PRODUCTION OF NATURAL PIGMENT WITH ANTIMICROBIAL ACTIVITY FROM A MARINE BACTERIUM, *Pseudoalteromonas rubra* BF1A IBRL

by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

February 2016

ACKNOWLEDGEMENT

With deep gratitude, I would like to express my appreciation to my honourable supervisor Professor Darah Ibrahim for her guidance, patience, encouragements and advices given to me in completing this research work. She has been my supervisor since in year 2005, where I began my Masters study, and I am glad to work under her supervision. Her guidance and her trust on me had encouraged me so much until I completed this study. Thank you very much to her.

I am also thankful to my co-supervisor, Dr. Wan Norhana Noordin for her suggestions, innovative ideas and also her assistance in collection of some samples used in this study. I genuinely appreciate her help. I am also thankful to Dr. Lim Sheh Hong for her time, guidance and also for sharing information and knowledge regarding this project.

Moreover, I acknowledge the financial supports provided by Ministry of Higher Education (MOHE) for MyBrain15 and also by Universiti Sains Malaysia (USM) for the Geran Penyelidikan Siswazah Penyelidikan (RU-PRGS).

Not forgetting the Industrial Biotechnology Research Laboratory (IBRL) members, primarily Dr. Tong Woei Yenn, and Miss Nor Afifah Supardy for their endless supports, care and valuable friendships.

My special thanks goes to my father, mother, hubby and son for their undyeing love, continuous supports, advices, inspiration and motivations.

Azlinah

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ACE	Acetone
ATCC	American Type Culture Collection
CCB	Centre for Chemical Biology
CFU	Colony Forming Unit
CHL	Chloroform
CV-I	Crystal violet-Iodine
DE	Diethyl ether
EtOAc	Ethyl acetate
FDA	Food and Drug Administration
H_2O_2	Sulphuric acid
HCL	Hydrochloric acid
HMDS	Hexamethyldisilazine
HPLC	High Performance Liquid Chromatography
Hx	Hexane
INT	p-iodonitrotetrazolium violet
LC_{50}	50% lethal concentration
MA	Marine Agar
MAP	2-methyl-3-n-amyl-pyrrole
MBC	Minimum Bactericidal Concentration
MBC	4-methoxy-2,2'-bipyrrole-5-carbaldehyde
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MM	Minimal medium
MRSA	Methicillin-resistance *Staphylococcus aureus*
NA	Nutrient Agar
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
OD	Optical density
P	Iso-propanol
P.I	Polarity index
PDA	Potato Dextrose Agar
r/t	Retention time
R_f	Retention factor
SDA	Sabouraud Dextrose Agar
SDC	The Society of Dyers and Colourists
SEM	Scanning Electron Microscope
TEM	Transmission electron microscope
TLC	Thin Layer Chromatography
UV/vis	Ultra-violet visible

PENGHASILAN PIGMEN SEMULA JADI DENGAN AKTIVITI ANTIMIKROB DARIPADA BAKTERIA MARIN, *Pseudoalteromonas rubra* BF1A IBRL

ABSTRAK

Pigmen semula jadi adalah alternatif kepada pewarna sintetik yang sering digunakan dalam industri tekstil, kosmetik, makanan, farmaseutikal dan juga industri akuakultur. Bakteria marin adalah salah satu sumber biologi yang produktif dalam menghasilkan pigmen semula jadi. Selain berfungsi untuk mewarna, pigmen semula jadi juga telah dilaporkan mempunyai fungsi antimikrob. Kajian untuk penemuan sebatian antimikrob masih berterusan dan dengan itu, kajian mengenai sebatian antimikrob berwarna turut dapat membantu mengembangkan lagi penyelidikan antimikrob tersebut di mana sebatian antimikrob yang berwarna dapat memberikan lebih manfaat kepada pelbagai industri. Hal ini dapat berlaku kerana sebatian tersebut bukan sahaja berfungsi untuk mewarna malah turut dapat menjadi bahan pengawet pada masa yang sama bagi sesuatu produk. Justeru itu, kajian ini dijalankan untuk menghasilkan pigmen semula jadi daripada bakteria marin yang dikulturkan di dalam sistem kelalang dan juga mengkaji aktiviti antibakteria yang dipamerkan oleh pigmen tersebut. Dalam kajian ini, sebanyak 30 bakteria berpigmen telah dipencilkan daripada sampel marin, termasuk 8 daripada makroalga, 8 daripada batu karang, 6 daripada sumber haiwan laut, 6 daripada pasir laut, 1 daripada air laut dan 1 daripada sangkar ikan. Kebanyakan bakteria yang dipencilkan adalah berpigmen kuning (53%), diikuti dengan jingga (30%), merah jambu (10%) dan merah (7%). Keputusan analisis gravimetrik bagi penghasilan pigmen menunjukkan bahawa semua pencilan dapat menghasilkan pelbagai jenis pigmen pada kuantiti yang berbeza, iaitu dalam julat 0.294 g / L hingga 1.542 g / L. Daripada 30 pencilan bakteria, didapati sebanyak 9 bakteria mempamerkan aktiviti antibakteria terhadap sekurang-kurangnya satu bakteria ujian. Daripada 9 jenis bakteria ini pula, 1 bakteria dipilih iaitu bakteria BF1A IBRL sebagai bakteria berpotensi kerana ia dapat merencat lebih banyak bakteria ujian dengan nilai MIC yang lebih rendah, iaitu di antara 0.28 -8.88 mg /mL. Pigmen ekstrasel yang dihasilkan oleh pencilan BF1A IBRL mempamerkan aktiviti antibakteria yang lebih baik berbanding dengan pigmen intrasel. Pencilan BF1A IBRL telah dikenalpasti sebagai *Pseudoalteromonas rubra* berdasarkan ciri-ciri fenotip dan genotip. Keputusan yang diperolehi daripada analisa UV/vis spektoskopi, ujian pigmen dan analisa kromatografi menunjukkan bahawa pigmen yang dihasilkan oleh *P. rubra* BF1A IBRL adalah jenis prodigiosin iaitu pigmen ini berwarna merah dan menunjukkan penyerapan serapan maksimum UV/vis pada 534 nm. Dalam kajian masa maut, aktiviti antibakteria ekstrak prodigiosin *P. rubra* BF1A IBRL adalah bergantung kepada kepekatan ekstrak. Keputusan daripada proses penyisihan dan penulenan mendedahkan bahawa pigmen prodigiosin yang tulen mempamerkan aktiviti antimikrob yang lebih baik berbanding dengan sebatian campuran. Fraksi aktif (Fraksi 4) yang diperolehi daripada kromatografi turus mempunyai nilai kepekatan perencatan minimum yang sama dengan pigmen prodigiosin yang ditulenkan menggunakan *preparative-TLC,* iaitu 13.75 µg / mL terhadap *B. subtilis, B. cereus,* MRSA, *S. aureus,* dan *A. anitratus*. Prodigiosin yang tulen adalah sangat toksik kepada *Artemia salina* bagi kedua-dua tahap akut dan kronik. Namun ekstrak prodigiosin tidak menunjukkan sifat toksik terhadap anak udang tersebut. Keupayaan pigmen prodigiosin dalam mewarnakan fabrik tekstil telah dikaji dan didapati prodigiosin mampu mewarnakan fabrik wol dengan lebih baik berbanding dengan fabrik kapas, satin sutera dan sutera. Kain wol yang telah dicelup dengan pewarna semula jadi menunjukkan daya ketahanan yang baik terhadap kesemua ujian ketahanan kecuali cahaya. Tambahan pula, fabrik wol yang dicelup dengan prodigiosin semula jadi menunjukkan 97.21% kepada 99.99% perencatan bakteria apabila fabrik yang ditambah dengan *B. subtilis, B. cereus,* MRSA, *S. aureus, A. anitratus* dan *S. epidermidis*, manakala 2.83% kepada 49.8% perencatan bakteria telah dicapai untuk *E. coli* dan *K. pneumoniae*. Secara keseluruhan, pigmen semula jadi yang diekstrak daripada *Pseudoalteromonas rubra* BF1A IBRL mempunyai potensi untuk menjadi bahan pewarna dan agen antibakteria sekaligus.

