigmen merah *Monascus* telah dikenalpasti mempunyai keupayaan untuk menghasilkan warna semula jadi yang memenuhi permintaan di dalam industri. Objektif kajian ini adalah untuk mengenal pasti keadaan yang optimum bagi pengeluaran pigmen merah oleh *Monascus Purpureus* FTC 5356 dalam proses penapaian pepejal dengan menggunakan hasil agrikultur tempatan sebagai substrat. Substrat yang digunakan dalam kajian ini adalah pisang, jagung, betik, isi kelapa, beras putih, labu dan jambu batu. Dalam menentukan substrat terbaik, kandungan kelembapan awal bagi setiap substrat telah ditentukan ditentukan pada 50% dan pH 6 dan dibiarkan di dalam inkubator selama 10 hari pada 30 °C. Pisang telah dikenalpasti sebagai substrat terbaik di antara hasil-hasil agrikultur tempatan yang lain dengan penghasilan pigmen merah sebanyak 0.3390AU/g.hari. Dalam pengkajian parameter, keadaan optimum untuk substrat bagi penghasilan pigmen adalah pada 50% kandungan kelembapan awal dan penambahan sumber nitrogen sebanyak 1.8%. 7 hari diperlukan bagi pertumbuhan optimum untuk penghasilan pigmen oleh *Monascus Purpureus* FTC 5356. Kaedah seterusnya adalah dengan menggunakan Design Pusat Komposit (CCD) oleh Kaedah Permukaan Respon (RSM) dengan menetapkan dua parameter iaitu kepekatan sumber nitrogen dan peratusan kandungan kelembapan substrat dalam penghasilan pengeluaran pigmen yang maksimum.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Color and flavor are the signals that are immediately received by the optical and chemical senses of humans and these determine whether a certain food is appealing (Babitha et al., 2006). In the past, synthetic colorant or dyes are widely used and popular because of their stability and low cost. In the international food processing industry, most of the food will be dye with attractive colorant to give impact in the color of the product. The attractive food color is usually to increase the consumption which is a normal human behavioral response (Babitha et al., 2006). Thus, these mean that the production of color is very important and profitable.

According to Velmuguran et al. (2009), A large amount of dyestuff being directly lost in the waste water were due to efficiencies in dyeing and the increased of dyes or colorants applications in dyeing industries. These consequent may affect the environment. In the case of reactive dyes, 50% of the initial dye load is present in the dye bath effluent and during the dyeing process, it is estimated that 10-35% of the dyes lost in the effluent (Rai et al., 2005; Velmuguran et al., 2009). These dyes or colorants also have a carcinogenicity to harm human's health. Hence, the increasing in the concerns on the health and environment has lowering the demand on the use of synthetic coloring. Thus, causes the demand for eco-friendly colorants to increase.

There is several numbers of natural colors that extracted from plant flowers, fruits, leaves and roots but only few are available in a sufficient quantity to be use in the industry. Therefore, an advantage to produce natural colors from microorganisms, and some pigment had been shown being produce from *Monascus* (Kim et al., 1999). *Monascus* sp produce three categories of pigments, orange, red and yellow. Among these pigments, the red pigment (monascorubramine and rubropunctamine) is of high demand (Gunjan Mukherjee and Sanjay Kumar Singh, 2010).

Most of the studies had been performed using liquid culture medium on general culture condition and substrate evaluation for pigment production. But, there is a need for developing low cost process than the currently used technology of pigment production on industrial scale (Babitha et al., 2006). According to Wook-jin et al.(2002), the solid-state culture technique is preferable technique than the submerged culture technique because the solid-state technique is simple, requires less capital investment, allows for better product recovery, and yield a high-quality product.

During optimization process, screening or selection of important parameters influencing fermentation productivity is initially carried out by Central Composite design, and finally using different advanced statistical techniques, the screened parameters are optimized. A widely used technique for optimization of fermentation process parameters is a Response surface methodology (RSM). RSM has its own advantages which it requires less experiment runs, and is suitable for multiple factor experiments, search for relationships between factors, and for finding the most suitable condition and prediction of response (Panda et al, 2010).

