PRODUCTION OF FLEXIRUBIN PIGMENT FROM *Chryseobacterium artocarpi* CECT 8497 USING BANANA PEEL WASTE AS SUPPLEMENT

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DEDICATION

This study is wholeheartedly dedicated to my beloved father and mother, Wahidin bin Md Tasar and Sarimah bt Abu Bakar, and also my better half, Rosyiela Azwa binti Roslan, who have been source of inspiration, strength when thought of give up, who continually provide their moral, spiritual, emotional, financial and very supportive through thick and thin for this meaningful years. Thanks for all the prayers, advices and guidance.

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ABSTRACT

As the present trend throughout the world is shifting towards the use of eco-friendly and biodegradable commodities, the demand for natural colorants is increasing. The production of bacterial pigments from agricultural wastes and application of the pigments as natural colorants have been investigated by many researchers. Banana peel waste is highly nutritious owing to its high levels of starch, crude protein, fibre and vitamins in nature that facilitates microbial growth. Flexirubin is a yellowish orange-colour pigment produced from *Chryseobacterium artocarpi* CECT 8497 that exhibits pharmacological properties including antibacterial activity and a potential anticancer when formulated with silver nanoparticles. Previous reports have shown that the pigment production by *C. artocarpi* CECT 8497 is largely influenced by chemical components, such as lactose, L-tryptophan, and potassium dihydrogen phosphate (KH_2PO_4). However, pigment production by physical means, such as controlling the bioreactor condition in order to obtain maximum pigment yield, has not been much reported. Hence, in this study, one-variable-at-a-time (OVAT) approach was employed to seek for the physical key determinants, which are agitation rate, aeration rate, and temperature that would impose a significant effect on the production of flexirubin pigment. The pigment production in a 2-L bioreactor was then optimized using the self-learning particle swarm optimization (PSO) algorithm. The OVAT results show that at a temperature of 30°C, aeration rate of 2 L/min, and agitation rate of 250 rpm, afforded highest flexirubin pigment yield of 0.144 g/L. However, under optimized conditions, namely temperature of 29.80°C, aeration rate of 2.23 L/min and agitation of 245 rpm, slightly higher pigment yield (0.176 g/L) was obtained compared to the predicted yield (0.151 g/L). The pigment produced has the potential to be used as ink for plastic food packaging and as an antibacterial detergent. The findings of this study would be useful for increasing the efficiency in producing natural pigment using a low-cost medium in a pilot scale or even in an industrial scale. The application of pigment as ink for plastic food packaging and as antibacterial detergent will be the development of safe products for better future.