PRODUCTION OF NATURAL PIGMENT WITH ANTIMICROBIAL ACTIVITY FROM A MARINE BACTERIUM, *Pseudoalteromonas rubra* BF1A IBRL

ABSTRACT

Natural pigments are important substitutes to the synthetic dyes in many industrial applications including textile, cosmetics, food, pharmaceutical and also aquaculture industry. Marine bacteria is one of the prolific biological sources for the natural pigments. Besides colouring benefits, many natural pigments posses antimicrobial properties. The search of antimicrobial agents is a continuing dialogue to fight different types of diseases. Thus, the finding of colorant antimicrobial agent can expand the current portrait of the research and can give benefits to many industries since the colorants may give both tinctorial and preservative value simultaneously to the industrial products. The aim of the study is to produce natural pigment by cultivating the marine bacteria in shake flask system, and evaluate the antimicrobial activity of the natural pigment. In this study, a total of 30 cultivable pigmented bacteria were isolated from marine samples, including 8 from macroalgae, 8 from corals, 6 from animal resources, 6 from sand sediment, 1 from seawater and 1 from fish net. Most of the isolated strains were yellow pigmented bacteria (53%), followed by orange (30%), pink (10%) and red pigmented bacteria (7%). Gravimetric analysis of pigment production revealed that all the isolated strains were able to produce different types of coloration with different quantities, where the crude pigment yield ranged from 0.294 g/L to 1.542 g/L. Out of the 30 isolated bacteria, 9 bacteria exhibited antibacterial property against at least one test bacteria. Out of the 9 selected strains, 1 isolate, namely BF1A IBRL was selected as potential strain since it was able to inhibit broader range of test bacteria with lower MIC value, which ranged from 0.28 to 8.88 mg/mL. The extracellular pigment of isolate BF1A IBRL showed higher pigmentation strength with greater antimicrobial activity compared to intracellular pigment. Isolate BF1A IBRL was identified as *Pseudoalteromonas rubra* based on its phenotypic and genotypic characteristics. The results obtained from UV/vis spectrophotometer, presumptive test and chromatographic analysis indicated that the pigment produced by *P. rubra* BF1A IBRL is prodigiosin-type. The pigment was red and showed a maximum absorption at 534 nm. Based on the time kill assay the antibacterial activity of prodigiosin extract from *P. rubra* BF1A IBRL was concentration dependant. The results of bio-guided purification analysis revealed that the purified prodigiosin pigment exhibited greater antimicrobial activity compared to the crude extract. The active fraction (Fraction 4) obtained from column chromatography had similar MIC values with TLC-purified prodigiosin which was 13.75 µg/mL against *B. subtilis, B. cereus,* MRSA*, S. aureus,* and *A. anitratus.* The purified prodigiosin was highly toxic towards the *Artemia salina* for both acute and chronic toxicities; however the crude extract of the prodigiosin did not cause any toxic effect towards the brine shrimp. The production and evaluation of microbial pigment as textile colorants was investigated. The wool fabrics showed greater affinity towards the prodigiosin extract compared to cotton, silk satin and silk. The naturally dyed wool fabric showed fair to excellent fastness property towards all colourfastness properties except for light colourfastness. Furthermore, the results also disclose that the wool dyed with natural prodigiosin showed 97.21% to 99.99% of bacterial reduction when the fabric was treated with *B. subtilis, B. cereus,* MRSA*, S. aureus A. anitratus* and *S. epidermidis,* whereas 2.83% to 49.8% of bacterial reduction was achieved for *E. coli* and *K. pneumoniae*. Overall, the pigment component isolated from *Pseudoalteromonas rubra* BF1A IBRL has potential as colorant and preservative agent.