1.2 Problem Statement

Many of the research had been done for producing the natural color because the synthetic colorants that been widely used in industry to give an attractive color for the food has high potential of carcinogenicity which can harm human health. *Monascus* pigment had been found to have capability to give natural color thus meet demand in the industries and among of all color, red colors are use commercially in the industries and have attracted the worldwide interest. Also, the red pigment of *Monascus* is the most stable and edible compared to other pigment. Production of pigment from fungi has its own advantages because fungi like *Monascus* species can grow rapidly which may lead to high productivity of the product. Therefore, this research is about to produce red pigments by *Monascus* sp.

Cost of production is the most critical and primary focus in the industries in order to produce high production of product using low value of cost. Therefore, solid state fermentation is the best result to lowering the cost of production in producing the red pigment of *Monascus*. By using local product such as banana, white rice, guava, pumpkin, coconut meat and corn which is economically viable and cheap as substrate in this research is essential to more lowering the cost of production.

In fermentation, there is variety of parameters that could affect the process performance. Fermentation is known to be dependent on moisture content of the substrate and nutrition (carbon or nitrogen source) concentration for the fungi. So, that is why optimization steps need to be done in order to maximize the production rate of *Monascus* pigment. The parameters need to be control in order to provide the perfect fermentation condition in this fermentation process.

1.3 Objective

To identify the optimize condition of red pigment production in solid state fermentation by *Monascus Purpureus* FTC 5356 using local agricultural products as a substrate.

1.4 Scope Of The Study

To achieve the objective, few scopes have been identified in this research:

i. To investigate the capability of red pigment production by *monascus purpureus* in solid state fermentation using local product such as white rice, corn, guava, pumpkin, banana and coconut meat as the substrates.

ii. To determine the best substrate based on pigment production and biomass estimation.

iii. To study the effect of percentage of substrate moisture content and concentration of nitrogen source on pigment production.

iv. To compare the production of the red pigment from local agricultural product with the production of red pigment from the white rice that had been widely used in the worldwide industries.

v. To applied Design of Experimental software to design experimental work for *Monascus* red pigment production.

1.5 Rationale And Significance

The aim of this study is to explore for new beneficial source of substrates from local agricultural product so that the cost of growth factor can be lower down. It was also to estimate the optimum parameter values in order to get high yield of red pigment production in solid state fermentation by using white rice, corn, banana, guava, coconut meat and pumpkin as media to *Monascus purpureus* culture. Thus, the capability of local agricultural product as the substrate can be commercialized and will bring new changes in the local industries. The application of Design of Experimental software via Response Surface Methodology (RSM) is a step development in recent technology and to expose others with new technology that can easier the experimental work.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Monascus species are known fungi which is the sources of various secondary metabolites.Pigments synthesized by *Monascus* sp. was traditionally used in Asia for coloring and as preservative of fermented foods, such as alcoholic beverage, red soybean curd, meat, and vegetables. According to Lee et al., (2002), *Monascus* sp. can produce six related pigments, which can be divided into three groups: Two are orange (rubropunctatin and monascorubin), two are yellow (monascin and ankaflavin) and two are red (Rubropunctamine and monascorubramine). Because red is the most popular food coloring and true red natural pigments that suitable for food use are really difficult to obtain, the red pigments are of particular interest among other pigments.

The production of *Monascus* pigments can be obtained in both solid-state and submerged culture. However, the solid-state culture technique is a preferable technique than submerged because it is simple, requires less capital investment, features lower levels of catabolite repression and end-product inhibition, produces lower amount of waste water output, allows for better product recovery, and yield a high-quality product (Lee et al., 2002).

The approach of using agricultural waste as substrate consumes a low cost of operation when compared with liquid fermentation and gives high pigment productivity (Nimnoi and Lumyong, 2009; Cavalcante et al. 2008). These reports indicated that utilization of cheaply available substrates in SSF could be a good strategy for attaining significant pigment production (Nimnoi and Lumyong, 2009).

