PERFORMANCE OF RED PIGMENT PRODUCTION BY *Monascus purpureus* AT DIFFERENT EXTRACTION METHODS

SHARIFAH SHUFIZA KHAZIRI BT TUAN HADI

UNIVERSITI MALAYSIA PAHANG

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SHARIFAH SHUFIZA KHAZIRI BT TUAN HADI

Thesis submitted in fulfillment of the requirements for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

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SUPERVISOR DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature

Name of Supervisor	Dr.Farhan Binti Mohd Said
Position	Lecturer
Date	1st February 2013

STUDENT DECLARATION

I declare that this thesis entitled ‘‘*Performance of Red Pigment Production by Monascus purpureus at Different Extraction Methods’’* is the result of my own research except as cited in the references. This thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature	
Name	Sharifah Shufiza Khaziri Bt Tuan Hadi
ID Number	KE09051
Date	1st February 2013

Special dedication to my beloved parents ; Tuan Hadi bin Tuan Long and Sharipah Suhaibi binti Tuan Dalam, my family members(Shuhada,Shutira,Shuzlia,Rahafi and Rabbi), my supervisor, my lecturers, staffs
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PERFORMANCE OF RED PIGMENT PRODUCTION BY *Monascus purpureus* AT DIFFERENT EXTRACTION METHODS

ABSTRACT

The purpose of this research is mainly to study the performance of red pigment production at different extraction methods. The strain used in this study is *Monascus purpureus* which produce red pigment and red yeast rice was used as the substrate in the solid state fermentation. The initial moisture content in fermentation would be 56% which was considered as optimal pigmentation to be occurred. It would then be inoculated with $1x10^7$ spore/ml suspension of inoculum. Extraction methods including solvent extraction, Soxhlet extraction, agitated extraction and ultrasonic extraction were being carried out after fermentation process complete. Each methods required several conditions to be fulfilled to achieve the objective of this research. In this study, the best solvent used was 60% ethyl alcohol. Uv-Vis spectrophotometer is used to analyze red pigments in the extracts which were quantified by reading the absorbance at wavelength of 500 nm for this pigment. Thus, the best performance of the methods was identified by maximum extracts achieved which was measured by concentration of extracted product obtained. Solvent extraction was used as the reference and this method produced 1.278AU/g.d pigment extract. The results were 1.268AU/g.d for ultrasonic extraction, 1.265AU/g.d for agitated extraction and 0.128AU/g.d for Soxhlet extraction. The best method was ultrasonic extraction as compared to solvent extraction in a separate experiment and it produced 0.920AU/g.d than in solvent extraction, 0.869 AU/g.d pigment extract. The increrment percentage was about 5.81%. Besides, an analysis of variance (ANOVA) was carried out to investigate the significance to use the methods, showing the p-value. The p-value for agitated extraction was 3.63x10-7, 8.35x10-8 for ultrasonic extraction and 3.79x10-7 for Soxhlet extraction. All of the methods were significant to be carried out since all of the p-value was lower than 0.05.

PRESTASI PENGELUARAN PIGMEN MERAH OLEH *Monascus purpureus* PADA KAEDAH PENGESTRAK BERBEZA

ABSTRAK

Tujuan kajian dilakukan adalah untuk mengkaji prestasi pengeluaran pigmen merah pada kaedah pengekstrakan yang berbeza. Mikrob yang digunakan dalam kajian ini adalah *Monascus purpureus* yang menghasilkan pigmen merah dan beras yis merah digunakan sebagai substrat dalam fermentasi keadaan pejal.Kandungan kelembapan dalam fermentasi akan menjadi 56% di mana pigmentasi optimum berlaku. Ia kemudian akan disuntik dengan $1x10^7$ spora/ml inokulum. Kaedah pengekstrakan termasuk pengekstrakan pelarut,pengekstrak Soxhlet,pengekstrakan pergerakan dan pengekstrakan ultrasonik dijalankan selepas proses penapaian tamat. Setiap kaedah memerlukan beberapa syarat yang perlu dipenuhi untuk mencapai objektif.Dalam kajian ini, pelarut terbaik yang digunakan adalah etil alkohol 60%, alcohol. Spektrofotometer UV-Vis digunakan untuk menganalisis pigmen merah dalam ekstrak yang kuantitinya dengan membaca keserapan pada panjang gelombang 500 nm untuk pigmen ini. Oleh itu, prestasi yang terbaik kaedah-kaedah ini telah dikenal pasti mengikut ekstrak maksimum yang dicapai, diukur dengan kuantiti produk diekstrak yang diperolehi. Pengestrakan pelarut digunakan sebagai rujukan dan ia menghasilkn 1.278AU/g.d pigmen. Keputusan menghasilkan 1.268AU/g.d untuk pengestrak ultrasonik, 1.265AU/g.d untuk pengestrak pergerakan dan 0.128AU/g.d untuk pengestrak Soxhlet. Kaedah yang terbaik adalah pengestrakan ultrasonik apabila dibandingkan dengan pengestrakan pelarut dalam eksperimen berasingan dan ia menghasilkan 0.920AU/g.d berbanding sebanyak 0.869 AU/g.d pigmen dalam pengestrakan pelarut. Peratus kenaikannya adalah sebanyak 5.81%. Analisis varians (ANOVA) dilakukan untuk mengetahui kepentingan penggunaan kaedah tersebut.. Nilai-p untuk pengestrak pergerakan adalah 3.63x10-7, 8.35x10-8 untuk pengestrak ultrasonik dan 3.79x10-7 untuk pengestrak Soxhlet. Semua kaedah adalah penting kerana nilai-p kurang daripada 0.05.

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LIST OF SYMBOLS

H_2O	Distilled water
C_3H_5OH	Ethyl alcohol
CH_3OH	Methyl alcohol
mL	Mililiter
g	gram
min	minutes
rpm	Revolution per minute
mm	milimeter

LIST OF ABBREVIATIONS

PDA	Potato dextrose agar
DMSO	Dimethyl sulfoxide
AU	Absorbance unit
DF	Dilution factors
ANOVA	Analysis of variance
Uv-Vis	Ultra-violet spectrophotometer

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

During this recent year, the use of natural additives in food technology has been well-known since the usage of artificial color additives cause several diseases and harmful to health due to their toxicity (Velrumugan *et al.*, 2009). Therefore, in order to give attractive color to food or textiles, industries of producing color additives and dyeing agents had been introduced. As a result of, the natural dyeing agent and natural additives had been in a great world demand for its non-toxic and environmental friendly behavior.

Biopigments are secondary products from fermentation with natural substrates. Since they are natural and produced rapidly compared to pigment from animal or vegetable, the fermentation process contributes to a great concern in food industries (Carvalho *et al.*, 2007). An example of microorganism which can produce this product is a strain of *Monascus purpureus*. The highest production of pigment and maximum biomass was observed in *the M. purpureus* (Velrumugan *et al.*, 2009).

Japan was the main consumer of *Monascus* pigment since its annual consumption increased to 600tonnes in 1992 from 100tonnes in 1981and the value was about $1.5million(Rosenblitt *et al*., 2000; Duffose *et al*.,2005).The colors of pigments analyzed by *Monascus* species are red pigment, yellow pigment and orange pigment. However, red pigment can also be produced by other species, for instance, *Paecilomyces sinclairii* which differed in some aspects (Lim *et al.,* 2000). Red biopigment is considered as the most important since this application of producing red biopigments in industries may substitute the usage of synthetic pigment (Carvalho *et al.*, 2007).

It is also crucial to select high potential natural substrates in order to produce high yield of red biopigment. Cassava bagasse, cassava and rice are among natural substrates that can be used. *Monascus purpureus* is cultivated on rice substrate to produce red yeast rice during fermentation. Red yeast rice produce high pigment yield compared to other substrate. Cassava bagasse is not preferred as it produce low pigment yield (Carvalho *et al.*, 2006). Fermentation can be carried out by several different approaches which are solid-state or submerged fermentation. In both ways, best conditions in fermentation broth need to be maintained. Solid state fermentation is chosen in this study.

This study will be continued with extraction methods after fermentation finish. This research aims to investigate the performance of red pigment production by *Monascus purpureus* FTC 5356 at different extraction methods which are solvent extraction, agitated extraction, ultrasonic extraction and Soxhlet extraction at different conditions. The efficiency of these methods will be compared.

1.2 PROBLEM STATEMENT

The needs for biopigments are higher compared to synthestic pigments as it is beneficial to human heatlh. Only several research investigated the use of red yeast rice during fermentation. There is also lack of study comparing the extraction methods of red pigment by *Monascus* species. As such, research need to be carried out in order to fulfill the requirement. Therefore, this study is carry out to compare the performance of extraction process in producing biopigment using strain of *Monascus* cultivated on rice. This study will stress more on the extraction methods use besides fermentation process where no contamination occurred. The results obtained can be applied in industrial applications to fulfill the demand.

1.3 RESEARCH OBJECTIVE

The main purpose of this study is to investigate the performance of red pigment production at different extraction methods.

1.4 SCOPE OF PROPOSED STUDY

In order to achieve the objective, there are several types of extraction methods will be carried out which are solvent extraction, agitated extraction, Soxhlet extraction and ultrasonic extraction. Different methods have their own conditions to be performed outlined as:

- Solvent extractions or known as static extraction will be performed by mixing the fermented substrate with different solvents at room temperature. This method will show which solvent is the best solvent and will in turn use in other extraction methods.
- Agitated extraction is performing with different ratio of solvent to substrate of the best solvent evaluated before in solvent extraction in similar speed. It will show which ratio is the best. Agitated extraction is repeating with other different speeds with the best ratio obtain.
- Soxhlet extraction is carrying out to enable the extraction of different fermented mass by the best solvent. This will provide the relation between extracted yields with mass of substrate.
- Ultrasonic extraction is performed at different times in order to investigate which time shows the maximum extraction.

1.5 SIGNIFICANCE OF STUDY

This study is believed to provide crucial information for the most important solvent can be used in different extraction methods to extract red pigment. On top of that, it also outline different extraction methods in red bio-pigments production at different conditions provided. As such, the best performance of this pigment at those methods can also be investigated and will in turn be applied in industrial applications.