ABSTRAK

Sejajar dengan haluan dunia kini yang beralih ke arah penggunaan komoditi yang mesra alam dan boleh terbiogradasi, permintaan terhadap pigmen semula jadi semakin meningkat. Penghasilan pigmen bakteria daripada sisa pertanian dan penggunaan pigmen itu sebagai bahan pewarna semula jadi telah dikaji oleh ramai penyelidik. Sisa kulit pisang mempunyai nilai nutrisi yang tinggi kerana kandungan kanji, protin mentah, serat, dan vitaminnya yang tinggi secara semula jadi yang membantu pertumbuhan mikroorganisma. Flexirubin ialah pigmen berwarna oren kekuningan yang dihasilkan oleh *Chryseobacterium artocarpi* CECT 8497 yang mempunyai sifat-sifat farmakologi termasuk aktiviti antimikrob dan berpotensi sebagai antibarah apabila diformulasikan dengan nanopartikal perak. Laporan terdahulu telah membuktikan bahawa penghasilan pigmen oleh *C. artocarpi* CECT 8497 dipengaruhi terutamanya oleh komposisi kima, misalnya laktosa, L-triptofan dan kalium dihidrogen fosfat (KH_2PO_4). Walau bagaimanapun, penghasilan pigmen dengan cara pengawalan fizikal, misalnya pengawalan keadaan bioreaktor untuk memperoleh hasil pigmen maksimum, masih kurang dilaporkan. Oleh itu, dalam kajian ini, pengunaan kaedah satu pemboleh ubah pada satu masa (OVAT) telah digunakan untuk mencari titik-titik penentu utama, iaitu kadar pengacauan, kadar pengudaraan dan suhu yang memberi kesan penting kepada penghasilan pigmen fleksirubin. Penghasilan pigmen di dalam bioreaktor 2 L telah dioptimumkan menggunakan algoritma pengoptimuman kawanan partikel (PSO) belajar sendiri. Kajian OVAT menunjukkan pada suhu 30°C, kadar pengudaraan 2 L/min dan kadar pengacauan 250 rpm mampu memberi hasil pigmen fleksirubin tertinggi 0.144 g/L. Walau bagaimanapun, pada keadaan optimum, iaitu suhu 29.30°C, kadar pengudaraan 2.23 L/min dan kadar pengacauan 245 rpm hasil pigmen yang lebih tinggi sedikit (0.176 g/L) telah dihasilkan berbanding dengan hasil yang diramalkan (0.151 g/L). Pigmen yang terhasil berpotensi untuk digunakan sebagai dakwat bagi pembungkus makanan plastik dan sebagai bahan pencuci antibakteria. Kajian ini boleh digunakan untuk meningkatkan kecekapan pembuatan pigmen semula jadi menggunakan bahan pengantara kos rendah dalam skala perintis atau juga dalam skala industri. Penggunaan pigmen sebagai dakwat bagi pembungkus makanan plastik dan sebagai bahan pencuci antibakteria merupakan pembangunan produk yang selamat untuk masa depan yang lebih baik.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANN	-	Artificial Neural Networks
BPW	-	Banana Peel Waste
EA	-	Ethyl Acetate
LPW	-	Liquid Pineapple Waste
PSO	-	Particle Swarm Optimization
MEK	-	Methyl Ethyl Ketone
NA	-	Nutrient Agar
NB	-	Nutrient Broth
OVAT	-	One Variable at A Time
HDPE	-	High Density Polyethylene
LDPE	-	Low Density Polyethylene
PP	-	Polypropylene
PET	-	Polyethylene Terephthalate
ASTM	-	American Society for Testing and Materials
ANOVA	-	Analysis of Variance

LIST OF SYMBOLS

mg L^{-1}	**-**	Milligram per litre
°C	-	Degree celcius
Rpm	-	Revolution per minute
v/v	-	Volume per volume
L*	-	The lightness of an object
a*	-	The measure of redness (positive value) or greenness (negative)
b*	-	The measure of yellowness (positive value) or blueness (negative value)
C*	-	Chroma value
h°	-	Hue angle, an angular measurement

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Nowadays, synthetic colorants have been widely used in foods, fabrics, cosmetics, and pharmaceutical products. However, various hazardous effects were encountered during their uses and this has increased the interest towards natural pigments (Venil and Lakshmanaperumalsamy, 2009). Natural pigments are expected to be non-toxic, non-carcinogenic, and biodegradable, thus play a vital role in the development of natural food colourings. Natural pigments can be obtained from living organisms, such as plants, animals, fungi, and microorganisms (Vargas et al., 2000). Bacteria offer certain advantages of pigment production based on their flexibility, short life cycle, easy propagation process, and simple extraction technique compared to other sources of natural pigments (Hendry and Houghton, 2013). Bacterial pigments also possess several pharmacological properties, such as antibacterial, antimicrobial, and antioxidant activities. Currently, there has been an increasing trend towards the replacement of synthetic colorants by natural pigments because of the strong consumer demand for natural products.

In recent years, environmental problems have boosted agriculture waste importance and a growing interest in the efficient use of various agriculture wastes. Banana (*Musa sapientum)* is the second largest produced fruit after citrus, contributing about 16 % to the world's total fruit production. Twenty seven percent of the world's banana production is contributed by India alone. Banana peel is a rich source of starch (3 %), crude protein (6-9 %), crude fat (3.8-11 %), total dietary fibre (43.2-49.7 %), and polyunsaturated fatty acids, particularly linoleic acid and α-linolenic acid, pectin, essential amino acids (leucine, valine, phenylalanine, and threonine), and micronutrients (K, P, Ca, Mg). Banana peels are also a good source of lignin (6-12 %), pectin (10-21 %), cellulose (7.6-9.6 %), hemicelluloses (6.4-9.4 %), and galactouroninc acid. Additionally, banana peels are affordable and renewable low-cost raw materials, which make them a potential feedstock for ethanol production (Bhatia et al., 2010).