2.2 *Monascus*

Monascus sp. is in the group of Ascomycetes and particularly to the family of Monascaceae. The genus *Monascus* can be divided into four species which they are *M. pilosus, M. purpureus, M. ruber* and *M. froridanus* that account for the majority of strains isolated from traditional oriental food. *M. purpureus* is spherical in shape of 5 microns in diameter or slightly ovoid and the mycelium is white in the early stage. However, it rapidly changes to a rich pink and subsequently to a distinctive yellow orange color (Pattanagul et al., 2007). Monascus has at least six molecular structures of pigment which is classified into three groups based on their color and each has two components of polyketide origin, that are secondary metabolites possessing a common azaphilone skeleton (Zhou et al., 2008). They are yellow pigments of monascin (C21H26O5) and ankaflavin (C23H30O5), the orange pigments of monascorubrin (C23H26O5) and rubropunctatin (C21H22O5) which possess the oxo-lactone ring and the red pigments of monascorubramine (C23H27NO4) and rubropuntamine (C21H23NO4) which are the nitrogen analogues of the orange pigments (Pattanagul et al. 2007; Nimnoi and Lumyong, 2009). The color specification depends on the associated amino acid or protein (Lian et al. 2007; Nimnoi and Lumyong, 2009). The pigments produced by Monascus are safe with their special characteristics that have high protein adhesion, thermal stability and wide-range-pH stability (Zhou et al., 2008)

2.2.1 Uses of *Monascus* Pigment

As far as industrial production is concerned recently, *Monascus* is one of the microbial resources which are used to produce pigments (Zhou et al., 2008). According to Lee et al. (1995), the red polyketide pigments of the fungus *Monascus* are widely used as natural food colorant in Oriental countries. These filamentous fungi were used to make rice wine, soy bean cheese and anka which are red rice in many Asian countries like Japan and China for centuries. Furthermore, according to Lee et al., (2002), the use of *Monascus* pigments as substitutes for synthetic colorants was promotes by their interesting features which is therapeutic properties and relatively high stability with respect to pH and temperature. The interest in red pigments produced by *Monascus* sp. in the food industry has been increasing because of their wide application in meat, fish, and ketchup processing (Hamano and Kilikian 2006). According to Mukherjee and Singh (2010), *Monascus* pigments have been used as traditional food additives in East Asia. While, *Monascus* red pigment have been use in meat products to substitute nitrites and also been reported to have the potential for therapeutic use particularly when produced in red rice.

2.2.2 Factor Influencing Red Pigment Production

Monascus red pigment fermentation has been mainly performed in solid cultures. Several factors such as gaseous environments, agitation, aeration and sources of carbon and nitrogen can influence the red pigment production by Monascus. In order to maximize and sustain the productivity of *Monascus* pigments, optimization of the culture conditions is critical. The effects of various environmental and nutritional factors need to be evaluated in order to determine their influence on pigment production in solid-state cultures. These including the initial moisture content, pH, inoculums size, air rate, sample size, and nutrient supplements (Lee et al., 2002)

2.2.2.1 Effect of Initial Moisture Content on Pigment Production

In solid state fermentation, moisture is a key parameter to control the growth of microorganism and metabolite production (Babhita *et al*., 2006; Pandey, 1992, 2003). The maximum pigment production was observed at 50% initial moisture content and a decrease in pigment yield was observed when the moisture level was higher, or lower than that. This result was similar to the research that conducted by Lee *et al*. (2002) which reported that with an initial moisture content of up to 50%, the production of red pigments by *M. purpureus* increased in proportion to the increase in the initial moisture content and with a 50% initial moisture content, the maximum production of biomass and pigments was obtained.

According to Lee *et al*. (2002) the red pigment production decreased drastically at initial moisture content of 60% and with moisture content greater than about 55%, separating particles of fermenting rice became very difficult. This aggregation of substrate particles resulted in oxygen starvation and a poor distribution of mycelia, thereby resulting in poor pigment production (Lee *et al*., 2002).

According to Babhita *et al*. (2006), higher initial moisture in solid state fermentation leads to suboptimal product formation due to reduced mass transfer process. Furthermore, decrease in initial moisture level results in reduced solubility minimizes heat exchange, oxygen transfer and low availability of nutrients to the culture (Babhita *et al*., 2006; Carrizales and Rodriguez, 1981). *Monascus* showed low alpha amylase and glucoamylase activity at an initial moisture content of 35 and 40% at a present study and higher alpha amylase and glucoamylase activity were observed at 50% substrate moisture content (Babhita *et al*., 2006). Activity of these hydrolyzing enzymes resulted in effective utilization of starchy substrate, which could be attributed to the high pigment yield at 50% initial moisture content.