On top of that, this study is also important for the extraction of bio-pigment which can be used as natural coloring agent instead of artificial color additives in food technology industries and as dyes in dyeing industries. The demand for natural color additives has been high nowadays due to the fact that artificial agent will give disadvantages for health. It was also investigated that natural coloring agent is more beneficial to health. It was not only used in food technology but also can be treated as a therapeutic agent. The red color of this bio-pigment from *Monascus purpureus* can be used to colorize meat and sauces while in therapeutic activities, it can ease digestion system. Therefore, this study is very important to be carried out.

CHAPTER 2

LITERATURE REVIEW

A review is performed to identify the performance of *Monascus purpureus* in producing red pigment by different extraction method. In order to identify the material for this study, this chapter will elaborate on five major topic reviews which involve background of fungi, secondary metabolite products, pigment of fungi, solid state fermentation and extraction methods. This research is basically discussed on the efficiency of different extraction methods in production of red pigment by this *Monascus purpureus*.

2.1 BACKGROUND OF *Monascus purpureus*

Monascus Purpureus is among the fungi that are generally involved in the production of pigments like yellow and red pigment. It can be cultivated on substrates which contains startch like rice. Table 2.1 show that highest production of pigment and maximum biomass was observed in *the M. purpureus* (Velrumugan *et al.*, 2009). Due to its effects in food industries, this species had been widely used as a

Chinese traditional fermentation fungus on food for more than thousands years in China. Actually, *Monascus* species is among the *Monascaceae* family which belongs to *Ascomycetes* group. *M.pilosus, M.purpureus, M.ruber and M.froridanus* are generally the genus of this *Monascus* species(Pattanagul *et al*.,2007;Lin *et al*.,2008). This species are called with several names according to the several languages in countries. Japanese call this fungi as benikoji while Chinese with a name of zhitai. In Europe, this fungal is known as rotschimmelreis, and red mould in USA. With the ascospores appeared to be in spherical shape of five microns diameter, *M. purpureus* can be easily differentiated from other species (Pattanagul *et al.*, 2007).

Besides that, cultivation of *Monascus* fungi with rice can produce angkak or red yeast rice which in turns converts starchy substrates into several metabolites such as alcohols, antibiotic agents, antihypertensive, enzymes, fatty acids, flavor compounds, flocculants, ketones, organic acids, pigments and vitamins. *Monascus* pigments, cell bound and hydrophobic species, contain an aminophilic moiety which will react with compound containing amino group on its substances to form water-soluble pigments (Mapari *et al.*, 2008). The examples of amino group-containing compound are proteins, amino acids, and nucleic acids.

Thus, the application of *Monascus* pigment as a coloring agent in food will provide an additional advantage of specific flavor in the products. It is possible to use it as food colorant in order to avoid allergic problem which occur from synthetic additives. However, many factors in *Monascus* production must be considered to ensure that angkak production could be carried out safely while maintaining its functional characteristics.

Table 2.1 Pigment production obtained by five filamentous fungi

Fungi	pH	X (gdm^{-3})	UA_{400}	UA_{550}	UA_{600}	Extract colour[a]
M.purpureus	5.2	6.02 ± 0.3	25.0 ± 1.6	20.1 ± 1.4	16.6 ± 1.8	R
Isoria spp.	4.6	5.10 ± 0.9	10.1 ± 1.2	7.2 ± 1.2	3.1 ± 0.3	P
Emerilla spp.	5.9	3.68 ± 0.6	16.0 ± 0.6	10.1 ± 1.4	6.2 ± 0.2	R
Fusarium spp.	5.6	4.16 ± 1.2	12.2 ± 0.2	8.2 ± 1.4	3.3 ± 0.4	RB
Penicillum spp.	4.6	5.20 ± 1.6	23.0 ± 0.3	19.0 ± 1.2	14.4 ± 0.2	Y

[a]R:red;P:pink,RB;reddishbrown,Y;yellow
(source: Velrumugan *et al.,* 2009)

2.2 SECONDARY METABOLITE PRODUCTS

Secondary metabolites are products in which the production is not linked directly to the cell growth. Actually, there are a lot of secondary metabolites synthesized from *Monascus purpureus* strain which are pigment, citrinin and lovastatin. Mevinolin (lovastatin and monakolin K) is a product which can be used as a dietary supplement since it inhibits the production of cholesterol by certain methods. Monacolin K reduces the amount of cholesterol by inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) (Chairote *et al.,* 2010). Critinin shows negative effects on liver function and metabolism. As such, this critinin need to be getting rid of in the production.

The pigment by *Monascus* can be used as natural coloring agent and natural additives in food industries. The pigments can be classified in three colors which are red pigment, yellow pigment and orange pigment. These pigments are stable to temperature up to $60^{O}C$ and pH2 to pH10. Its solubility in water and lipids,

production process and its applications can be affected by its polar structure (Carvalho *et al*., 2006).

2.3 MAJOR PIGMENT OF *Monascus*

Several studies showed *Monascus* species produce six major pigments which can be classified according to the color (Arunachalam and Narmadhapriya, 2011). The most important major pigments are red pigment, yellowish pigment and orange pigment. Orange pigment consists of rubropunctatin and monascorubrin, yellow pigment consists of monascin and ankaflavin while red pigment consists of monascorubramine and rubropuntamine. Several studies show that orange pigments had been found to undergo antibiotic activity against bacteria, yeast, and filamentous fungi. The factors that affect stability of pigments are acidity, temperature, light, oxygen, water activity and time (Dufosse *et al.,* 2005).

It also can be concluded that red pigment is the most interest pigment than other pigment color because it is reported to be beneficial to health as it can be used in therapeutic purpose besides being used as coloring agent. In western countries, red biopigment is used in meat processing industries while it is use as traditional food additives in East Asian countries (Pattanagul *et al.,* 2008;Mukherjee and Singh, 2010).

2.4 FERMENTATION

2.4.1 Solid State Fermentation of *Monascus Purpureus*

According to Shuler and Kargi (2002) in Bioprocess Engineering Basic Concepts, solid-state fermentation involves the fermentation of solid substrates at lower moisture level which is in the range of 40% to 80%. The usage of agricultural products in fermentation involves rice, corn, wheat and so on. It is important to use solid-state fermentation rather than submerged fermentation since solid-state fermentation can reduce the possibility of fermentation media by bacteria or yeast to contaminate. This is because they cannot tolerate lower water activity. On top of that, solid-state fermentation also important because spore formation is carried out in proper state and easy to obtain. Besides, this state of fermentation produce more pigment production than in submerged fermentation (Jiefeng *et al.*,2010). Teng and Feldheim(2000) also stated that solid state cultivation is much better than submerged fermentation.

The variables involve in solid-state fermentation are moisture content or can be known as water activity, inoculum density, temperature, pH, particle size and aeration or agitation. All of these parameters need to optimize in order to produce high product yield and increase the rate of pigment formation. Fermentation of *Monascus* species on rice to produce red yeast rice will synthesize high yield of pigments than other species as stated in the table 2.2 (Carvalho *et al.,* 2007). Red yeast rice is chosen also because of the optimal fermentation day for rice is only 7 days compared to cassava baggase which around 10 to 11 days (Carvalho *et al.,* 2007).

Table 2.2 Biopigment Production (AU/g dry substrate) and Substrate Composition

Substrate	Approximate composition(g/kg dry basis)			Average specific absorbance (AU/g dry substrate)
	carbohydrate	protein	phosphorus	
Rice	820	90	1.14	216
Wheat	820	140	3.63	79
Corn	770	130	3.19	60
Soy	780	400	6.00	13
Soy bran	330	480	7.00	22
TSP	400	-*	-*	12.6
Cassava	864	19	10.99	119.6
Cassava starch	900	20	3.3	38.5
Cassava flour	910	14	2.2	98.1
Cassava bagasse	660	11	-*	15.7
potato	800	100	9.6	4.7

*(-) indicates data not known

(source : Carvalho *et al.,* 2007).

Solid state fermentation had been used as a traditional method for centuries ago due to the fact that it gives benefits to obtain high pigment yields at low cost consumption. In classical Chinese method, inoculation of steamed rice grains spread on big trays with a strain of *Monascus* species is involved. After that, the inoculated rice will be incubated in an aerated and temperature controlled room for 20 days. The important parameters to be controlled and managed including the moisture content, oxygen level, carbon dioxide level in the surrounding and cereal medium composition when observing in this type of culture. In plastic bags consist of rice grains, red pigments also can be produced (Dhale, 2007).

2.4.2 Fermentation Parameters

2.4.2.1 Moisture Level Content

In solid state fermentation, one of the most important parameter need to be optimized is moisture content. It is defined as the weight of water contained in rice which is expressed in percent. The unique characteristic of solid-state fermentation is the operation at low moisture level. This in turn will provide for a selective environment when dealing with mycelial organism like molds. Molds are fungi that grow in form of multicellular filaments. At 30% of moisture content, the microbial activity will become inhibit. At more than 60% of moisture level which is considered high to rice as the substrate, will cause solid substrate become sticky and in a result of, large aggregation will be formed (Babitha *et al*.,2007).

Therefore, it is important to obtain optimal moisture level in this research to avoid these conditions from happened. Duffose *et al* (2005) stated that optimal pigmentation was found at initial moisture content of 56% on rice. In this study, a trial and error method will be carried out in order to achieve 56% moisture content for the purpose of carrying out fermentation.

The application of measuring of rice moisture content is in managing and marketing of rice or paddy. According to Post harvest Unit of the International Rice Research Institute (2010), inaccurate tests in calculating moisture level will lead to extra drying cost and harvesting loss if paddy is harvested wetter than necessary. In addition, it will lead to spoilage if the grain is too wet in storage. Post harvest Unit of the International Rice Research Institute (2010) also indicated the formula to

calculate dry basis moisture content as shown in equation 2.1 below.

moisture content ={(initial weight- final weight)/final weight}x100 (2.1)

2.4.2.2 Gas Exchange in Aeration

During fermentation in solid state, oxygen needs to be supply enough in order to enable the growth of pigment mean while, carbon dioxide produced will be removed out from the conical flask. Sufficient oxygen content required for fermentation is very important so that optimal pigmentation occurred. Besides, carbon dioxide level need to be reduced as it will affect the pigment growth by *Monascus purpureus* strain. As such, membrane filter 0.45µm can be used in contact with silicon tube for that purpose. On top of that, membrane filter may also avoid contamination. Cotton wool and cotton dressing will therefore allow sterile gas exchange.