CHAPTER 1.0 INTRODUCTION

1.1 Problem statement

Synthetic pigments have been widely used as colouring agent in many industries including food, textile, cosmetics and also aquaculture. The synthetic pigments are created through chemical manufacturing, and exhibit toxic, carcinogenic and mutagenic properties, thus create lots of adverse effect on human and environments (Pathak & Chauhan, 2013). Recently, consumers awareness on natural products are alarming as a consequence of proven toxicological effect of some synthetic compounds, limited chemical diversity and structural complexity, and also the great success of natural products on the market in the last years. This consumer awareness has necessitated the need to explore for natural pigments from natural sources as an alternative to the synthetic pigments.

It is well known that the number of drug resistance pathogens have increased over time. The survival of these antibiotic-resistance pathogens in hospitals environments, hospitals fabrics and hospital worker's uniforms are the growing concern particularly in units in which patients are immunosuppressed either intentionally (as for transplantation) or as a result of trauma (severe burns) or disease (such as acquired immunodeficiency disease). Fabrics are known to be the vector for spreading the harmful bacteria (Neely & Maley, 2000). The most common harmful bacteria are *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae,* and *Enterobacter aerogenes* (Lee *et al*., 2009). As more bacteria have the ability to survive on fabrics, and some even become resistant to antibiotics, hence the ability to control the spread of these bacteria with antibiotic treatments decreases. Therefore, the research and investigation to develop fabrics with eco-friendly antibacterial finishing is vital in recent years.

1.2 Rational of study

Pigments are colourful compounds consisting of chromaphores, which either absorb or reflect light in varying wavelengths of the visible region (Delgado-Vargas *et al*., 2000). It is known that colour is the crucial factor of quality in some industrial product to be commercialized and also an important attribute that determine the consumer's acceptance. Hence, pigments are used as colouring agent for colouring the paints, plastics, inks, fabrics, cosmetics, food and other materials.

Natural pigments are safer, healthier, biodegradable, and exhibit higher compatibility with the environment. Hence, the screening process for biological source for new pigment is expected to be continued in the future. The natural sources of colorants have emerged at recent times, where the natural pigments can be either produced by plants (higher and lower plants) and also microorganisms.

Among the natural sources of pigments, microorganisms offer great advantages for the pigment production of commercial activities. Microorganisms are ubiquitous and an indispensable component of our industries. Moreover, microbial pigments provide a safer alternative to chemical synthetic dyes and an economical substitute for natural pigments obtained from plants and animals. Although microorganisms are distributed in both terrestrial and marine environments, and most of the bioactive compounds have been isolated from both environments, but still the marine microorganism are more attractive to researchers. This is because the marine microorganisms, particularly marine bacteria have potential to produce compounds with unique biological properties, this serves as an attractive choice for commercial scale production. Moreover, one of the main goals of the marine biotechnology recently is the finding of natural substances originated from marine microorganisms (Darabpour *et al.*, 2011). The various advantages of producing pigments from bacteria include ease of cultivation, colours of different shades, easy to manipulate genes, structural complexicity suits for industrial needs, and growth on cheap substrate with bulk production. Consequently natural pigments isolated from microorganism inhabiting environments other than terrestrial are an attractive research tool, not only for biochemist and microbiologist, but also for pharmaceutical and clinicians. This is because the natural pigments not only have capacity to increase the market acceptability, but also display various advantageous biological activities which includes antioxidants and antimicrobial activities.

Despite thousand of marine bioactive compounds have been isolated and identified previously, this study will focus on the pharmacologically active pigmented compounds produced by marine bacteria. Even though pigmented compounds produced by terrestrial samples are beyond the scope of the study, some brief explanation and examples will still be mentioned for comparative purposes, in order to outline common sources of natural pigments and its functions.

Among natural pigments, prodigiosin is an alkaloid group of pigments which poses wide variety of biological activities which includes antibacterial, anticancer, antimalarial, antiplasmodial, immunosuppressant, antifouling and many more (Campas *et al.*, 2003; Sertan-De-Guzman *et al.*, 2007; Park *et al.*, 2012). Hence, this pigment is desired for many applications including for pharmaceutical and non-pharmaceutical industries (such as aquaculture, cosmetics, food, textile etc). The prodigiosin pigments, that poses antimicrobial potential is an additional advantages, as the pigments can be simultaneously used as colouring agent and preservative agent in many industrial applications. This is consequent with the increasing demand for effective and non-toxic antibacterial therapeutics.

Colours are vital characteristics of textile products, where it is the first characteristic perceived by the senses and aided in determining acceptability, judging quality and increase basic aesthetic value of fabrics. Textile industry is one of the rapidly growing industries world wide which utilizes enormous amounts of synthetic dyes. In recent years, the demand of colorants from natural sources is alarming worldwide due to the adverse impact of synthetics dyes. Moreover, the dyeing of fabrics using prodigiosin exhibiting antibacterial agent have sponsored the production of protective cloths especially to the hospitals fabrics, since the fabrics has reported to be the vector of spreading many harmful bacteria from one person to another person (Gupta & Laha, 2007).

1.3 Research objectives

The general objective of the present study was to focus on production of natural pigments from a marine bacterium, and the pigments were evaluated on its colouring and antimicrobial properties, which would be used as a colouring agent and antimicrobial agent, simultaneously in textile industry. In order to achieve those, there were specific objectives that had been planned for as the following:

1. To isolate pigmented bacteria from different marine environments and to screen their pigment production as well their antimicrobial activity
2. To characterize and identify the potential strain of marine bacteria
3. To optimize the culture condition for growth, pigment production and antimicrobial activity
4. To characterize and study the chemical profiling of the pigment
5. To evaluate the application of prodigiosin pigment from *P. rubra* BF1A IBRL as colouring agent and antimicrobial agent in textile industry