Microbial pigments can be produced either by a solid-state fermentation or submerged fermentation. Aruldass et al. (2016) had successfully carried out a submerged fermentation, in a pilot-scale of *Chryseobacterium artocarpi* CECT 8497 to produce flexirubin pigment using liquid pineapple waste as the growth medium. Although with efficient and rigorous control of some parameters of the culture condition in a bioreactor, there are few drawbacks (an uncontrolled bioreactor condition) to the fermentation, which resulted in a minimum yield of pigment production. Thus, it is necessary to achieve a balance between parameters, such as pH, temperature, and oxygen transfer in order to maximize the production and the yield of the pigment (Buffoa et al., 2016). However, the optimization of the operating conditions of bioreactors can be complicated to be achieved, mainly due to the highly nonlinear nature and the substantial unmodeled dynamics. Besides that, optimal operating conditions in bioreactors, i.e., the effect of the external perturbations or small changes in the bioreactor environment can lead to undesirable operating conditions (Lara-Cisnerosa et al., 2014).

The optimization of parameters for fermentation is crucially needed to obtain a high yield of pigment in a short time. Hence, a predictive model to illuminate the complicated law between various parameters in a bioreactor of the fermentation process is necessary. The particle swarm optimization (PSO) is an animal activity-based meta-heuristic optimization algorithm, inspired by the cooperative social behaviour of bird flocking and fish schooling (Xia et al., 2017). It has received much attention in solving optimization problems as it shows a better performance in computing speed, accuracy, and memory size compared to other methods, such as machine learning, neural network learning, and genetic computation (Zheng and Liao, 2016). In this technique, the experimental responses to experiment designs are fitted to a quadratic function. However, the application of PSO for bacterial pigment production has still not much yet reported.

In this study, fermentation parameters, such as agitation speed, aeration rate, and fermentation temperature were optimized using the PSO in order to maximize the yield of flexirubin pigment from *Chryseobacterium artocarpi* CECT 8497 in submerged 2 L bioreactor. The application of the PSO as the optimization tool minimized the number of experiments needed to study the relationship between experiment dependent variables (yield of flexirubin) and its independent variables (fermentation parameters). In order to achieve a low-cost production, liquid pineapple waste (LPW) and banana peel waste (BPW) were used as natural carbon and nitrogen sources for the growth of the bacteria during the fermentation as a substitution for expensive synthetic growth medium, such as nutrient broth (NB). Both LPW and BPW can be obtained easily and mostly free or at low prices, and as such it leads to cost effective in a large-scale production of bacterial secondary metabolite. Finally, the possible use of the flexirubin pigment as an antibacterial agent in the detergent formulation and as a component of printing ink were assessed in this study.

1.2 Problem Statement

Most commercially available culture media are expensive, such as nutrient broth (NB) and luria-bertani (LB), of which this factor has motivated the search for new formulations that support microbial growth (Tzintzun-Camacho, 2016). The utilization of agriculture organic waste as a cheap growth medium in production media is known as ecologically sound and economically advantageous. Table 1.1 shows the comparison in the production of 50 L of bacterial pigment prodigiosin from Serratia marcescens UTM1 using commercial and agricultural-based growth mediums (Venil et al., 2013). Replacing culture media with liquid pineapple waste (LPW) and banana peel waste (BPW) is expected to counter the high cost production of flexirubin pigment. Scaling-up in fermentation of bacterial pigment is needed as there is a growing demand for natural pigments in products. LPW and BPW are commonly chosen because of their availability and abundance compared to other agriculture wastes. However, the usage of BPW as a supplement and the optimization of flexirubin production in submerged bioreactor are still not widely researched.

Table 1.1 Price comparison to produce 50 L of bacterial pigment prodigiosin from Serratia marcescens UTM1 using commercial and agricultural-based growth mediums.