2.4.2.3 Temperature

In growing of red yeast rice for several days, the temperature involve is $30^{O}C$ where growth of pigment by *M.purpureus* occurred. Higher temperature may affect the growth while lower temperature will make the pigment is unable to start.

2.5 EXTRACTION METHODS

Extraction is among process of separating solute from sample by supplying liquid solvent. It can be known as solid-liquid extraction or industrial cases, namely leaching. Leaching process is divided into several parts. Firstly, solvent will make a contact with the solid pores and diffuse. Secondly, the solute will be transferred to liquid form by dissolving the solute into solvent. Lastly, leaching involves the transferring of the solution from porous solid to the main bulk of the solution. The solution will then be recovered by centrifugation.

2.5.1 Solvent Extraction

This method of extraction involves the basic step of extraction process. It can also be known as static extraction. Static has no movement involve. The sample is allowed to be extracted by certain solvent for certain time. Solvent like ethanol will dissolve fermented powder which contains pigment act as the solute desired.

2.5.2 Agitated Extraction

Agitated extraction will involve extraction of solute from fermented powder in the presence of movement. The movement is considered as agitation speed. Usually, the unit of speed is known as revolution per minute. Certain sample will only require optimum speed to be extracted. Lower agitation speed will not extract

the sample properly while higher speed will cause disruption of sample cell. The equipment can be used for this purpose is orbital shaker.

2.5.3 Ultrasonic Extraction

As agitated extraction involve agitation speed, ultrasonic extraction will involve the presence of ultrasonic wave to extract the solute or pigment from fermented powder. Ultrasonic wave can also disrupt biological cell walls (Velickovic *et al.,* 2006). Thus, it will fasten the diffusion of solute from solid into solution required (Wang and Weller, 2006). In this method, there are several parameters can be analyzed. There are sonication times, sonication power, mass of sample and volume of solvent. While investigating the effect of sonication time to the concentration of solute, other parameters will be kept constant. This method can be carried out in ultrasonic bath.

2.5.4 Soxhlet Extraction

Soxhlet extraction also uses the principle of solid-liquid extraction. Generally, it involves the boiling process of large amount of solvent so that the solvent may vaporize and condense into thimble which contains the fermented powder. After certain period of time, the solvent will diffuse into the powder and change into solution. The desired solution will then be kept in the round bottom flask. Soxhlet extraction requires larger time compared to ultrasonic extraction. The

parameters that can be analyzed are volume of solvent, mass of sample and time of extract.

Wang and Weller (2006) stated that the main disadvantages for this type of extraction was including time-consuming of extraction process and usage of large amount of solvent evethough it brought a direct contact of solid and the solvent.

2.6 ULTRA-VIOLET SPECTROPHOTOMETER (Uv-Vis)

Substances which produce colored solutions usually absorb visible light. The specific groups or chemical bonding present is the factor of light absorption. Light absorption is important to measure the concentration of substances using spectrophotometer. In spectrophotometer, amount of light being absorbed by a solution is related to its solute concentration and the path length of the light within the sample. Absorbance (A) is a quantified term used to represent light absorption amount (Ghosh, 2006). In this study, the solute involved is red pigment. Red pigment absorbs light at 500 nm wavelength while yellow pigment absorbs light at 400 nm wavelength (Carvalho *et al.*,2007).

Figure 2.3 shows the operation of Uv-vis spectrophotometer. When light passes through monochromater, a small band of wavelength will be selected, followed by transferring the light through sample and the light will be detected by a detector, transferred to the amplifier to readout.

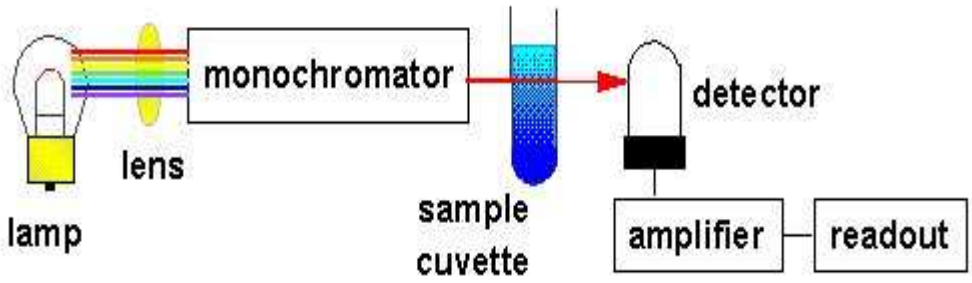


Figure 2.3 Absorption of Light
(source :Department of Chemistry, The University of Adelaide,Australia)

CHAPTER 3

METHODOLOGY

This chapter would be discussing on the materials and techniques that were used to conduct the experiment investigating the efficiency of different extraction methods to produce red pigment by *Monascus purpureus*. The substrate used was non-glutinous rice. This research provided for design research which was divided into three major sections which were materials, apparatus and equipment and procedures involved to achieve the objective. On top of that, sterile technique was implemented to avoid contamination from disturbing the process.

3.1 MATERIALS

The materials that were used in this study were *Monascus purpureus* FTC 5356 strain, non-glutinous rice, potato dextrose agar (PDA), distilled water (H_2O), deionized water, 60% ethyl alcohol (C_3H_5OH), 95% ethyl alcohol (C_3H_5OH), methyl alcohol (CH_3OH) and dimethyl sulfoxide (DMSO).

3.2 APPARATUS AND EQUIPMENTS

3.2.1 Apparatus

The apparatus used were 250mL Erlenmeyer flask, measuring cylinder, beakers, agar plates, universal bottles, inoculating loop, Schott bottles, cuvettes, droppers, Bunsen burner, rubber tube, filter membranes, filter paper, tissue for cuvettes, aluminium foil, 50mL centrifuge tubes and parafilm to wrap agar plates.

3.2.2 Equipments

The equipment used were a set of Soxhlet extractor consisting of condenser, thimble and round bottom flask, incubator, orbital shaker (Certomat), hemocytometer, refrigerated centrifuge (Eppendorf Centrifuge 5810R), ultrasonic cleaning bath and UV-Vis spectrophotometer.

Figure 3.1 Uv-Vis Spectrophotometer (U-1800 Spectrophotometer Hitachi)

Figure 3.2 Orbital Shaker (Certomat)

Figure 3.3 Refrigerated centrifuge (Eppendorf Centrifuge 5810R)

3.3 PROCEDURES

The methods outlined to investigate the red pigment production was:

3.3.1 PDA medium preparation

19.5 gram of potato dextrose agar (PDA) powder was weighed on an electronic balance and then was inserted into 500ml of distilled water in a Schott bottle. After that, it was heated and stirred with magnetic stirred on a hot plate. Then, the medium was then brought to autoclave about 15 minutes at temperature of 121^0C.

The medium was transferred into universal bottle in preparing for agar slant and agar plate. Sterile technique was performed under laminar fume hood by swaping the working area with 70% ethanol. Then, agar slant and agar plate containing medium will be preserved in a freezer at 4^0C.

Figure 3.4 PDA medium preparation on hot plate with stirrer

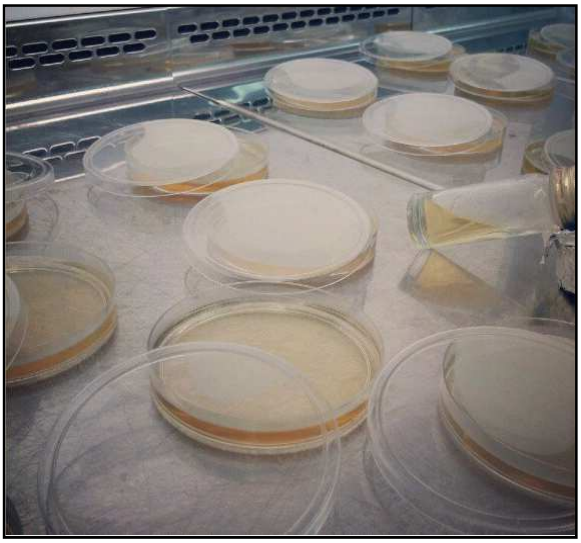


Figure 3.5 Preparation of agar slant and agar plate medium

3.3.2 Preparation of seed culture

A strain of *Monascus purpureus* FTC5356 was inoculated about 1mm x 1mm on PDA agar slant and PDA agar plate prepared before under laminar fume hood. The requirement to be in sterile state was important to avoid contamination from being existed. On top of that, parafilm was used to wrap the plate and universal bottle. Both slant and plate PDA medium containing the strain was incubated for 7 to 8 days at 30^0C in incubator under static conditions for culturing to be existed. The agar plate culture was considered as pure stock culture.

Figure 3.6 Plate culture of *Monascus purpureus*

Figure 3.7 Slant culture of *Monascus*

3.3.3 Inoculum preparation

For fully sporulated (6 to 8 days old)agar slope culture, 10ml of sterile distilled water was added. Then the spores were scrapped under aseptic conditions to avoid contamination. The spore suspension obtained was used as the inoculum

($1.0x10^7$ spores/ml) which also contained mycelium. Mycelium was the vegetative part of fungus. Hemocytometer and microscope was used to study the spore suspension.

3.3.4 Substrate preparation and solid state fermentation

Normal rice available from market in Kuantan was used to make red yeast rice as the substrate in fermentation process. Before fermenting, the non-glutinous rice grains were dried overnight in oven at temperature of 60^OC to remove the moisture content presented in rice. After that, the rice was ground to powder using grinder and followed by sieving with size of 1.00mm using sieve shaker. The range wanted was 0.8 to 2.00mm.

100gram of rice powder was kept into conical flask by mixing it with 56gram of distilled water in order to get 56% moisture content for optimal fermentation on rice occurred. The flask was wrapped with aluminium foil and 0.45µm membrane filter was used to avoid contamination but allowed oxygen transfer. It was then sterilized. After that, the sterilized rice was being inoculated with 10ml culture of $1x10^7$/ml spore suspension in laminar hood. The sample was incubated in incubator for 8 days for growing occurred. The optimal fermentation day for rice as the substrate was 7 days.