1.4 Scope of study

This study was carried out to assess the ability marine bacteria in producing pigment with antimicrobial activity which can be potential candidate for natural coloring agent. The pigmented bacteria were isolated from marine samples, which was then cultured and maintained in laboratory conditions for further studies. The isolates were then screened for their potential in pigment production and antibacterial activity. The screening for the bioactivity was done thoroughly where the solvent effectiveness in extraction and partitioning was evaluated in order to select the potential isolate with greater bioactivity. Screening for pigment production was done using spectrophotometric analysis, whereas the screening for antimicrobial activity was performed using disc diffusion assay and microdilution method. The potential isolate was then characterized and identified based on its morphological, cultural, microscopical, physiological and 16S rRNA molecular analysis. Next, several physical parameter including light, pH, temperature, agitation rate and inoculum size were optimized to improve the culture condition of the isolate for higher production of pigment and antimicrobial activity. The chemical profiling of the pigment produced was done using thin layer chromatography (TLC), preparative-TLC, column chromatography, and high performance liquid chromatography (HPLC). The toxicity level of the pigment was determined using brine shrimp (*Artemia salina*) lethality test. In this analysis, the toxicity level of crude extract, partitioned and fractionated extract was compared. The pigment extract exhibiting the antimicrobial activity was then used to dye different types of fabric including wool, cotton, silk satin and silk. The dyeability of the biopigment was evaluated using different types of mordants. The colourfastness property of the naturally dyed fabric was then evaluated towards light, washing, rubbing, perspiration and water. The antibacterial property of the naturally dyed fabric was also determined.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of pigments and its classification

Pigments are colourful substances consisting of chromatophores, which either absorb or reflect light in varying wavelengths of the visible region (Delgado-Vargas *et al.*, 2000). The absorbed light is dissipated in the pigment, and the reflected light is visible as colours. The colours are also the result of a mix of residual wavelengths that are reflected as stated by Mlodzinska (2009). There are different nature of pigments which includes synthetic pigment, natural organic pigments, and also natural inorganic pigments, which are classified based on their properties and origin.

Synthetic pigments (or artificial pigments) are organic pigments which contain carbons and are chemically synthesized in laboratories. They have lower toxicity effect compared to inorganic pigments and usually applied for colouring agent for textiles, plastics, synthetic fibres, surface coating paints, inks and also added to fish feed for pigmentation of the fishes (Ni *et al.*, 2008). Examples of synthetic pigments were diazo pigments, monoazo pigments, phthalocyanine pigments, quinacridone pigments, astaxanthin and chantaxanthin (Mortensen, 2006). Synthetic pigment is expensive, for example the price of synthetic astaxanthin is approximately USD 2000 per kg (Ni *et al.*, 2008). Nguyen (2013) stated that the synthetic pigment cost for aquaculture industry is approximately 15 to 20% of the total cost incurred and the potential market value for astaxanthin (carotenoid-type of pigment) to be over 15 billion dollars for 2020.

On the other hand, natural inorganic pigments are mineral-earth pigment and also can be termed as heavy metal pigments, which is derived mainly from minerals such as gold and silver. Even though there are many naturally occurring pigments, but the toxicity level of the natural pigments is always too high which finally limiting its usage. Moreover, inorganic pigments commonly lack the intensity and brightness of colour compared to organic pigments (Christie, 2001; Allam & Kumar, 2011). Some examples of inorganic pigments are cobalt blue, chromium oxide, cadmium yellow, molybdate orange and nickel titanate (Crisea & Vilarem, 2006).

Demands for pigments are increasing drastically due to the wide function to industrial application and also to human health. The new awareness in human safety and environmental conservation has kindled fresh enthusiasm for natural colorants. The natural organic pigments are obtained from natural resources such as plants, animals and microorganisms. The sources and distribution of natural pigments will be discussed in detailed in Section 2.3. Natural pigments are also termed as bio-pigments which are chemically and physically the most diverse group of pigments (Salaudeen *et al.*, 2010).

2.2 Disadvantages of synthetic pigment

The synthetic colorants have been used extensively in foods, medicines, textile and cosmetics but the demand for the synthetic pigment has decreased through the years due to its toxicological effect. The world production of colorants is 1 million tonnes per year (Christie, 2007) and it is estimated that over 0.7 million tonnes of synthetic pigments are produced each year (Robinson *et al.*, 2001). One of the challenges of using synthetic pigment is the dependence on non-renewable oil resources and sustainability (Venil *et al.*, 2013).

2.2.1 Effect of synthetic pigments on environment

The majority of synthetic pigments are the azo derivatives (carcinogenic), and thus the toxicity effect of the synthetic pigments is terrifying. The major environmental problem of colorants is the removal of dyes and its intermediate compounds from the effluent. Since the synthetic pigments represent large group of organic chemicals, hence it is possible that such chemicals have undesirable effects on environment and human health (Zollinger, 1987).

According to Delgado-Vargas *et al*. (2002), the synthesis of synthetic pigments involves many types of intermediate organic compounds (including aromatic and heterocyclic compounds) and also involves many reactions (nitration, sulfonation, halogenations and amination). All the reaction involved in the manufacturing processes are contributing to the release of the waste of the hazardous organic compounds which lead to the environmental contamination when the waste drained into the effluent. For example in textile industry, up to 200 000 tonnes of synthetic pigments are lost to effluents every year during the dyeing and finishing processes as a result of inefficient or incomplete dyeing processes (Zollinger, 1987). Unfortunately, most of the dyes escape to the wastewater treatment plant and persist in the environment as a result of high stability towards light, temperature, and microbial attack.

Chemical toxicity in the environment also leads to the damage of aquatic ecosystem, e.g soil fertility and aquatic organisms. The chemicals can affect the gas solubility in water bodies and subsequently decrease photosynthetic activity in aquatic life by reducing the light penetration (Banat *et al*., 1996). Moreover, the chemicals can reduce the seed germination and plant growth, and inhibit the elongation of shoot and roots of the plants associated with the untreated effluents (Nirmalarani & Janardhanan, 1988).