Prodigiosin production	Medium/raw materials	Quantity used (kg)	Cost (USD)	Total cost (USD)
Synthetic medium	Nutrient broth	0.4	58.61	58.61
Agricultural based	Agricultural-Based substrate	0.2	0.10	5.91
Substrate	Nutrient broth	0.044	5.81	

Fermentation parameters can be optimized by using OVAT and PSO. One-variable-at-a-time (OVAT) is an experimental design, of which experiment is designed by testing one factor at a time instead of multiple factors simultaneously. It can also be used in the preliminary screening of bioreactor parameter. However, the OVAT method cannot study the interactions between factors and is time consuming (Khadiga and Galal, 2018). Therefore, the optimization of physical parameters for maximum pigment production by using PSO is of utmost importance in making the process cost-effective and economically viable. However, lack of information on flexirubin pigment production using a bioreactor prompted studies on the optimization of this pigment to use PSO. PSO is a computational intelligence-based technique that is not largely affected by the size and nonlinearity of the problem. In addition, it can converge to the optimal solution in many problems where most analytical methods fail to converge. Simultaneously, it has a better performance in computing speed, computing accuracy, memory size, and requires a minimum number of experiments to predict the experimental designs for a maximum pigment production in a submerged fermentation in a 2-L bioreactor.

1.3 Hypothesis

Agriculture waste can be used as a growth medium and a maximum yield of flexirubin pigment can be obtained using an optimized temperature, aeration and agitation rates of fermentation setup as suggested by PSO.

1.4 Objectives

The objectives of this study are as follows:

(a) To study the application of banana peel waste for production of flexirubin pigment from *C. artocarpi* CECT 8497.

(b) To optimize the parameters, such as temperature, aeration and agitation rates using OVAT and PSO for the fermentation of *C. artocarpi* CECT 8497.

(c) To incorporate the flexirubin pigment as an antibacterial agent in the detergent formulation and as a colouring agent for coating of plastic materials.

1.5 Scope of Study

This research focuses on the optimization of the growth of *C. artocarpi* CECT 8497 in a 2-L bioreactor using the submerged fermentation technique. The range of the aeration and agitation rates and temperature of the bioreactor for optimum growth of *C. artocarpi* CECT 8497 were evaluated using one-variable-at-a-time (OVAT) method.

Next, a self-learning PSO algorithm was used to optimize the aeration rate, agitation rate, and temperature in a 2-L bioreactor. Based on the predicted value generated by PSO, a comparison between experimental and predicted values of the optimized condition for production of flexirubin pigment from *C. artocarpi* CECT 8497 could be made.