2.2.2.2 Effect of Supplementation of Nitrogen Source

According to Babhita *et al*. (2006), monosodium glutamate was found to be outstanding for red pigment production, followed by peptone, soybean meal and chitin powder. Jackfruit seed powder without any addition of nitrogen source was not able to produce any water-soluble pigment which was showed in the study conducted by Babhita *et al*. (2006), in spectral analysis of water extract. It has been reported that the addition of monosodium glutamate could give rise to water-soluble red pigments. In the present study, a water-soluble pigment with maximum absorbance was resulted in the addition of nitrogen sources such as monosodium glutamate, soybean meal, peptone and chitin powder. Although the monascorubrin-rubropunctatin mixture that constitutes the orange pigment produced as the direct fermentation product of *Monascus* species is water-insoluble and therefore of limited utility as a food colorant, it has been reported that these materials react with primary amines to afford red colorants, many of which are water-soluble. According to Babhita *et al*. (2006), jackfruit seed powder supplemented with required organic nitrogen sources could be considered as a potential substrate for the production of water-soluble *Monascus* pigments.

2.3 Solid State Fermentation (SSF)

According to Pandey (2008), solid state fermentation is the fermentation process that occurs in the absence or near free of water. This process generally employs a natural raw material as carbon and energy source and also employs an inert material as solid matrix which requires an addition of nutrient solution containing necessary nutrient as well as a carbon source. SSF however must contain enough moisture and this is depending on the nature of the substrate. In order to allow higher rate of biochemical process, the amount of water absorbed must be one or more times than its dry weight which leads relatively high water activity (a_w) on the solid/gas interface.

In SSF there are two parameter which, physicochemical and biochemical parameters such as particle size, initial moisture, and pH. According to Ng et al. (2004), the medium pH will be adjusted using 1M HCl or 1M NaOH. Other than those parameter, pre-treatment of the substrate, relative humidity, temperature of incubation, agitation and aeration, age and size of the inoculum, supplementation of nutrients such as N, P and trace elements, supplementation of additional carbon source and inducers, extraction of product and its purification are also the parameter need to be considered in SSF.

Solid substrates that been used in SSF generally provide a good dwelling environment to the microbial flora comprising bacteria, yeast and fungi. Among these, filamentous fungi are the best studied for SSF due to their hyphal growth, which have the capability to grow on the surface of the substrate particles and also penetrate through them (Pandey, 2008).

2.3.1 Solid State Fermentation Is Preferable Technique than Submerged Fermentation

Lately, *Monascus* had been successfully cultured in submerged fermentation. However, there are a few technical difficulties in the cultivation of *Monascus* (Lee et al., 1995) and extensive submerged culture studies have revealed that the yield is markedly affected by many factors, such as the medium composition, pH and agitation. In addition, the pigments produced are mainly retained intracellularly, which causes inhibition of further production (Lee et al., 2002). In this regard, Solid-state fermentation could solve these difficulties.

In solid-state fermentation process, the solid substrates supplies the nutrients to the microbial culture growing in it and also serves as an anchorage for the cells (Pandey, 2003 ; Babitha et al., 2006). In recent years, solid-state fermentation has gained much interest for the production of primary and secondary metabolites and presents a more adequate habitat for fungus, with high pigment productivity in a relatively low-cost process by using agro-industrial residues as substrates (Pandey et al., 2000, 2001; Babitha et al., 2006).

The cultivation environment and cultivation methods would be directly or indirectly affected the biomass growth, types and production of metabolites. According to Vidyalakshmi et al, (2009), concerning in the cultivation methods, solid state cultivation results in higher pigment yield than cultivation in shake culture and this is caused by the fact that pigments are accumulated in the mycelium under submerged cultivation and the pigments are released into grains under solid state culture.

At present, since the cost of technology used is still high, it showed that the pigment production at an industrial scale is not really economical. Therefore, there is a needed for development of low-cost processes. Till now, in solid-state fermentation (SSF), several materials such as jackfruit seed powder, sesame oil cake, coconut oil, wheat bran, palm kernel cake, and grape waste have been studied as the substrates. This approach consumes a low cost when compared with liquid fermentation and gives high pigment productivity (Nimnoi and Lumyong, 2009; Cavalcante et al. 2008). These reports indicated that utilization of cheaply available substrates in SSF could be a good strategy for attaining significant pigment production (Nimnoi and Lumyong, 2009).