Figure 3.8 Solid state fermentation with sterile oxygen transfer

3.3.5 Pigment extraction

In order to proceed with extraction methods, the fermented rice was dried overnight at temperature of $60^{O}C$ and followed by grinding into powder.

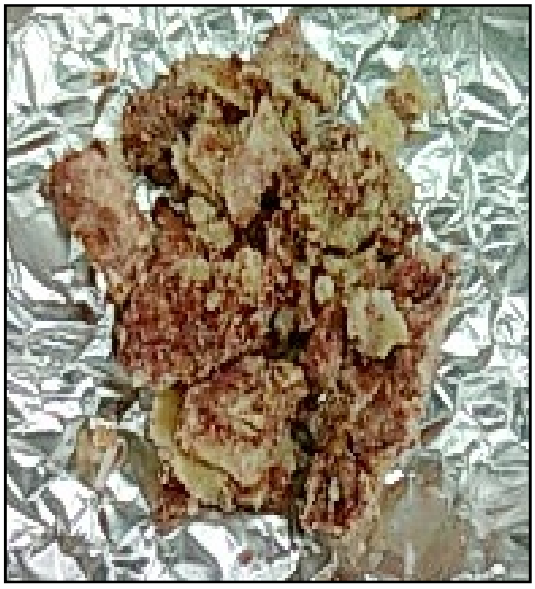


Figure 3.9 Sample after drying

Figure 3.10 Sample after grinding into powder using mortar and pestle

3.3.5.1 Solvent extraction

Solvent extraction was performed with 7 gram of fermented substrate in 250 mL Erlenmeyer flasks extracted with 35 gram of different solvents which were deionized water, methanol, 60% ethanol, 95% ethanol and dimethyl sulfoxide in room temperature for 24 hours. The ratio used was 5 gram solvent to 1gram substrate as in the previous study. The objective in carrying this method was to know the best solvent to be used to extract red pigment.

Figure 3.11 Solvent extraction at static condition

3.3.5.2 Agitated extraction

Agitated extraction was performed by using the best solvent obtained in solvent extraction method on a rotary shaker at room temperature in order to extract 7 gram of fermented sample. The agitation speed was set at 110 revolution per minute as stated in previous study and the ratio of solvent to substrate was varied at 2:1,3:1,4:1,5:1 and 6:1.This method was carried out in order to investigate which ratio showed the highest extract for the best solvent.

Agitated extraction was continued to study the effect of different speed to the pigment extract by using the best ratio of solvent to substrate. The experiment was repeated for 100rpm, 150 rpm, 200 rpm and 250 rpm.

Figure 3.12 Agitated extraction of red pigment

3.3.5.3 Soxhlet extraction

Soxhlet extraction was performed by extracting 6, 7, 8 and 9 gram of fermented mass with the best solvent resulted from solvent extraction. Volume solvent is kept constant with 250ml.The temperature used was the boiling point of the solvent and it was allowed to be extracted for 12 hours.

Figure 3.13 Soxhlet extraction of red pigment

3.3.5.4 Ultrasonic extraction

In ultrasonic extraction, the best solvent from solvent extraction was used accompanied by the best ratio of solvent to substrate from agitated extraction. 7 gram of fermented sample was extracted at different times which were 30minutes, 60minutes, 90 minutes and 120 minutes.This method was also carried out to study

the effect of time with applying of sonication to the extracted concentration of red pigment.

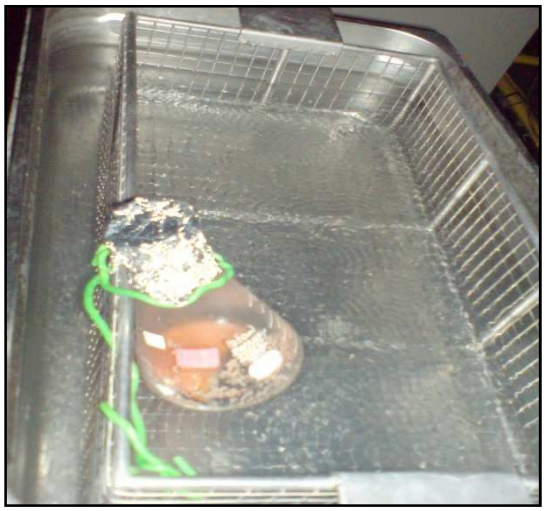


Figure 3.14 Ultrasonic extraction of red pigment

In all cases, the extracts were centrifuged at 3000 rpm at 4^{o}C for 10 minutes in refrigerated centrifuge to get the supernatant (Chairote *et al*., 2007).

Figure 3.15 Centrifugation took place

3.3.6 Analyzing the pigment

The supernatant obtained from centrifugation was then be used to analyze the red pigment production. It was quantified by reading the absorbance with a Uv-Vis spectrophotometer at 500 nm which was corresponding to red pigments (Johns and Stuart., 1991). The value was measured three times to obtain the average in order to reduce the error. This was converted in specific absorbance (in AU/g) relative to substrate dry mass, multiplying the absorbance by dilution factors and dividing by the substrate dry mass on fermentation medium as shown in equation 3.1 and 3.2(Carvalho *et al.*, 2007).

Specific absorbance(AU/g.d) = (Absorbance x DF)/ substrate dry mass (3.1)

Where ;

Dilution factors (DF) can be calculated from this formula

DF = (volume sample + volume of solvent)/ volume of sample (3.2)