2.2.2 Effect of synthetic pigments on human health

The effect of synthetic pigment towards human health can be categorised into two types that are acute (short-term) and chronic (long-term) toxicological effect. The acute toxicity involves oral ingestion and inhalation, skin irritation, skin sensitisation and eye irritation (Christie, 2007). Whereas, the chronic toxicity of synthetic pigment involves the genotoxicity effect when humans are exposed to the synthetic pigments, which exhibits mutagenic, carcinogenic and teratogenics characteristics (Christie, 2007). Some frequently used synthetic dyes that poses negative effect towards human health are Reactive brilliant red, acid violet 7, reactive black 5 and disperse blue 291 (Sudha *et al.*, 2014).

The workers involved in the manufacturing of the synthetic dyes usually become exposed to the dyes as well as the intermediate toxic chemicals used in their manufacturing plant. Many years ago, it became apparent that workers involved in the manufacturing of certain dyes, such as fuchsine, auramine, benzidine and 2-naphthylamine had developed a high incident of bladder cancer. There is also evidence that some reactive synthetic pigment caused contact dermatitis, allergic conjunctivitis, rhinitis and occupational asthma to the workers dealing with the synthetic pigments (Christie, 2007). The problem is caused by the ability of the reactive dyes to combine with human serum albumin (HSA) to give dye-HSA complex, which acts as an antigen. The antigen produces specific immunoglobulin E

(IgE) and cause allergic reactions through the release of chemicals such as histamine (Luczynska & Topping, 1986).

A total of 50% of the world colorant production are the textile colorants. The effects of synthetic textile colorant to human health are too obvious since its being encountered in almost every aspect of our lives. For example, humans are in direct contact with textile dyes because of the wearing cloths, and indirect contact with the dyes because of the furnishing, such as carpets, curtains lounge suits, etc.

In terms of food colorant effect on human health, there are only few permitted food colorants available (Delgado-Vargas *et al.*, 2000), and indeed, both permitted and non-permitted synthetic colorants are known to pose adverse effects. Ashfaq & Masud (2002) stated that some non-permitted mutagenic and carcinogenic food colours are still being used as food colorants, which includes auramine, methanol yellow, lead chromate, orange-1 and malachite green. Tartrazine, a permitted synthetic yellow colour that has been frequently used in sugar confectioneries has been reported to be associated with irritability, restlessness and sleep disturbance in hypertensive children (Rowe & Rowe, 1994).

2.3 Sources and distribution of natural pigments in nature

Pigments are distributed naturally in terrestrial and marine environment. Marine and terrestrial sources differ from each other due to the influence of their respective environmental conditions. In both terrestrial and marine environments, the natural pigments are produced by biological sources which includes higher plants (terrestrial), lower plants (marine) and microorganisms (both terrestrial and marine) (Rivera & Canela-Garayoa, 2012; Ibrahim, 2008). Godinho & Boshle, 2008 divided the biological materials containing pigments into two categories that are from photosynthetic organisms (such as plants and some bacteria) and non-photosynthetic organisms (such as bacteria, yeasts and fungi).

Humans and animals are not able to synthesize pigments *de novo*, hence they need to acquire the pigment through their diet. Although animals do not synthesis the pigments *de novo*, but they have been one of the pigment sources for human. Animals that obtained pigments from their feed or diet, are either being metabolised by the animal or the pigments accumulate inside their body especially in the skin. Hence the animal still can be source of natural pigment for human, where the pigment can act as an antioxidants and provitamin A to human health.

Plants and animal pigments are not suitable to be used as colouring agent for industrial application because of the limited supply and vulnerable to damage of the biodiversity (Shatila *et al*., 2013). Microorganisms are essential components of earth's biosphere (Whitman *et al*., 1998) which include fungi, yeasts and bacteria. The study of pigment production from microorganisms has been started as early as 1926 (Snow & Fred, 1926) and now is one of the emerging fields of research. Moreover, a number of research works have been reported over the last few years on microbial pigments. Pigment is one of the secondary metabolite in microorganisms. Microorganisms that are able to synthesis pigments are known as chromogenic microbes.

2.3.1 Terrestrial environment (plants, animal, microbes)

2.3.1.1 Terrestrial plants

Plant pigmentation is among the oldest interest of botanist and plant was the main source of pigments in 19th century (Christie, 2007). Plants can produce a variety of pigments including indigo, chlorophylls, carotenoids, anthocyanins and betalains (Davies, 2009; Mlodzinska, 2009). Generally, the parts of higher plants that contain pigments are fruits (Susilowati, 2008; Nugraheni *et al*., 2010; Al-Sayed & Kishk, 2011), vegetables, leaves (Biswas *et al*., 2013), roots and flowers as well (El-Refai *et al*., 2010). Among the plant pigments, chlorophyll and carotenoid pigments can be found in all terrestrial plants, which are also a major photosynthetic pigment. This is because the primary function of pigments in plants is photosynthesis. Chlorophyll is the green pigments that responsible for the green colours of the leaves. Whereas, the variety of bright and attractive colours of fruits vegetables and flowers are due to the carotenoid pigments (Corol *et al*., 2002). On the other hand, the red, blue and purple coloured foliage are also caused by anthocyanin pigments (Alkema & Seager, 1982), whereas the heartwood colour in certain plants is caused by the quinine pigments (Delgado-Vargas *et al*., 2002). Table 2.1 lists some pigments produced by plants in relative to its colour. The natural pigments from plants have been extracted to be used for industrial application, for example in food industry (Rymbal *et al*., 2011). Joshi *et al*. (2003) stated that most of plant natural pigments have been extracted from grapes, paprika, and beet, previously.

2.3.1.2 Terrestrial animal

Very few studies have been reported about the distribution of natural organic pigments in terrestrial animals. Amato *et al*. (2003) had studied the pigmentation effect in terrestrial isopods, *Atlantoscla floridana*. Cochineal pigment from cochineal insect (*Dactylopius coccus* costa) has been used since time immemorial in India, Persia and Europe for colouring the clothes (Delgado-Vargas *et al*., 2002).