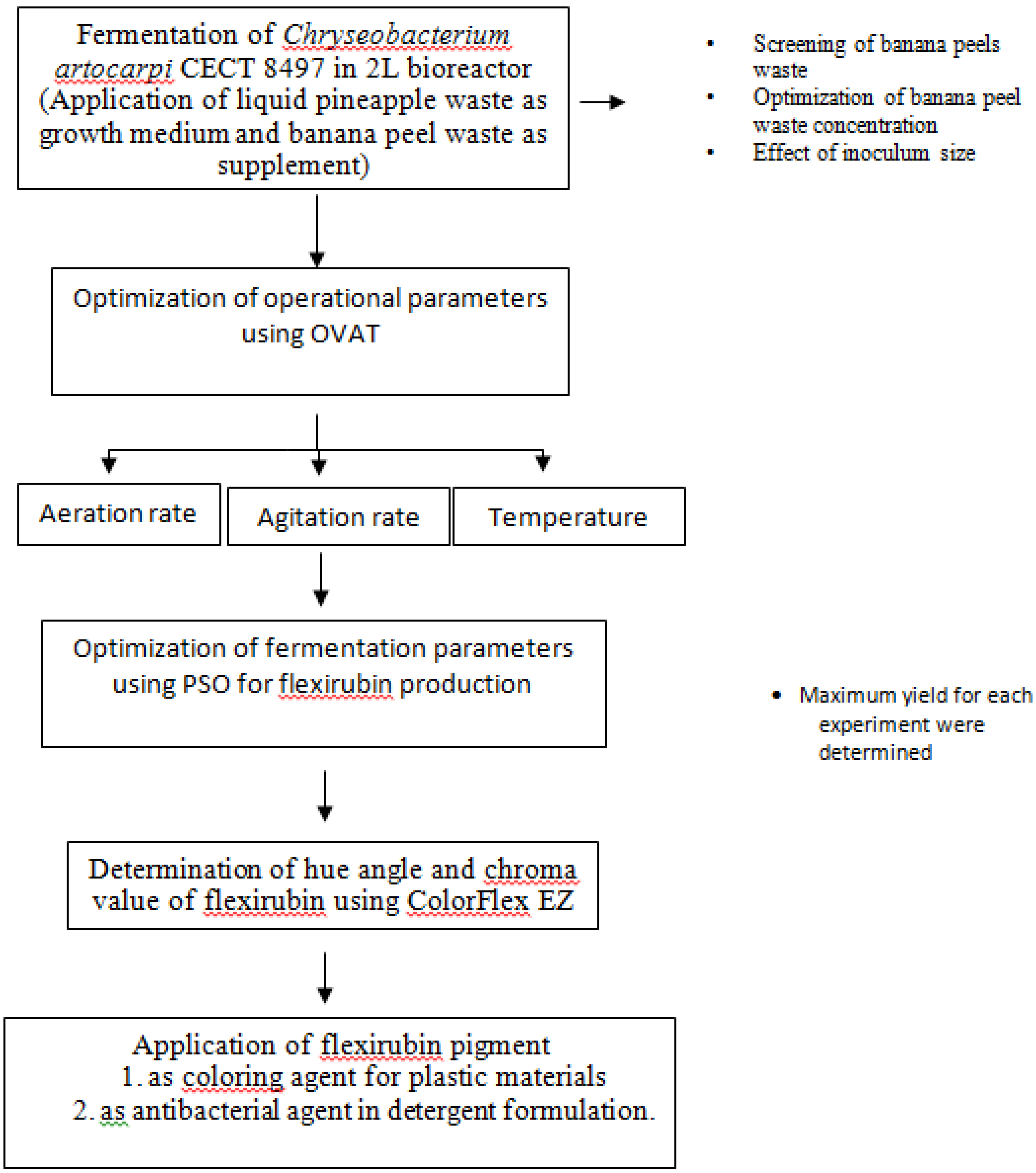
The flexirubin pigment was used as a colouring agent for plastic materials and as an antibacterial agent in detergent. Several types of plastics were tested, which were High Density Polyethylene (HDPE), Low Density Polyethylene (LDPE), Polypropylene (PP), and Polyethylene Terephthalate (PET). The flexirubin pigment was incorporated into a detergent solution using an ultrasonicator and its dipersion stability towards surfactants solution was tested. The disc diffusion method was used to determine the antibacterial activity of the detergent.

An overview of experiments conducted in this study is presented in section 1.7.

1.6 Significance of Study

The use of liquid pineapple waste as an alternative growth medium and banana peel waste as a growth supplement reduces the production cost of flexirubin. Besides that, the use of fruit wastes for the production of microbial pigments contributes to a lower waste generation and better waste management. In addition, the relationship between the fermentation parameters, which are the temperature, aeration and agitation rates as well as the production of flexirubin pigment were studied. A maximum yield of flexirubin pigment can be obtained based on the optimized conditions suggested by PSO. The application of the flexirubin pigment as a colouring agent of plastic materials and as an antibacterial agent in the detergent formulation was reported for the first time.

1.7 Overview of Study



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1. Venil, C. K., **Wahidin, M. A.**, Aruldass, C. A., Ahmad, W. A. (2017). Production of bacterial pigments in low cost medium and formulation of biodegradable. *Indian Journal of Experimental Biology*, *55*, 441-447. **(Q2, IF: 0.934)**

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1. **Wahidin, M. A.**, Aruldas. C. A, Mohd Hamzah, M. A. A., Setu, S. A., Ahmad, W. A. (2017). Flexirubin-type Pigment Production from Chryseobacterium artocarpi CECT 8497 and its Application as Natural Ink. *Journal of Energy and Environmental Sustainability, 3*, 62-65.