PRODUCTION OF PRODIGIOSIN BY *Serratia marcescens* IBRL USM84 FOR LIPSTICK FORMULATION

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PRODUCTION OF PRODIGIOSIN BY *Serratia marcescens* IBRL USM84 FOR LIPSTICK FORMULATION

by

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LIST OF ABBREVIATIONS

BUT	Butanol
CFU	Colony Forming Unit
DCM	Dichloromethane
EA	Ethyl acetate
EC_{50}	50% effective concentration
FDA	Food and Drug Administration
HAI	Hospital-acquired infection
HCL	Hydrochloric acid
HEX	Hexane
HMDS	Hexamathyldisilazine
INT	p-iodonitrotetrazolium violet salt
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
MRSA	Methicillin-resistance *Staphylococcus aureus*
NA	Nutrient Agar
NaOH	Sodium Hydroxide
OD	Optical density
P.I	Polarity index
PDA	Potato Dextrose Agar
r/t	Retention time

R_f	Retention factor
SDA	Sabouraud Dextrose Agar
SEM	Scanning Electron Microscope
TEM	Tranmission Electron Microscope
TLC	Thin Layer Chromatography
UPLC	Ultra Performance Liquid Chromatography
UV-vis	Ultra-violet visible

PENGHASILAN PRODIGIOSIN OLEH

***Serratia marcescens* IBRL USM84 UNTUK**

PEMFORMULASIAN GINCU BIBIR

ABSTRAK

Mikroorganisma menawarkan pelbagai pigmen semula jadi sebagai satu alternatif selamat kepada pewarna sintetik dalam kebanyakan aplikasi perindustrian termasuk industri kosmetik, makanan, tekstil, farmaseutikal dan akuakultur. Penyelidikan ini dijalankan bertujuan untuk mengkaji pigmen merah semulajadi prodigiosin dengan aktiviti antimikrob oleh bakteria marin. Penemuan pewarna semula jadi dengan aktiviti antimikrob boleh memberi banyak faedah kepada kebanyakan industri dan bertindak sebagai agen pewarna semula jadi dengan kesan pengawetan secara serentak kepada produk perindustrian. Dalam kajian ini, *Serratia marcescens* IBRL USM84 telah dipencilkan daripada span laut tempatan *Xetospongia testudinaria* dari Pulau Bidong, Terengganu. Analisis tentang kegiatan agen antimikrob daripada pigmen intrasel dan ekstrasel pencilan IBRL USM84 telah dilakukan dan mendapati aktiviti antimikrob daripada pigmen intrasel adalah lebih tinggi. Analisis penghasilan pigmen mendedahkan bahawa pigmen yang diekstrak dari intrasel dan ekstrasel boleh menghasilkan keamatan warna yang berbeza dalam kuantiti yang berbeza, iaitu pigmen daripada intrasel menghasilkan 7.02 g/L pigmen berbanding dengan pigmen ekstrasel sebanyak 0.25 g/L sahaja. Daripada proses peningkatan, *S. marcescens* IBRL USM84 telah menghasilkan pigmen prodigiosin yang lebih tinggi dan aktiviti antibakteria yang lebih baik pada 48 jam tempoh pengkulturan, pH 7 bagi pH awal medium pengkulturan, dieramkan pada 25^{o}C, dengan 150 psm kelajuan pengocakan, manakala medium pengkulturan ditambahkan dengan 0.2 % agar-agar dan 1% maltosa