Table 2.1: Examples of pigment producing terrestrial plants

Pigments	**Types**	**Example of typical colours**	**Plants**	**References**
Flavonoids	Anthocyanin	Blue, purple and red	*Beta vulgaris* *Hibiscus sabdariffa* *Solanum melongena*	El-Refai *et al.* (2010)
Carotenoids	Carotenoids	Yellow and orange	*Spinacia oleracea* *Curcuma longa* *Curcuma reticula*	El-Refai *et al.* (2010)
Cucuminoids	Curcumin Demethoxy-curcumin Bisdemethoxy-curcumin	Yellow	*Curcuma longa*	Kulkarni *et al.* (2012)
Betalains	Amaranthin	Red-violet	*Amaranthus tricolor*	Biswas *et al.* (2013)

2.3.1.3 Terrestrial microorganisms

Terrestrial fungi are one of the significant microorganisms that are well known in producing a wide range of pigments. This is because the fungal pigments could be easily produced in high yields via cultivation technology (Mapari *et al.*, 2009; Qiu *et al.*, 2010). There were over 1000 pigments being extracted from fungi (Delgado-Vargas *et al.*, 2002). Some of the fungi that are known to synthesize pigments are *Blakeslea trispora* and *Penicillium* sp. (Gunasekaran & Poorniammal, 2008), *Penicillium purpurogenum* (Mendez *et al.*, 2011) and *Monodictys castaneae* (Visalalakchi & Muthumary, 2010). Among the natural pigments, β-carotene is the major pigment produced by fungi. The other pigments produced by terrestrial fungi are monascus pigment, rubropunctatin and monoscorubin (Joshi *et al.*, 2003).

Besides, pigmented terrestrial yeasts are *Phaffia* sp. (Ni *et al.*, 2006), *Sporobolomyces* sp. (Maldonade *et al.*, 2006 and Maldonade *et al.*, 2007), *Rhodotorula* sp. (Maldonade *et al*., 2006), *Rhodotorula gaminis, Rhodotorula glutinis, Rhodotorula mucilaginosa, Rhodotorula minuta, Rhodotorula roseus* (Maldonade *et al*., 2007,) and *Rhodobacter* (Chen *et al*., 2006). There are also studies that mutate the yeasts to increase the yield of pigment production. Chew (2004) had mutated the yeasts *Xanthophyllomyces dendrorhous* to produce carotenoids-type pigment, which was later used to formulate fish feed. Some reporters has established metabolic engineering of a non-chromogenic yeasts to produce pigment, as done by Misawa & Shimada (1998).

Other terrestrial source of pigmented microorganisms is bacteria, which have been isolated from different type of terrestrial samples. Most pigmented terrestrial bacteria are isolated from soil samples. Lins *et al*. (2014), and Goswami *et al*. (2010) had isolated bacteria from soil samples, which were capable in producing prodigiosin and carotenoid pigments, respectively. The fact that pigmented bacteria were predominant in soil was further confirmed by Indra Arulselvi *et al*. (2014), who had isolated 24 of yellow pigmented bacteria from different types of soil under different condition and climates. Similarly, Rashid *et al.* (2014) had isolated red, brown, pink, black, blue, green, orange and yellow pigmented bacteria from 8 types of soil of Dhaka City, Bangladesh.

Besides, many pigmented bacteria are also isolated from clinical samples. For example, a clinical isolate of *Pseudomonas aeruginosa*, previously obtained from a clinical laboratory was reported to produce pyocyanin pigments (Karpagam *et al*., 2013). *Serratia marcescens* is another clinical isolate that has been isolated from clinical sample (Samrot *et al*., 2011).

Moreover, Shatila *et al*. (2013) had isolated a bacterium, *Exiguobacterium aurantiacum* FH from the air, and the bacterium is known to produce carotenoids-type of pigment, whereas *Serratia rubidaea* was isolated from an agricultural farm, which was known to produce pigment with antibacterial, antiproliferative and immunosuppressive properties (Darshan, 2013). The terrestrial pigmented bacteria also can be found in freshwater (Hardjito *et al*., 2002), and toilet water sources (Gulani *et al*., 2012) confirming their widespread diversity.

2.3.2 Marine environment

Up to date, there are more than 20, 000 structurally diverse marine natural compounds that have been isolated from the marine environment including the pigmented compounds (Rocha-Martin *et al*., 2014) and there are hundreds of new compounds that have been discovered every year from the marine environment (Pabba *et al*., 2011). Surprisingly, the discovery rate of natural bioactive compounds from marine microorganisms has surpasses that of the terrestrial counterparts (Attimarad *et al*., 2012). However it is stated that the research into marine environment is still in the early phase, and many mysteries associated with aquatic fauna and flora have yet to be discovered (Soliev *et al*., 2011). In marine environment the animals produced more bioactive compound compared to plants, and this is in sharp contrast to the terrestrial environment, where the plants by far exceeds animals in terms of production of natural bioactive substances (Proksch *et al*., 2002).

The privileges and special condition of marine environment that differ from other ecosystem is reflected by the physiology and biochemical properties of marine organisms especially the microorganisms.

2.3.2.1 Marine plants (Algae)

In macroalgae, the pigments are stored in chloroplast and chromoplast (Romero *et al.*, 2012). Structures exhibited by the compounds produced by macroalgae ranges from acyclic entities with a linear chain to complex polycyclic molecules and included biogenic compounds such as terpenoids, phenolic compounds, alkaloids, aldehydes, alcohols (Abad *et al.*, 2011, Bagawathy *et al.*, 2011). Likewise the higher plants, the green colour of the macroalgae are also contributed by chlorophyll pigment. On the other hand, the xanthophylls and fucoxanthin pigments are responsible for the brown coloured algae, whereas phycoerythrin and phycocyanine pigments contributed to the red colour characteristics of algae (Abad *et al*., 2011).

2.3.2.2 Marine animal

Some sessile and non-sessile marine invertebrates are brilliantly coloured especially the species inhabiting the shallow water (Banranayake, 2006) which are also biological source of pigments. Donia & Hamann (2003) stated that the majority of marine based bioactive compounds including pigments have been identified from marine invertebrates especially the sponges (Hamid & Ahmad, 2013). Devi *et al*. (2012) reported that one of the sessile invertebrates with brilliant coloration was sea-anemones (*Hetaractis magnifica* and *Stichodactyla haddoni*), which the coloration is due to the photosynthetic pigment of symbiotic zooxanthellae (a photosynthetic algae) present in different tissues of the organisms (Bandaranayake, 2006).