2.3.2 Local Agricultural Product as a Substrate

Several agro crops such as cassava, barley, and agro-industrial residues such as wheat bran, rice bran, sugarcane bagasse, cassava bagasse, various oil cakes like coconut oil cake, palm kernel cake, soybean cake, ground nut oil cake, fruit pulps like apple pomace, corn cobs, saw dust, seeds like tamarind and jack fruit, coffee husk and coffee pulp, tea waste, and spent brewing grains are the most often and commonly used substrates for SSF processes (Pandey, 2008). According to Carvalho et al. (2006), rice was the best substrate for cultivation of the culture in solid state fermentation, but some of the other substrates used also produce good production of biopigment. There are many natural substrates that are presumably good sources of C and N for the fungus development which show similar or even higher quantities of carbohydrates and proteins. Carvalho et al. (2006) states that there are several cheap by-products and residues from food processing industry which might have the potential to be the substrates for *Monascus* fermentation and have shown promising results in the production of other metabolites in SSF. According to Babitha et al. (2006), various agro-industrial residues such as rice bran, wheat bran, cassava, etc. have been used for pigment production and there are reports describing some other raw materials used as substrate for *Monascus*, which is cassava starch, prickly pear juice, and dairy milk (Carvalho et al. 2006). However, in some cases, the supplementation with nutrients such as vitamins and organic nitrogen supplements for these substrates is necessary (Carvalho et al., 2006).

2.3.3 Local Agricultural product Contain Nutrient in promoting Red Pigment Production

Monascus red pigment is dependent on the nitrogen and carbon nutrition in the local agricultural products which act as a substrate for solid state fermentation. Nitrogen source and carbon source is exist in the form of protein and carbohydrate. From the nutrition data, local agricultural product does have high percent of carbon and nitrogen source (percentage of nutrition is taken over 100g as basis). Guava, which is pear in shape, 2-4 inches long with granular flesh, 1/8 to 1/2 in (3-12.5 mm) thick (Morton, 1987) has high carbohydrate and protein content. Rice had been known to be the best substrate for the *Monascus* red pigment production. Recent research by Carvalho et al. (2006) had proved that rice can promote to the higher pigment production than corn.

According to wise geek, coconut meat is the flesh of the coconut fruit. Recent study, by Chang et al. (2004) had carried out nata as the substrate. Nata is the product of coconut. Like guava, coconut meat has high carbohydrate and protein content which 5% and 7% respectively. As for the banana, the protein and carbohydrate content are 2% and 8% respectively. Corn has the highest percentage of protein and carbohydrate which they are 19% and 25% respectively. While pumpkin contain 2% of carbohydrate and 1% of protein.

According to Morton (1987), papaya is regarded as a fair source of iron and calcium and also has a good source of vitamins A, B and G and an excellent source of vitamin C. Papaya has 3% and 1% content of carbohydrate and protein respectively. Below is the table of the nutrition data of local agricultural product which was retrieved from http://nutritiondata.self.com

Table 2.1: Nutrition Facts of Local Agricultural Products

	Banana	Papaya	Guava	Rice	Pumpkin	Coconut	Corn
Carbohydrates (%)	8	3	5	9	2	5	25
Protein (%)	2	1	5	4	1	7	19
Magnesium (%)	7	2	5	1	2	8	32
Phosphorus (%)	2	1	4	4	3	11	21
Potassium (%)	10	7	12	-	7	10	8
Zinc (%)	1	-	3	2	5	22	16

(Retrieved from: http://nutritiondata.self.com)

*basis is over 100 g

2.3.4 Particle Size of the Substrate in Solid State Fermentation

According to Pandey (2008), solid substrates must have large surface area per unit volume. Smaller substrate particles do provide larger surface area for microbial attack but small area pose difficulty in aeration or respiration due to limitation in inter-particle space availability. Larger particles provide better aeration or respiration opportunities but it also provides lesser surface area. For the reason of cost effectiveness, it may be necessary to use a compromised size of particles in bioprocess optimization. For example, wheat bran, which is the most commonly used substrate in SSF, is obtained in two forms, fine and coarse. For optimal production, most of SSF processes use a mix of those fine and coarse form at different ratios.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter includes all the methodology that was applied in the optimization of red pigment by *Monascus purpureus* (FTC 5356) in solid state fermentation. This study was carried out in the laboratory of chemical engineering in University Malaysia Pahang. All the chemical, raw material and apparatus was decided and had been set up before running this experiment.