dalam kondisi cahaya. Ekstrak diklorometana daripada pigmen intrasel yang dihasilkan oleh *S. marcescens* IBRL USM84 menunjukkan kekuatan pigmentasi yang lebih tinggi dengan aktiviti agen antimikrob yang lebih tinggi berbanding dengan ekstrak lain. Ekstrak ini mampu merencat pertumbuhan bakteria ujian dalam julat yang lebih besar dengan nilai kepekatan perencatan minimum yang sama ke arah semua bakteria ujian iaitu 250 µg/mL. Berdasarkan kajian masa maut, aktiviti antibakteria ektrak prodigiosin *S. marcescens* IBRL USM84 adalah bergantung kepada kepekatan. Keputusan yang diperolehi daripada analisa UV/vis spektoskopi, ujian jangkaan dan analisa kromatografi menunjukkan bahawa pigmen yang dihasilkan oleh *S. marcescens* IBRL USM84 ialah daripada kumpulan prodiginin. Pigmen merah lembayung ini menunjukkan penyerapan maksimum UV/vis pada 535 nm. Keputusan daripada analisa penulenan mendedahkan bahawa pigmen prodigiosin yang tulen menunjukkan aktiviti antibakteria lebih rendah berbanding dengan ekstrak diklorometana dan ini disebabkan oleh kesan sinergisme antara sebatian yang hadir dalam ekstrak. Fraksi aktif (Fraksi 4) yang diperolehi daripada kromatografi turus mempunyai nilai kepekatan perencatan minimum lebih tinggi iaitu 1000 µg/mL terhadap *S. aureus, B. cereus, B. subtilis,* MRSA and *A. anitratus*. Ekstrak prodigiosin adalah sangat tidak toksik kepada *Artemia salina* bagi kedua-dua tahap akut dan kronik. Penghasilan pigmen prodigiosin sebagai satu agen pewarna dalam perumusan gincu dinilaikan. Aktiviti antibakteria gincu diwarnakan dengan prodigiosin semula jadi menunjukkan lebih daripada 99.0% daripada penurunan pertumbuhan bakteria apabila gincu diuji dengan *S. aureus, B. cereus, B. subtilis,* MRSA and *A. anitratus*. Penerimaan pengguna dikaji dengan menggunakan Ranking Test (Skala Likert) dan mendapati gincu merah semulajadi lebih diminati dalam perbandingan kepada pewarna sintetik dalam produk kosmetik berdasarkan kecenderungan memilih warnanya. Kesimpulannya, perhatian telah bertambah ke arah penggunaan sumber warna asli sebagai satu pewarna berpotensi dan agen pengawet semula jadi.

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PRODUCTION OF PRODIGIOSIN BY

***Serratia marcescens* IBRL USM84 FOR**

LIPSTICK FORMULATION

ABSTRACT

Microorganisms provide plenty of natural pigments as a safe alternative to the synthetic dyes in many industrial applications including cosmetics, food, textile, pharmaceutical and aquaculture industries. This research was aimed to study the natural red pigment prodigiosin with antimicrobial activity of a marine bacterium. The finding of natural colorant with antimicrobial activity can give benefits to many industries and acts as a natural coloring agent with preservative value simultaneously to the industrial products. In this study, *Serratia marcescens* IBRL USM84 was isolated from a local marine sponge *Xetospongia testudinaria* from Pulau Bidong, Terengganu. The analysis of antimicrobial activity from intracellular and extracellular pigments was performed and found that the antimicrobial activity from intracellular pigment was greater. The analysis of pigment production revealed that intracellularly and extracellularly extracted pigments were able to produce different color intensity at different quantity, where the pigment from intracellular yielded 7.02 g/L compared to extracellular pigment that was 0.25 g/L only. From the enhancement process, *S. marcescens* IBRL USM84 produced higher prodigiosin pigment and better antibacterial activity after 48 hours of cultivation period with medium initial pH at 7, incubated at 25^{o}C and with 150 rpm of agitation speed, the addition with 0.2 % of agar and 1% of maltose under light condition. The dichloromethane extract from intracellular pigment produced by *S. marcescens* IBRL USM84 showed higher pigmentation strength with greater antimicrobial activity compared to other extracts.

This extract was able to inhibit broader range of test bacteria with the same MIC value towards all the tested bacteria which was 250 µg/mL. Based on the time kill assay the antibacterial activity of prodigiosin extract from *S. marcescens* IBRL USM84 was concentration dependent. The results obtained from UV/vis spectrophotometer, presumptive test and chromatographic analysis indicated that the pigment produced by *S. marcescens* IBRL USM84 is of prodiginine group. The pigment was purplish red and showed a maximum absorption at 535 nm. The results of purification analysis revealed that the purified prodigiosin pigment exhibited lower antibacterial activity compared to the dichloromethane extract and this could be due to the synergism effect between compounds present in the extract. The active fraction (Fraction 4) obtained from column chromatography had higher MIC value which was 1000 µg/mL against *S. aureus, B. cereus, B. subtilis*, MRSA and *A. anitratus*. The prodigiosin extract was not toxic towards *Artemia salina* for both acute and chronic toxicities. The production of prodigiosin pigment as a colouring agent in lipstick formulation was evaluated. The antibacterial activity of lipstick dyed with natural prodigiosin showed more than 99.0% of bacterial reduction when the lipstick being treated with *S. aureus, B. cereus, B. subtilis,* MRSA and *A. anitratus.* The consumer acceptance was investigated using the Ranking Test (Likert Scale) and found that the natural red lipstick was more preferred in comparison to synthetic colorant in cosmetic product based on its colour preferences. As conclusion, due to the possible toxicity of the synthetic dyes, an increasing attention has been directed to natural color resources as a potential colorant and natural preservative agent.