Often, carotenoid types of pigments are incorporated into the animal feeds. Steven (1948) had studied the carotenoids distribution in brown trout and found a significant amount of carotene was stored in the liver and ovary of the fish.

Khanafari *et al*. (2007) had extracted a number of carotenoids compounds from shrimp waste (comprising of head and carapace). Among the pigments, carotenoid-types of pigment are the most valuable pigment for human health, where the pigment acted as antioxidant (Dutta *et al*., 2005). Hence, animals including fish, crabs, shrimps, and lobsters have been the pigment (carotenoids) sources for human health. However, Rao & Rao (2007) stated that the main source of carotenoids for human is from fruits and vegetables.

2.3.2.3 Marine microorganisms

Although the pigmented microorganisms is widely spread in terrestrial nature, a number of microorganism distributed in marine environment is much higher (Jensen & Fenical, 1994). Also, the marine ecosystem is presumed more heterogeneous than the soil ecosystem at the bacterial level (Kim, 2013). Marine microorganisms can be divided into three categories on the basis of habitat which are psychrophiles (living at low temperature), halophiles (living at high salinity) and barophiles (living under high pressure).

Marine microorganisms continue to be a major focus compared to other marine macroorganism, with 10% increase in the number of compounds reported from 2011 to 2012 (Blunt *et al*., 2004). It is estimated that marine oceans contains the highest percentage of prokaryotic cells on earth that is 4-6 x 10^{30} cells (Whitman *et al*., 1998), while in seawater, there are about 10^6 bacterial cells per millilitre (Pabba *et al*., 2011).

There are five groups of marine microorganisms including fungi, yeast, microalgae and bacteria. Marine fungi grow and sporulate exclusively in seawater, and their spores are capable of germinating in seawater. Some of them are facultative and there are over 800 obligate marine fungal strains reported so far. The fungus, *Monodictys* sp. has been isolated from the surface of sea-urchin and is known to produce antimicrobial anthraquinone pigment (El-Beih *et al*., 2007). Moreover, marine yeasts, *Aureobasidium pullulans* had been isolated from marine sediment of Southern Sea, China, which was able to produce an alkaline protease (Chi *et al*., 2007).

Dunaliella sp., *Haematococcus* sp. (Phromthong *et al*., 2012), and *Chlorella vulgaris* are some examples of the chromogenic microalgae (Gouveia & Empis, 2002) in marine environment. The production of pigment from *Haematococcus pluvialis* has been developed in large scale in 1990 (Lorenz & Cysewski, 2000). Marine microalgae such as diatoms and cyanobacteria are known to produce various bioactive compounds including natural pigmented compounds and novel metabolites (Abad *et al*., 2011), but have attracted little attention among researchers. Some examples of the pigments produced by microalgae are β-carotene, asthaxanthin, and canthaxanthin (Jissa, 2008; Guedes *et al*., 2011).

2.3.2.3.1 Marine bacteria

According to Jensen and Fenical, (1996), the general definition of marine bacteria is “the microorganisms which are isolated from the marine habitat and which are functionally reproductive under typical marine condition”.

The awareness of the role of marine microorganisms in biotechnology has started over the past 30-40 years and the research regarding the marine microorganisms is still growing where hundreds of new compounds are being discovered every year (Proksch *et al*., 2002). However, at the end of 2008, there were only 3000 microbial bioactive compounds had been reported from the marine environment (Rahman, 2008). Hence, since the earth is covered by seas at approximately 71% of its surface, and the microorganisms are distributed widely from ocean shores to the deep sea floor, there are still many resources that crucially need to be explored to isolate many more marine novel bioactive compounds.

One of the marine bacteria that is widely distributed in marine environment is pigmented marine bacteria. Due to the enormous diversity of marine bacteria, it is impossible to give a general number of pigmented bacteria to total of marine heterotrophic bacteria. Marine pigmented bacteria have different mode of living, which include free-living (referred to as pelagic or planctonic), attached to animate or inanimate materials and also attached to internal space of invertebrates. However, previous study stated that the bacteria with symbiotic relationship (attached to animate resources) are likely to be advantageous to produce beneficial secondary metabolites. The detailed explanation of symbiosis relationship is stated in section 2.3.2.3.1.

The important microhabitats for the prolific marine bacteria includes animate (internal tissues and surface of invertebrates) and inanimate (sediments, stone particles, seawater etc) resources. The marine microorganism exhibits a huge ability to produce various kinds of bioactive compounds (secondary metabolites). Carte (1996) stated that bioactive metabolites produced by marine bacteria have more novel and striking structures compared to terrestrial microorganisms.

Pigment synthesis by marine bacteria is definitely dependent on the pH, light, temperature of the nature, and the marine bacteria is capable in producing pigments with almost all colours of the rainbow including black, white, brown, golden, silver, florescent green, yellow or blue (Kim, 2013). Pigments produced by bacteria mostly by the quorum sensing mechanism (Thomson *et al*., 2000; Slater *et al*., 2003).

Among the marine bacteria that are able to synthesize pigments are *Bacillus* (Perez-Fons *et al.,* 2011), *Flavobacterium* (Courington & Goodwin, 1955; Weeks and Garner, 1967), *Micrococcus* (Sobin & Stahly, 1941; Courington & Goodwin, 1955; Kaiser *et al*., 2007; Ibrahim, 2008), *Erwinia* sp and *Sarcina* (Sobin & Stahly, 1941), *Serratia* (Teh Faridah, 2012), *Pseudoalteromonas* (Feher *et al*., 2008) *Pseudomonas* (Angell *et al*., 2006) and *Vibrio* (Allihosseini *et al*., 2008). Table 2.2 shows the list of marine bacteria that have been reported to be able to produce various pigmented bioactive compounds.