Figure 3.16 Analyzing the red pigment

3.4 FLOWCHART IN PRODUCTION OF RED PIGMENT

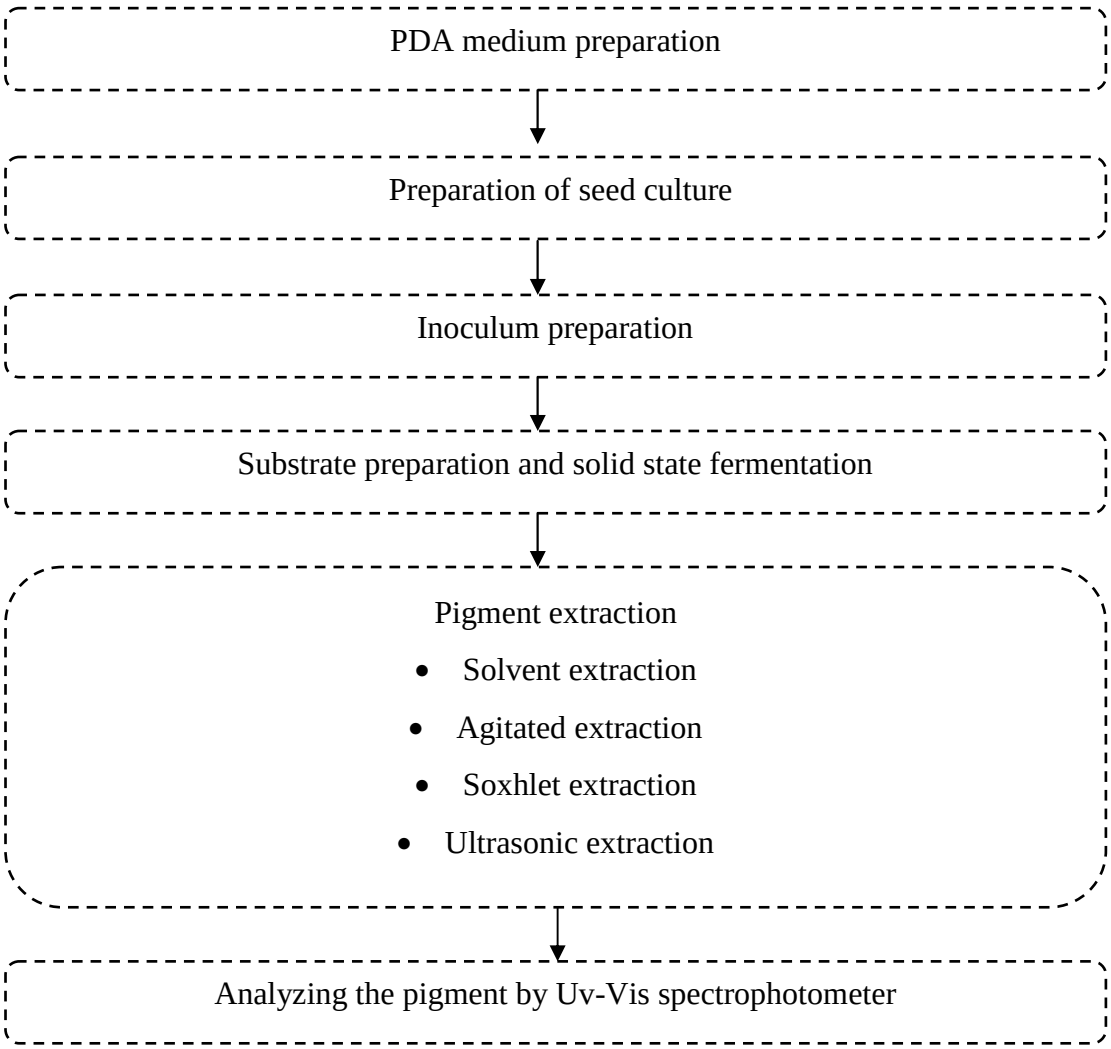


Figure 3.17 Overview of Methodology

CHAPTER 4

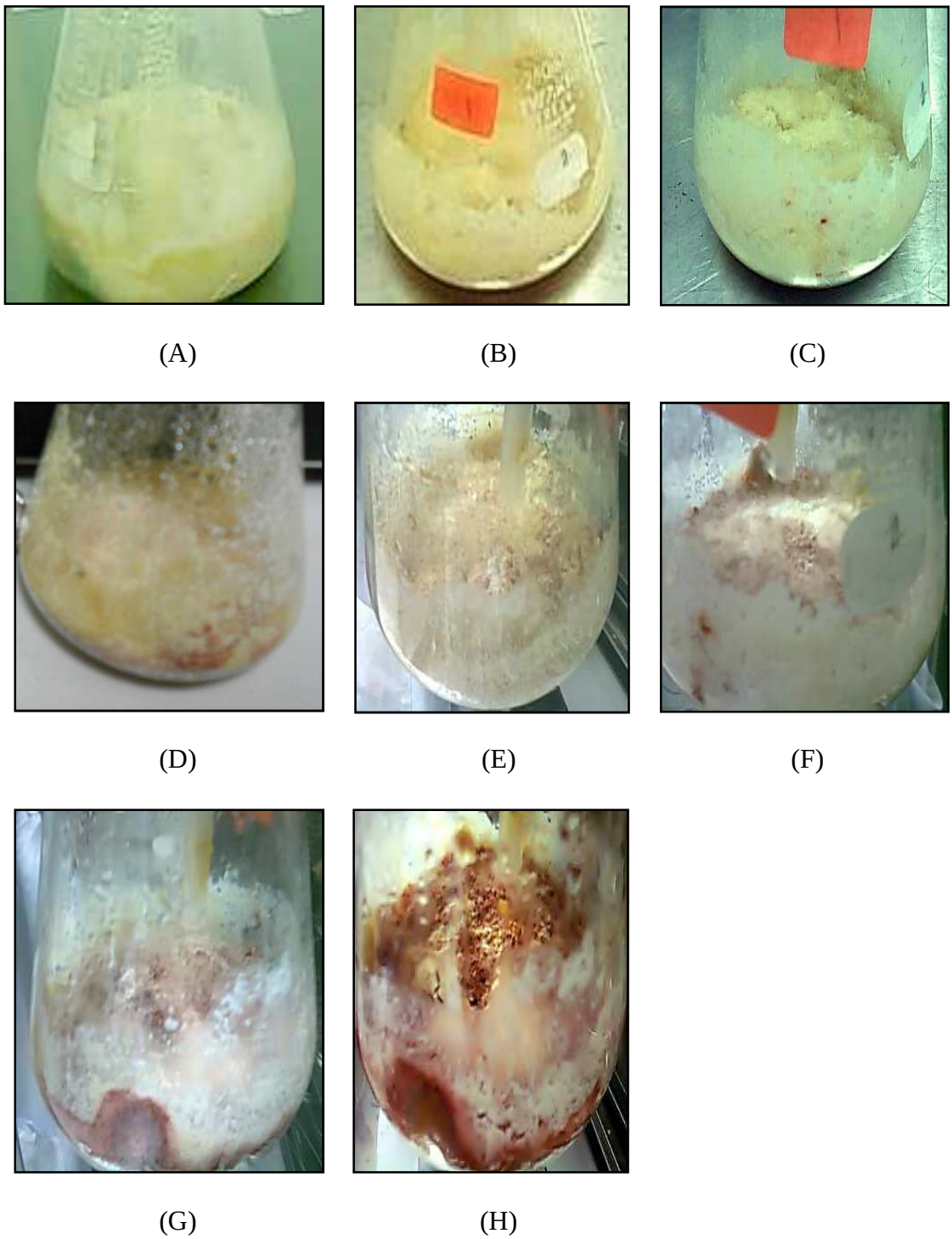
RESULT AND DISCUSSION

This chapter discussed on the results of this study which generally involve two parts,fermentation and extraction. Fermentation showed the growth of red pigment on rice substrate while extraction part shows result obtained in excel and analyzing the extraction method by ANOVA.

4.1 FERMENTATION

Fermentation process for *Monascus purpureus* FTC 5356 was considered optimum for more than 7 days. This study required 8 days for optimal growth of red yeast rice after cultivated on rice as the substrate as shown in figure 4.1. The substrate would gave nutrient for the fungal strain to consume in presence of sufficient amount of sterile oxygen. The growth indicated red spot formed in the flask used to ferment it. The first day involved the solid state fermentation started. After incubated at 30^{O}C for one day, the rice changed to yellowish color as the

growth took place. Third day showed that red spot had occured. The spot grew rapidly in fourth day until eighth where optimal pigmentation occured.



(A) (B) (C)

(D) (E) (F)

(G) (H)

Figure 4.1 Red pigment production of *Monascus purpureus* FTC5356 SSF

A) Day 1,B) Day 2,C) Day 3,D) Day 4,E) Day 5,F) Day 6,

G) Day 7,H) Day 8

4.2 EXTRACTION

4.2.1 Solvent extraction

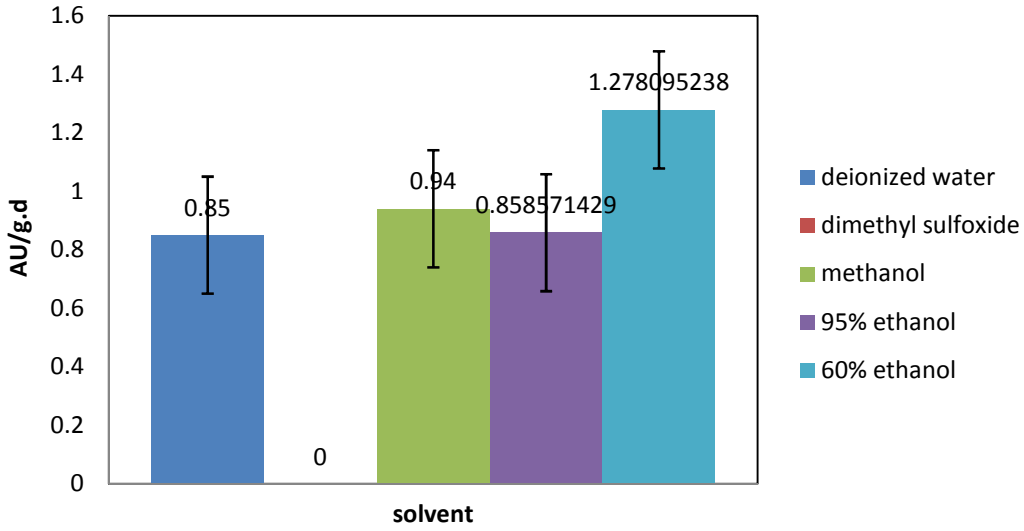


Figure 4.2 The extraction of different solvents

Figure 4.2 showed the result of pigment yield for different solvents in static extraction.The ratio of solvent to fermented mass used was 5:1 following the previous study (Carvalho *et al*., 2007). The resulting values were 0.850AU/g.d,0.0,0.940 AU/g.d, 0.859 AU/g.d and 1.278 AU/g.d when using deionized water, dimethyl sulfoxide, methanol, 95% ethanol and 60% ethanol respectively.The highest yield of red pigment was analyzed when using 60% ethanol. In other words, it meant that the pigment was extracted efficiently when using mixture of 40% water and 60% ethanol.

Therefore, 60% ethanol could be considered as the best solvent to extract pigment or solute from the fermented powder. The solubility of red pigment in less organic ethanol caused it to extract efficiently compared to other solvents. . Sivakumar *et al*(2009) stated that ethanol-water mixture produced better result in dye

extraction and ethanol would be used because of its polarity. Besides, ethanol is cheaper, volatile, and non-toxic solvent(Carvalho et al.,2007).

Previous study showed that the extraction of red pigment on cassava bagasse with anhydrous ethanol(95%) at static conditions gave 0.916 AU/g.d when using 1 gram of fermented mass with 5 gram of solvent-water mixture(Carvalho *et al.*,2007), while in this study, 95%ethanol could extract 0.859AU/g.d. Both of these were lower as compared to when using 60%ethanol.In this study, dimethylsulfoxide could not be used to extract the solute as red pigment on red yeast rice was very soluble in this solvent, caused it could not be separated.

4.2.2 AGITATED EXTRACTION (110RPM)

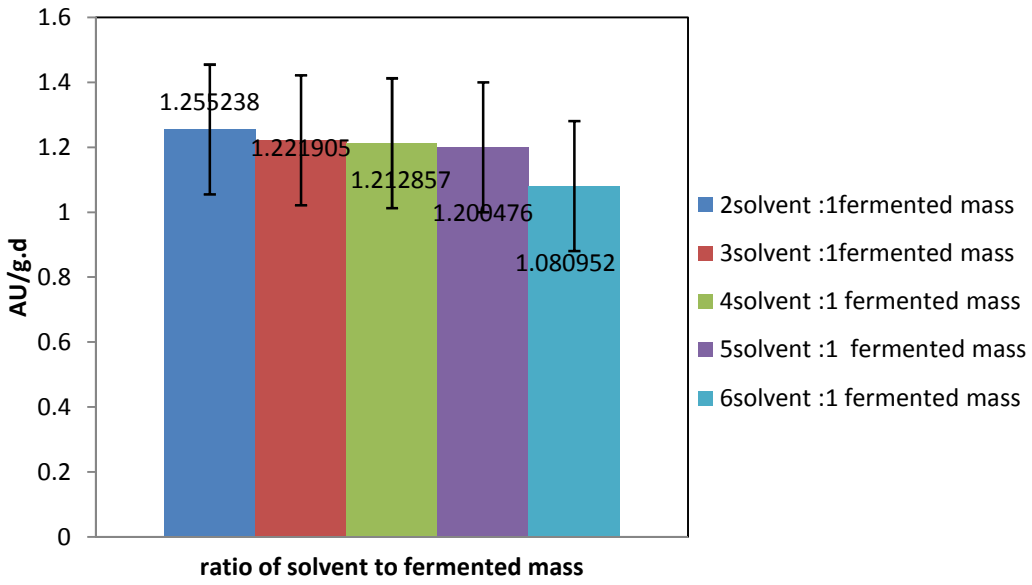


Figure 4.3 Extraction with different ratio of solvent to substrate at 110 rpm

Figure above showed that the results of pigment extraction was 1.255 AU/g.d, 1.222 AU/g.d,1.213 AU/g.d,1.091 AU/g.d for ratio of solvent to substrate were 2:1,3:1,4:1,5:1 and 6:1 respectively. The highest yield of pigmentation occured when

using ratio of solvent to substrate, 2:1 compared to other ratio. Red pigment being extracted and dissolve properly at that ratio. This was approved in the result showing that less solvent produce higher pigment concentration. Therefore, concentration of red pigment was inversely proportional to volume of solvent used in constant mass of fermented sample.

However, ratio 1 solvent to 1 substrate was not being able to extract the pigment as it was fully dissolved and cannot be separated after being centrifuged. Carvalho *et al*(2007) indicated that agitated extraction was conducted in order to study whether there was a saturation of pigment with certain amount of solvents and claimed that lower amount of solvent could be used. This might be led to use of less solvent amount in industrial process. The study showed that higher ratio of solvent to 1gram substrate would gave lower absorbance unit per dry mass which indicated the concentration of pigment.