3.2 Materials

The fungal culture of *Monascus purpureus* (FTC 5356), potato dextrose agar, sodium hydroxide, ethanol 99.5%, zinc sulfate, pepton, ehrlich reagent, glucosamine, sodium carbonate and acetyl acetone.

3.3 Procedure

3.3.1 Culture

A culture of *Monascus purpureus* (FTC 5356) (Appendix, Figure 3.1) was maintained on Potato Dextrose Agar medium at 4^{o}C. It will be sub-cultured once in every three week as mentioned in Babitha et al. (2007)

3.3.2 Inoculum preparation

As described in Babitha et al. (2007) 10 mL of distilled water had been added to fully sporulated (6-8 days old) agar slope culture (Appendix, Figure 3.2). The spores then been scrapped under aseptic condition and the spore suspension obtain will be used as the inoculum.

3.3.3 Substrates preparation

Seven different local agricultural products (white rice, pumpkin, papaya, banana, guava, coconut meat, and corn) were used as substrate in solid state fermentation. According to Carvalho et al. (2006) the substrates must be dried at 60°C overnight, ground and sieved. With some modification on the method, the substrate was dried at 60°C in the oven (Memmert) until it result in approximately 2% of water content in the substrate. Then, the dried substrates were blended to form powder and sieved to achieve the size particle of less than 315µm.

3.3.4 Solid state fermentation

An amount (Table 3.1) of 0.128M of zinc sulfate solution was added into 250mL conical flask to achieve 40% of zinc composition in those seven substrates. Then, deionized water was added to achieve the initial moisture content of approximately 50% for each substrate. The medium pH was adjusted using 1M HCl or 1M NaOH to get the pH 6 and pepton was added to achieve 2% of the nitrogen content in substrates. Then, 10 g of seven substrates which is banana, papaya, guava, pumpkin, coconut meat, corn and also white rice powder were added into the medium in conical flask. The conical flasks then were covered with 2 layer of aluminium foil to prevent any changes on the moisture content and autoclave at 121°C for 20 minutes in the Hirayama Hiclave HVE 50 autoclave machine. Each of the conical flasks had been inoculated with the spore suspension (10%) of *Monascus purpureus* (FTC 5356) and the fermentation had been incubated at 30°C for 10 days.

Table 3.1: volume of zinc, deionized water and weight of pepton (nitrogen source)

Substrate	Volume of zinc (mL)	Volume of deionized water (mL)	Weight of pepton (g)
Rice	3.97	4.6	0.16
Pumpkin	3.98	4.5	0.19
Banana	3.99	4.62	0.18
Guava	3.98	4.6	0.15
Coconut	3.93	4.6	0.13
Corn	3.85	4.5	0.01
Papaya	4	4.55	0.19

3.3.4.1 Growth study

The pigment growth study was conducted on two substrate which is rice (as reference) and coconut (the best substrate). From the growth study, the pigment growth curve had showed the optimum day for pigment production. The further study had been conducted based on the optimum day for pigment production. The pigment production (per day) on both substrates was calculated and compared.

3.3.4.2 Study on moisture and nitrogen source content value

Banana was chose as the best substrate as it yields the highest red pigment production among other substrate. The range of moisture content (30% to 70%) been added in different conical flasks that contain 10 g of coconut powder to find the optimum range value that yields the highest red pigment production. The other condition such as amount of zinc (3.99 mL, 0.128M), pH 6, concentration of nitrogen source (addition of 1.8% of pepton) and incubation temperature of 30°C was set up to be similar as the earlier experiment.

In separate experiment, nitrogen source concentration (0.05% to 3%) been added in different conical flasks which contain 10g of substrate (50% moisture content) and the other condition were maintain throughout this experiment.

3.3.4.3 Experimental Design

The investigation of the effects of initial moisture content of banana and the concentration of pepton on the red pigment production and biomass growth was conducted in Response Surface Methodology (RSM) by using central composite design. The value of initial moisture content of substrate was combined with various concentration of nitrogen source. The combination of the two variables for the experimental run will be design by the Experimental design.