Table 2.2: Marine bacteria that were able to produce various pigmented bioactive compounds

Marine bacteria	**Pigments**	**Activity**	**References**
Pseudomonas aeruginosa	Pyocyanin and pyorubrin	Antibacterial	Angell *et al.* (2006); Saha *et al*. (2008)
Bacillus sp.	Phenazine	Cytotoxic	Li *et al*. (2007)
Pseudoalteromonas tunicata	Tambjamines	Antibiotics, anticancer	Franks *et al*. (2005); Pinkerton *et al*. (2010)
Shewanella colwelliana	Melanins	Protection from UV radiation	Fuqua & Weiner (1993); Kotob *et al*. (2005)
Agrobacterium auranticum	Astaxanthin	Antioxidant	Misawa *et al*. (2005)
Pseudoalteromonas luteoviolacea, *Pseudoalteromonas* sp.	Violacein	Antiprotozoan, antibiotic, anticancer	Novick & Teyler (1985); Yada *et al*. (2008)
Serratia marcescens	Anthocyanin	Colouring agent	Nerurkar *et al*. (2013)
Pseudomonas sp.	Melanins	Antioxidant	Tarangini & Mishral (2013)
Streptomyces coelicolor, *S. violaceusruber,* *S. lividans*	Actinorhodin	Indicator compounds in laboratory	Palanichamy *et al.* (2011)

2.3.2.3.1.1 Advantageous of microorganisms as pigment sources over plants and animals

Among microorganisms, bacteria have immense potential to produce various bioactive compounds including the pigments. The single-celled organisms are the most likely commercial source of pigments with biotechnological techniques as tools for their exploitation (Delgado-Vargas *et al.*, 2002). Practically, fermentation of microorganisms could be a precious source of pigments but some have stated that the pigment production via fermentation will generate cost (Maldonade *et al.*, 2007). However the cost can be reduced by cultivating the pigment producers in cheap industrial by-products (e.g. agro-industrial residues) or waste products as nutrient source or growth medium (Aksu & Eren, 2005) which will provide a profitable means (Latha & Jeevarathnam, 2010).

Mata-Gomez *et al.* (2014) stated that industrial wastes such as chicken feathers, whey, and crude glycerol have been used as substrates for yeasts cultivation to produce carotenoids pigments (Valduga *et al.*, 2009; Taskin *et al.*, 2011; Saenge *et al.*, 2011). Further, Tarangini & Mishra (2013) also have used vegetable wastes to cultivate a bacterium *Pseudomonas* sp. to produce melanin-type of pigment.

Venil *et al.* (2013) reported that microorganisms is mostly preferred biological source of pigments compared to plants and animals owing to its ability to produce more stable pigments, higher yields and also lower residues . Proksch *et al.* (2001) also stated that the concentration of bioactive compounds present in the marine invertebrates (macroorganisms) were very little, accounting for less than a millionth of the wet weight. Likewise, Parthiban & Thilagavathi (2012) stated that only per grams of pigment yielded from 1 kg of dried plant materials, causing the current market price of plant pigments about US$ 1/g. The use of large amounts of biomass of these invertebrates and plants, on the other hand will lead to extinction of the respective species subsequently, damaging the ecosystem. Similarly, Maldonade *et al*. (2007) stated that the natural pigment by plants may suffer from diminishing or unstable supply of raw materials, subject to climate conditions, as well as varying colorant level and subsequently affecting the quality of the final product.

There are facts stated that the bacteria produced pigments in very low quantities, however the technologies to overcome this challenges is already in place, which provide a route for introducing the bacterial pigments to a cost sensitive-world. For example, the requirement for large number of Petri dishes for cultivating certain bacteria can be overcome by using fermentation vessels. On the other hand, the molecular approach can be used to clone the genes responsible for the biosynthesis of pigments in order to increase the production of pigments from the bacterial cells (Venil *et al*., 2013). The cloning and DNA recombinant technology have been harnessed to overproduce the pigments since ancient time (Malpartida & Hopwood, 1984).

The molecular approach also can be used to manipulate the biosynthetic pathways of pigment production to engineer a structure of pigment and consequently its colour. For example, *Streptomyces coelicolor,* which produced blue pigment (actinorhodin) can be genetically modified to produce bright yellow pigment (kalafungin), and alternatively actinorhodin pigment biosynthesis can be engineered to produce orange or yellow-red anthraquinones pigment (Bartel *et al*., 1990; McDaniel *et al*., 1993). The metabolic engineering of microorganisms for natural pigment synthesis has been described in detailed by Venil *et al*. (2013).

On the other hand, microorganisms can be cultivated using various laboratory medium which can be propagated easily compared to plant tissue culture. Therefore, the microorganisms have been selected to as a sustainable resource for the biological production of active compounds including the pigment.

2.3.2.3.1.2 Symbiosis

Marine plants and animals in marine environment are well known to have developed relationship (beneficial interaction) with numerous microorganisms and this association is known as epibiosis. The attaching microorganisms known as ephibiotic, whereas the marine living organisms known as epibionts (Wahl, 1989). Symbiosis is one of the common associations between the marine bacteria (symbiotic) and its attaching host (simbionts) (Haygood *et al*., 1999).

According to Armstrong *et al*. (2001), the symbiotic bacteria living on the surface of higher marine organisms could acquire the necessary nutrition from their animal or plant host such as vitamins, polysaccharides and fatty acids. The bacteria in return could excrete secondary products such as amino acid, antibiotics and toxin propitious for the development, metabolism and also for chemical defence of the host (Armstrong *et al*., 2001; Hamid *et al*., 2013). There are also studies reported that marine symbiotic bacteria are important source of fixed nitrogen for the associated algae (Goecke *et al*., 2010). The symbiotic bacteria that are able to protect its host from settlement of other pathogenic microflora is known to poses antimicrobial activity and will be great source for discovery of new drugs in pharmaceutical industry and also can be great source of antifouling agent in aquaculture industry.