4.2.3 AGITATED EXTRACTION

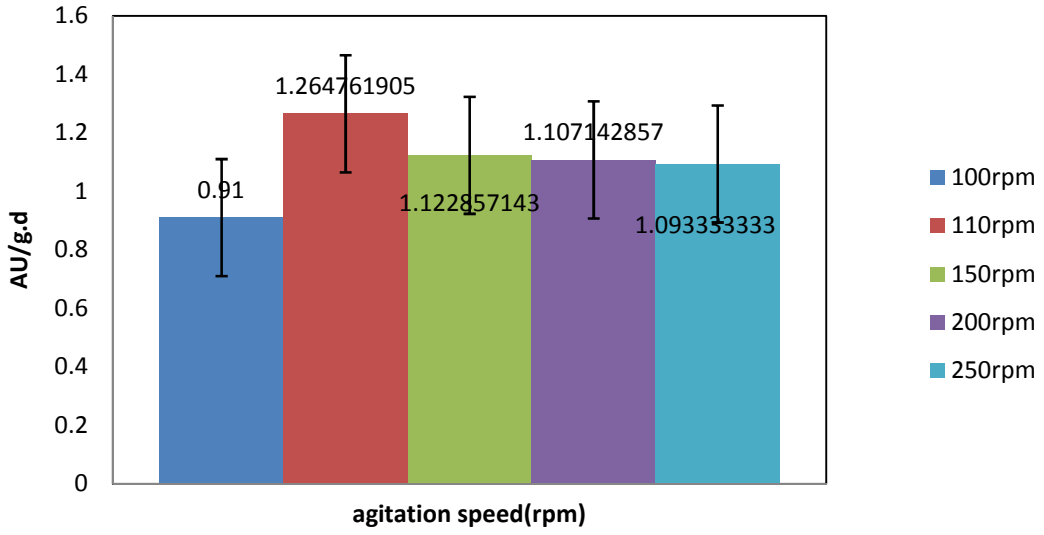


Figure 4.4 Effect of different agitated speed to absorbance(AU/g.d)

Figure 4.4 showed the effect of solute concentration analyzed at different agitation speed. The results were 0.91AU/g.d,1.265 AU/g.d, 1.123 AU/g.d, 1.107 AU/g.d and 1.093 AU/g.d at 100rpm,110 rpm, 150rpm, 200 and 250 rpm respectively. The highest result obtained was at speed of 110 rpm when analyzed using Uv-Vis spectrophotometer. This was because 110rpm speed showed better solubility and dissolving rate of red pigment into 60% ethanol.

However, higher speed was not suitable for red pigment extraction as the pigment cannot dissolve properly while lower speed than 110rpm cannot separate solute from the solid properly. Agitation speed was inversely proportional to pigment concentration of extract. According to Carvalho *et al* (2007), the agitation speed used was only 110 rpm to extract *Monascus* pigment.

The efficiency of this method could be compared with the reference which was the solvent extraction. In solvent extraction for 60% ethanol, 1.278 AU/g.d of red pigment had been extracted. This was higher than in agitated extraction which was 1.265AU/g.d. However, this happened because solvent extraction was conducted first in order to investigate the best solvent to be used in other extraction methods.

An analysis of variance (ANOVA) was analyzed by using the data of this agitated extraction at different speed as shown in table 4.1.It indicated that the p-value was 3.63E-07 which was less than 0.05. It showed that there was a significance effect on pigment extraction when using different speed to extract red pigment.

Table 4.1 ANOVA table for agitated extraction at different speed

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
100rpm	3	2.73	0.91	0.002016
110rpm	3	3.794286	1.264762	0.000188
150rpm	3	3.368571	1.122857	0.000263
200rpm	3	3.321429	1.107143	0.000284
250rpm	3	3.28	1.093333	0.00085

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.191591	4	0.047898	66.49953	3.63E-07	3.47805
Within Groups	0.007203	10	0.00072			
Total	0.198794	14				

4.2.4 ULTRASONIC EXTRACTION

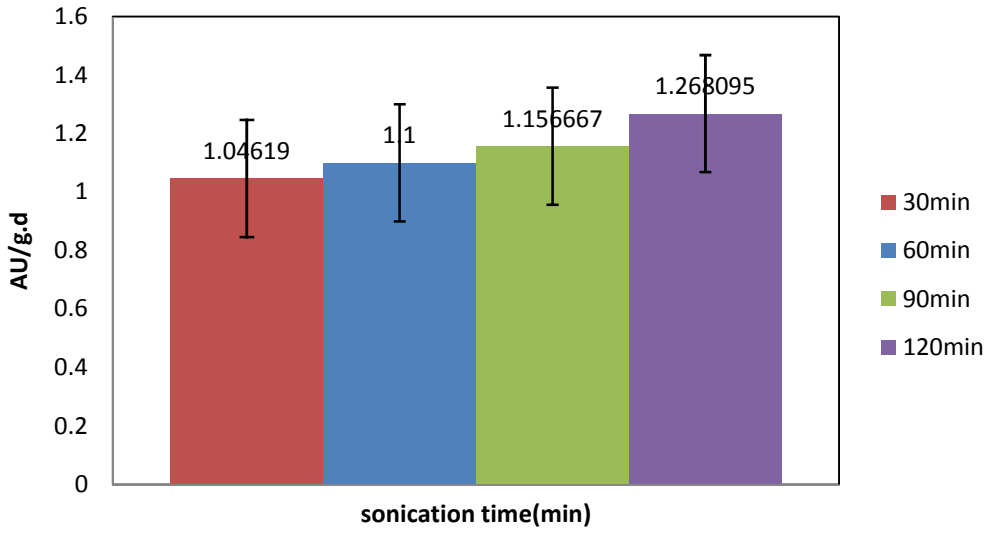


Figure 4.5 Effect of sonication time to absorbance(AU/g.d)

The figure above showed the effect of different sonication time to absorbance or red pigment concentration. The results were 1.046 AU/g.d, 1.100 AU/g.d, 1.156 AU/g.d, and 1.268 AU/g.d at 30 minutes, 60 minutes, 90 minutes and 120 minutes respectively. The highest pigment extraction was at 120 minutes or 2 hours duration.

It generally showed that higher time would increase the concentration of solute analyzed. Therefore, sonication time was directly proportional to concentration of red pigment. Since ultrasonic extraction involved ultrasonic wave, the microbial cell was disrupted (Mason *et al*., 1996; Dolatowski *et al.,* 2007). The disrupted cell would dissolve properly in time, thus increasing the concentration of extract.

In addition, this ultrasonic extraction could also be compared with solvent extraction. 1.268 AU/g.d of red pigment had been extracted in ultrasonic extraction while 1.278AU/g.d of red pigment was recorded in solvent extraction.

In ANOVA table 4.2 of this ultrasonic extraction, the significant of this method was 8.35E-08 where this p-value was less than α=0.05. Therefore, it showed a significance effect when using different sonication time in ultrasonic extraction in order to extract red pigment.

Table 4.2 ANOVA table for ultrasonic extraction

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
30min	3	3.138571	1.04619	8.84E-06
60min	3	3.3	1.1	0.00021
90min	3	3.47	1.156667	9.05E-05
120min	3	3.804286	1.268095	0.00025

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.081169	3	0.027056	193.5422	8.35E-08	4.066181
Within Groups	0.001118	8	0.00014			
Total	0.082288	11				

4.2.5 SOXHLET EXTRACTION

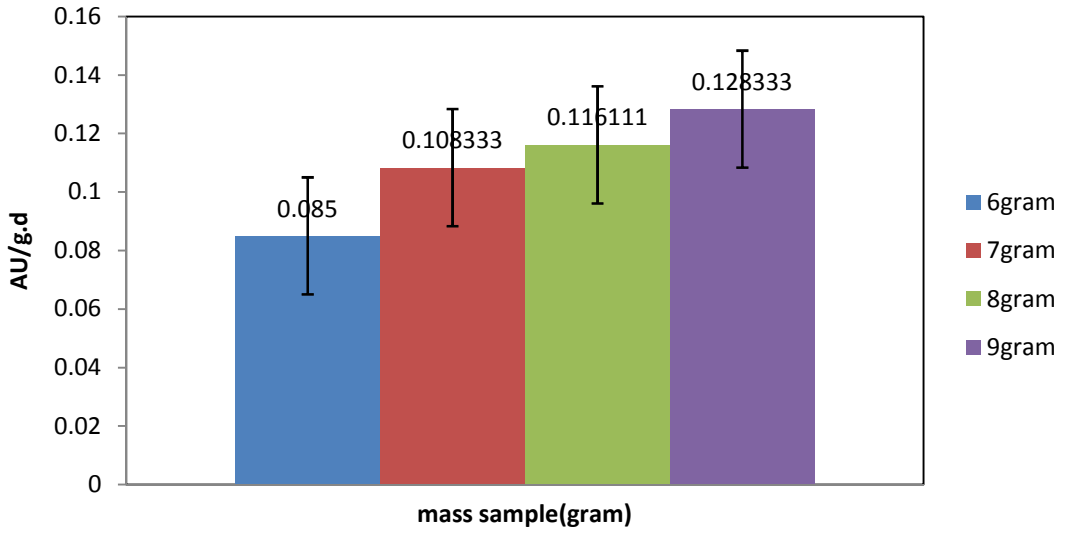


Figure 4.6 Effect of mass sample to the absorbance(AU/g.d)

The figure above showed the effect of amount of different fermented mass to the pigment extraction. The values were recorded as 0.085AU/g.d with 6 gram substrate, 0.108 with 7gram substrate, 0.116AU/g.d when using 8 gram of fermented substrate and 0.128AU/g.d when using 9 gram fermented substrate.

The figure showed that the highest pigment extract was analyzed when using 9 gram of fermented substrate. The absorbance increased with increasing fermented mass. This was because higher amount of powder would contain higher amount of solute. Therefore, this in turn increased the concentration analyzed when performing Soxhlet extraction at constant condition.