3.3.5 Pigment extraction

Before extraction, the fermented solid was dried for 24 hours at 60°C in the oven. The dried fermented solids then were grind by using pastel and mortar to powder form.

According to Babitha et al. (2006) a known amount of fermented had been taken and mixed with 90% ethanol (5ml ethanol per gram of fermented matter on dry basis). With some modification on the method, 10 mL 95% ethanol was mixed with 2g of the dried powder fermented solids. The mixed then was mixed by a rotary shaker (Infors HT Ecotrons) at 200 rpm for 1hour, allows standing for 15 minutes and filtered through Whatman No.1 filter paper. This process was carried out in dark condition.

3.3.6 Pigment estimation

Pigment estimation had been done by measuring maximum absorbance of pigment extract by spectral analysis using a double beam spectrophotometer taking in to consideration of the dilution factor of the sample (Babitha et al., 2006). Only extracellular pigment was considered and pigment yield was expressed as optical density at its wavelength per gram dry of substrate (Babitha et al, 2006). On this research, the Uv-Vis spectrophotometer was used instead of using double beam spectrophotometer to measure the pigment concentration at 500nm. Ethanol extract of substrate was used as blank for the analysis.

CHAPTER 4

RESULT AND DISCUSSIONS

4.1 Screening for the Best Substrate

To select the best substrate for the highest red pigment production, seven different local agricultural products were evaluated. Table 4.1 shows the amount of pigment produce on each of the substrate which expressed as specific absorbance at 500 nm over gram of extracted solid fermented and days (by considering the dilution factor). 10 gram of substrate powder with initial moisture content of 50%, addition of zinc so as the zinc composition will be 40 %, and addition of pepton as nitrogen source so as the nitrogen content will be 2% were maintain for each of those substrates. The Solid State fermentation was conducted for 10 days. The figure of pigment production on each of the substrate was placed in the appendix in Figure 4.11.

Table 4.1: Red Pigment production for different substrates (AU/g dry substrate. days of fermentation)

Substrate	Specific Absorbance (AU/g.day)
Corn	0.0234
Coconut	0.0173
Rice	0.0420
Pumpkin	0.0363
Guava	0.0209
Papaya	0.0010
Banana	0.3390

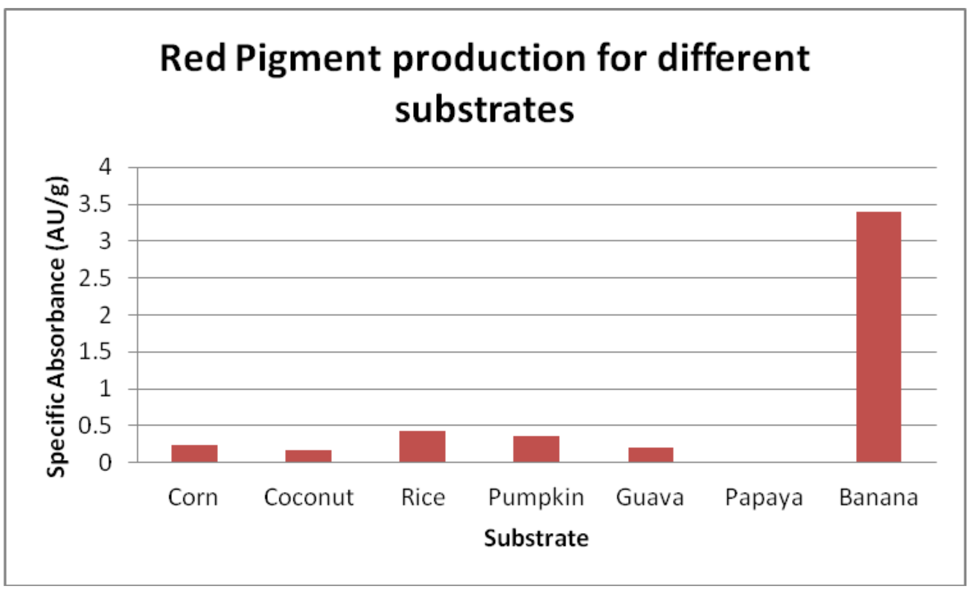

Figure 4.1: Red Pigment production for different substrates