Besides, the performance of this method would also be compared to solvent extraction as the reference method. In this Soxhlet extraction, the highest extract was only 0.128AU/g.d as compared to solvent extraction which was 1.278AU/g.d. This was because this method cannot use the ratio as in other methods since the boiling point of 60% ethanol as the solvent would not be achieved. Therefore, the volume of

solvent used was 250mL in order to complete the extraction process. As such, this method was not considered efficient to be used in laboratory scale.

The ANOVA of this method showed that the p-value was 3.79E-05 which was lower than the $\alpha=0.05$ as indicated I table 4.3. The extraction at different mass of fermented substrate was significant to be conducted. In conclusion, this method was significant but less efficient to be conducted.

Table 4.3 ANOVA table for Soxhlet extraction

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6gram	3	0.218571	0.072857	1.84E-05
7gram	3	0.278571	0.092857	3.27E-05
8gram	3	0.298571	0.099524	4.76E-06
9gram	3	0.33	0.11	1.84E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002204	3	0.000735	39.63303	3.79E-05	4.066181
Within Groups	0.000148	8	1.85E-05			
Total	0.002352	11				

4.3 Comparison of Methods

The result of these methods can be concluded as in the table below.

Table 4.4 Result summary of extraction methods

Type of method	Pigment extract (AU/g.d)	Type of substrate
Solvent extraction(reference) -24 hours, static condition	1.278	A
Ultrasonic extraction -120minutes	1.268	B
Agitated extraction -110rpm	1.265	C
Soxhlet extraction -12 hours	0.128	D

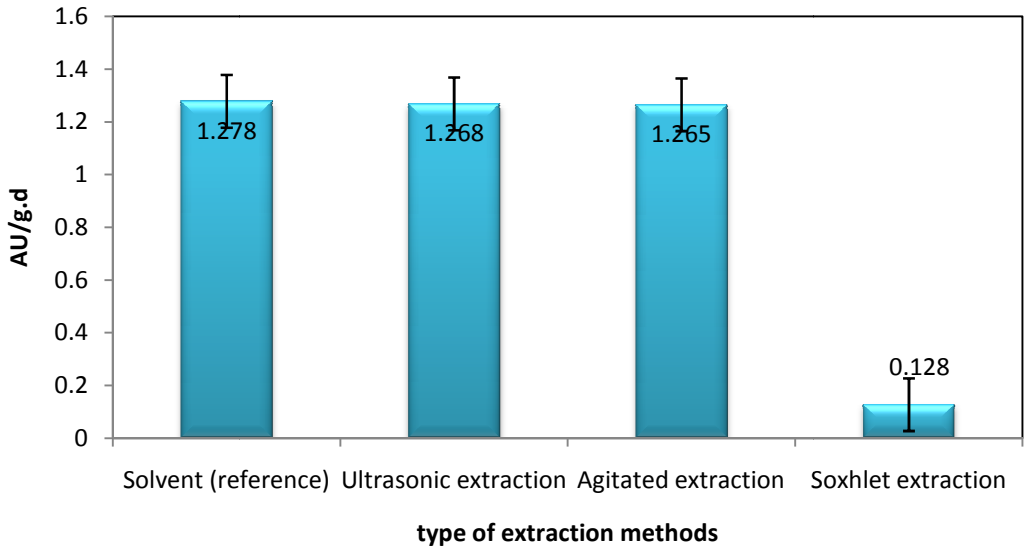


Figure 4.7 Performance of extraction methods to extract red pigment

Figure above showed that the recorded data were 1.268AU/g.d for ultrasonic extraction, 1.265AU/g.d for agitated extraction and 0.128AU/g.d for Soxhlet extraction. The highest pigment extract would be generated when using ultrasonic extraction. Therefore, it could be concluded that ultrasonic extraction was the most efficient method compared to other methods.

These results were compared with the reference which was the solvent extraction showing an extract of highest value,1.278AU/g.d since this method was conducted first to investigate the best solvent and then would be followed by other methods.

The results could not be comparable although the values were closed to each other. This was because all of the samples were fermented in different Erlenmayer flask and were considered as different source of fermented substrate. Therefore, in order to validate the data, a separate experiment was carried out to compare the most efficient method with solvent extraction. The experiment would be conducted with both methods used ratio of solvent to substrate was 2:1 with the similar source of fermented substrate.

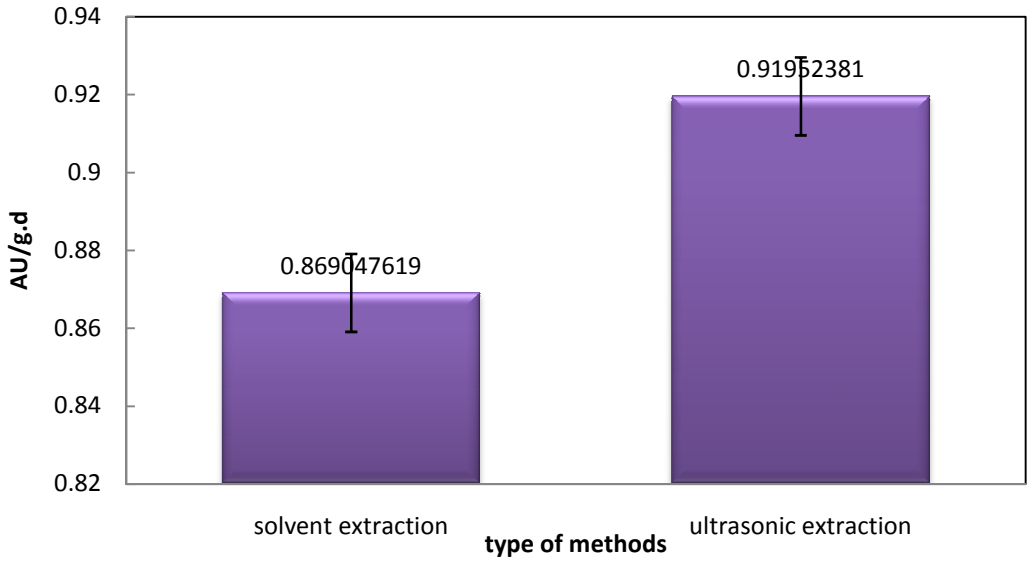


Figure 4.8 Comparison of extraction methods

Figure 4.8 showed that the extracted products were higher when using ultrasonic extraction, 0.920AU/g.d than in solvent extraction, 0.869 AU/g.d. This meant that ultrasonic extraction was the most efficient method used to extract red pigment by *Monascus purpureus.* However,the value of extract seemed to be lower

than in the first experiment conducted before. This might be because this separate experiment was conducted last where the pigment concentration reduced.

Ultrasonic extraction was carried out for 2 hours with 60% ethanol since this sonication time produced the highest extracted product before, while solvent extraction was conducted with 60% ethanol for 24 hours. The duration time of extract was longer. Besides, the increment value when using ultrasonic extraction was 5.81%. In conclusion, ultrasonic extraction would be selected since it produced higher extract of red pigment at shorter time and showed an aincrement in pigment extract. This would contributed well in industrial applications.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, this research is carrying out to accomplish the demand for natural color additives in food technology industries and lessen the use of artificial coloring agents which give negative side effects to health. Besides, the objective of this study is to analyze the performance of red pigment production by *Monascus purpureus* using different extraction methods. The extraction methods used are static, agitated, Soxhlet extraction and ultrasonic extraction which performed at different conditions to compare their quantity of extracted product.

Solvent extraction was used as the reference and this method produced 1.278AU/g.d pigment extract. The results were 1.268AU/g.d for ultrasonic extraction, 1.265AU/g.d for agitated extraction and 0.128AU/g.d for Soxhlet extraction. The best method was ultrasonic extraction since it produced higher

extract, 0.920AU/g.d as compared to solvent extraction which produced 0.869 AU/g.d pigment extract. The increrment percentage was about 5.81%.

An analysis of variance (ANOVA) was carried out to investigate the significance in using the methods. The p-value for significance is less than 0.05. The p-value for agitated extraction was 3.63x10-7, 8.35x10-8 for ultrasonic extraction and 3.79x10-7 for Soxhlet extraction. Therefore, all of the methods were significant to be carried out. It is hoped that this research will give benefits to industrial applications in future.

5.2 Recommendation

In order to analyze more about extraction method in extracting red pigment by *M.purpureus* on red yeast rice, an optimization of parameters in ultrasonic extraction need to be investigated in the future. This is in order to investigate the optimum value which shows the highest or lowest extracted product. In this study, it only provided the value of sonication time showing the highest extracted product. Future study need to be done to investigate the optimum sonication time. Other parameters can be investigated are for instance, ultrasonic power, mass of solvent and volume of sample. This will in turn provide for more important information in order to apply in industrial applications.

REFERENCES

Arunachalam,C.,and Narmadhapriya,D.(2011).*Monascus* Fermented Rice and Its Beneficial Effects: A New Review.Asian Journal of Pharmaceutical and Clinical Research,4(1),2011.

Babitha, S.,Soccol,C. R.,and Pandey,A. (2006). Solid-state fermentation for the production of *Monascus* pigments from jackfruit seed. Bioresource Technology, 98(8),1554-1560.

Carvalho,J.C.,Oishi,B.O.,Woiciechowski,A.L., Pandey,A., Babitha,S.& Soccol1,C.R. (2007). Effect of Substrates on the Production of *Monascus* Biopigments by Solid-state. Indian Journal of Biotechnology,6, 194-199.

Chairote,E., Chairote, G., Wongpornchai,S., & Lumyong,S.(2007). Preparation Of Red Yeast Rice Using Various Thai Glutinous Rice and *Monascus Purpureus* Cmu001 Isolated From Commercial Chinese Red Yeast Rice Sample. KMITL Sci. Tech., 7, 28-37.

Dhale, M.A.(2007). Physiology of *Monascus purpureus* in Relation to Metabolite Production and Application as Functional Food. PhD thesis,University of Mysore.

Dolatowski,Z.J.,Stadnik,J.,and Stasiak,D.(2007).Application of Ultrasound in Food Technology. Acta Sci. Pol., Technol. Aliment.,6(3), 89-99.

Dufosse,L.,Galaup,P.,Yaron,A.,Arad,S.M.,Blanc,P.,Murthy,K.N.C.and Ravishankar,G.A. (2005). Microorganisms and Microalgae as Sources of Pigments for Food Use: A Scientific Oddity or an Industrial Reality? Trends in Food Science & Technology, 16,389-406.

Ghosh.R.(2006). Principles of Bioseparations Engineering,World Scientific,Canada.

International Rice Research Institute. (2010). Measuring the Moisture Content . Rice Knowledge Bank.

Jiefeng,L.,Yiran,R.,and Shanjing,Y.(2010).Repeated-batch Cultivation of Encapsulated *Monascus purpureus* by Polyelectrolyte Complex for Natural Pigment Production. Chinese Journal of Chemical Engineering,18(6),1013-1017.

Johns,M.R and Stuart,D.M.(1991).Production of pigments by *Monascus purpureus* in solid culture.Journal of Industrial Microbiology,8,23-28.

Lim,H.S.,Yoo,S.K..,Shin,C.S.,Hyun,Y.M.(2000).*Monascus* Red Pigment Overproduction by Coculture with Recombinant *Saccharomyces cerevisiae* Secreting Glucoamylase. Journal of Microbiology, 38,48-51.

Lin,Y.L.,Wang,T.H.,Lee,M.H.,and Su,N.W.(2008).Biologically Active Components and nutraceuticals in the *Monascus*-fermented rice :a review. Appl Microbiol Biotechnol, 77,965–973.

Mapari,S.A.S.,Hanse, M. E.,Meyer,A.S.,and Thrane,U.(2008).Computerized Screening for Novel Producers of *Monascus*-like Food Pigments in *Penicillium* Species. Journal of Agricultural and Food Chemistry, 56,9981-9989.

Mason,T.J.,Paniwnyk,L.,Lorimer,J.P.(1996). The Uses of Ultrasound in Food Technology.Ultrasonics Sonochemistry,3(3),S253-S260.

Mukherjee,G. and Singh, S.K. (2011). Purification and Characterization of a New Red Pigment from *Monascus purpureus* in Submerged Fermentation. Process Biochemistry, 46(1),188-192.

Pattanagul,P.,Pinthong, R.,Phianmongkhol,A.,and Leksawasdi,N. (2007). Review of Angkak Production (*Monascus purpureus*). Chiang Mai Journal Science, 34(3),319-328.

Rosenblitt,A.,Agosin,E.,Delgado,J.,and Correa,R.P.(2000).Solid Substrate Fermentation of *Monascus purpureus*: Growth,Carbon Balance,and Consistensy Analysis. Biotechnol. Prog. ,16, 152-162.

Sivakumar, V.,Lakshmi,A.J. ,Vijayeeswarri,J.,and Swaminathan,G.(2009). Ultrasound assisted enhancement in natural dye extraction from beetroot for industrial applications and natural dyeing of leather. Ultrasonics Sonochemistry, 16(6),782-789.

Shuler,M.L. and Kargi,F.(2002).Bioprocess Engineering Concepts, Prentice Hall,USA.

Teng,S.S. and Feldheim,W.(2000).The fermentation of Rice for Anka Pigment Production. Journal of Industrial Microbiology & Biotechnology,25,141-146.

Velickovic, D.T., Mileovic, D. M., Ristic, M.S., and Veljkovic, V.B.(2006). Kinetics of ultrasonic extraction of extractive substances from garden (Salvia officinalis L.) and glutinous (Salvia glutinosa L.) sage. Ultrasonics Sonochemistry, 13,150-156.

Velmurugan,P.,Kannan,S.K.,Balachandar,V.,Lakshmanaperumalsamy,P.,Chae,J.C., and Oh,B.T. (2010). Natural Pigment Extraction from Five Filamentous Fungi for Industrial Applications and Dyeing of Leather. Carbohydrate Polymers, 79,262-268.

Wang,L. and Weller,C.L.(2006).Recent Advances in extraction of nutraceuticals from plants.Trends in Food Science and Technology,17(6),300-312.

APPENDIX A

Table A.1 Solvent extraction (before dilution)

Solvent	**reading 1**	**reading 2**	**reading 3**	**average of reading**
deionized water	2.701	2.703	2.7	2.701333333
dimethyl sulfoxide	0	0	0	0
methanol	2.797	2.797	2.824	2.806
95% ethanol	2.721	2.769	2.797	2.762333333
60% ethanol	2.958	2.958	2.957	2.957666667

Table A.2 Solvent extraction (After dilution)

solvent	**reading 1**	**reading 2**	**reading 3**	**average of reading**	**standard deviation**
deionized water	0.595	0.596	0.594	0.595	0.000816497
dimethyl sulfoxide	0	0	0	0	0
methanol	0.605	0.688	0.681	0.658	0.037585458
95% ethanol	0.605	0.6	0.598	0.601	0.00294392
60% ethanol	0.895	0.894	0.895	0.894666667	0.000471405

Table A.3 Summary of result for solvent extraction

solvent	**AU/g.d**
deionized water	0.85
dimethyl sulfoxide	0
methanol	0.94
95% ethanol	0.858571429
60% ethanol	1.278095238

Table A.4 Agitated extraction at 110rpm (Before dilution)

ratio	**reading 1**	**reading 2**	**reading 3**	**average reading**
2:01	2.745	2.721	2.721	2.729
3:01	2.598	2.509	2.52	2.542333333
4:01	2.46	2.466	2.465	2.463666667
5:01	2.294	2.303	2.271	2.289333333
6:01	2.222	2.222	2.224	2.222666667

Table A.5 Agitated extraction at 110rpm (After dilution)

ratio	**reading 1**	**reading 2**	**reading 3**	**average reading**	**standard deviation**
2:01	0.869	0.884	0.883	0.878666667	0.006847546
3:01	0.857	0.855	0.854	0.855333333	0.001247219
4:01	0.846	0.846	0.855	0.849	0.004242641
5:01	0.804	0.823	0.894	0.840333333	0.038732702
6:01	0.743	0.776	0.751	0.756666667	0.014055446

Table A.6 Summary result of agitated extraction at 110rpm

Ratio of solvent to substrate	**AU/g.d**
2:01	1.255238
3:01	1.221905
4:01	1.212857
5:01	1.200476
6:01	1.080952

Table A.7 Agitated extraction (Before dilution)

speed(rpm)	**reading 1**	**reading 2**	**reading 3**	**average reading**
100	2.231	2.238	2.250	2.2396667
110	2.745	2.721	2.721	2.729
150	2.62	2.553	2.537	2.57
200	2.444	2.432	2.442	2.439333333
250	2.268	2.284	2.293	2.281666667

Table A.8 Agitated extraction (After dilution)

speed(rpm)	**reading 1**	**reading 2**	**reading 3**	**average reading**	**standard deviation**
100	0.659	0.651	0.601	0.637	0.025665
110	0.887	0.875	0.894	0.88533	0.00785
150	0.799	0.781	0.778	0.786	0.00927
200	0.785	0.762	0.778	0.775	0.00963
250	0.77	0.783	0.743	0.76533	0.01666

Table A.9 Summary result for agitated extraction

speed(rpm)	AU/g.d(1)	AU/g.d(2)	AU/g.d(3)	AU/g.d(average)
100rpm	0.941429	0.93	0.858571	0.91
110rpm	1.267143	1.25	1.277143	1.264762
150rpm	1.141429	1.115714	1.111429	1.122857
200rpm	1.121429	1.088571	1.111429	1.107143
250rpm	1.1	1.118571	1.061429	1.093333

Table A.10 Ultrasonic extraction (Before dilution)

effect of sonication time(min)	reading 1	reading 2	reading 3	average reading
30	2.41	2.479	2.389	2.426
60	2.473	2.489	2.5	2.487333333
90	2.62	2.62	2.537	2.592333333
120	2.678	2.678	2.675	2.677

Table A.11 Ultrasonic extraction (After dilution)

effect of sonication time(min)	reading 1	reading 2	reading 3	average reading	standard deviation
30	0.73	0.733	0.734	0.732333333	0.001699673
60	0.772	0.779	0.759	0.77	0.008286535
90	0.814	0.813	0.802	0.809666667	0.005436502
120	0.876	0.889	0.898	0.887666667	0.009030811

Table A.12 Summary result for ultrasonic extraction

effect of sonication time(min)	**AU/g.d(1)**	**AU/g.d(2)**	**AU/g.d(3)**	**AU/g.d(average)**
30	1.042857	1.047143	1.048571	1.046190476
60	1.102857	1.112857	1.084286	1.1
90	1.162857	1.161429	1.145714	1.156666667
120	1.251429	1.27	1.282857	1.268095238

Table A.13 Soxhlet extraction(Before dilution)

mass sample(gram)	**reading 1**	**reading 2**	**reading 3**	**average reading**
6	0.444	0.449	0.458	0.450333333
7	0.493	0.506	0.509	0.502666667
8	0.549	0.548	0.544	0.547
9	0.557	0.551	0.563	0.557

Table A.14 Soxhlet extraction (After dilution)

mass sample(gram)	**reading 1**	**reading 2**	**reading 3**	**average reading**	**standard deviation**
6	0.054	0.051	0.048	0.051	0.00244949
7	0.065	0.069	0.061	0.065	0.003265986
8	0.071	0.07	0.068	0.069666667	0.001247219
9	0.077	0.074	0.08	0.077	0.00244949

Table A.15 Summary result for Soxhlet extraction

mass sample(gram)	AU/g.d(1)	AU/g.d(2)	AU/g.d(3)	AU/g.d(average)
6	0.077143	0.072857	0.068571	0.072857
7	0.092857	0.098571	0.087143	0.092857
8	0.101429	0.1	0.097143	0.099524
9	0.11	0.105714	0.114286	0.11

APPENDIX B

Total volume =volume sample + volume of solvent

=1ML + 9ML

=10 ML

Dilution factors (DF) can be calculated from this formula :

DF = total volume/ volume of sample

$$= \frac{10ML}{1ML}$$

= 10

Fermented dry mass : 7 g dry solid.

Specific absorbance(AU/g.d) = (Absorbance x DF)/ substrate dry mass

In table A.1,for solvent extraction after dilution, the average absorbance reading : 0.894666667

Specific absorbance = $\frac{0.894666667*10}{7}$

=1.2781 AU/g.d