CHAPTER 1.0: INTRODUCTION

1.1 Problem statement

Nowadays, humans are more inclined towards natural resources for survival. Consciousness of risks and undesirable effects from the use of chemicals, prompted consumers prefer to choose something more safe and natural. Land and marine resources provide variety of natural resources that can meet the requirements of various aspects of life such as food and beverages, medicines, health, economy and beauty. A wide variety of diseases and medical problems pose a challenging threat to humans, who since ancient times have searched for natural compounds from plants, animals, and other sources to treat those problems (Kumar *et al.*, 2015).

Many artificial synthetic colorants, which have broadly been used in food and beverages, dyestuffs, cosmetics and pharmaceutical manufacturing processes, may lead to various hazardous effects. To curb the harmful effect of synthetic colorants, there is worldwide interest in production of pigments from natural sources. Hence, the deployments of natural pigments in food and beverages, dyestuffs, cosmetics and pharmaceutical manufacturing processes have been increasing in recent years (Unagul *et al*., 2005). Moreover, natural pigments not only provided a good appearance to increase the marketability of products, but also have biological properties such as antibiotic, antioxidant, and anticancer activities (Dufosse, 2009).

In the cosmetic industry for instance, the beauty product that has been produced may not be completely safe for the consumer’s health. Unsuitable ingredients and additives formulated in cosmetic products cause side effects to the skin. Some side effects may be seen after long term usage by appling the cosmetic products directly on the skin or body part of the user (Vinensia, 2012). In addition, the safety of synthetic colorants has been questioned hence leading to decrease in number of permitted colorants by Food and Drugs Administration (FDA). The limitation of permitted colorants conducted by FDA also increased the worldwide interest in uses of natural colorants in production of consumer goods (Huck & Wilkes, 1996; Azwanida *et. al.,* 2015).

Besides, the number of drug resistance pathogens has been increasing over the years. Some of factors that lead to the emergence of new diseases are microbial adaptation becomes resistant to medicine, misuse of antibiotics, human migration worldwide, poor sanitation lifestyle, and those who often exposed to disease vectors and reservoirs (Racaniello, 2004; Wan Norhana & Darah, 2005). All these problems lead to the increasing number of pathogenic bacteria that are resistant to multiple antibiotic treatments including methicillin-resistant *Staphylococcus aureus* (MRSA) (Sakoultas & Moellering, 2008) and vancomycin-resistant *Staphylococcus aureus* (VRSA) (Courvalin, 2008).

Undeniable, the products will have good market value if they are coloured with natural pigment (Venil *et al.,* 2013). Therefore, the research in natural pigment is important to develop industrial products with good appearance, attractive colour and eco-friendly protection.

1.2 Rationale of study

Color plays an important role in our daily life. Color is a basic element for visual communication. Some examples of colour application in our daily life are the coloured goods, cosmetic products, food appearances, textiles and decorative items.

Color is the main characteristics evaluated and is used as target by the industries to attract customers.

The production of the synthetic colorants is more economically and practically advanced with colors covering the whole color spectrum. However, synthetic colorants are facing some trouble such as dependence on non-renewable oil resources and sustainability (Venil *et al.*, 2013) of current operation, environmental toxicity, and human health concerns of some synthetic dyes. Plants also have been used in production of natural colorants before synthetic dyes were invented, but in very low yields, low eco-efficiency and less economic (Raisainen *et al*., 2002). For example the natural colorants from several plants including *Hylocereus polyrhizus* (deep purple), *Pandanus amaryllfolius* (green), and *Clitorea ternatae* (blue) have been used as cosmetic coloring agents (Azwanida *et al.*, 2015).

Among the natural pigments resources, the natural pigments from microorganisms are preferred for production at commercial scale because of their stability (Raisainen *et al*., 2002) and the availability of their cultivation technology throughout the year (Parekh *et al*., 2000). Microbial natural pigments can be produced through fermentation of microbial cells in bioreactor for industrial scale (Dharmaraj *et al*., 2009). Technically, it can be valuable sources of natural pigment production as they are able to produce higher yields of pigment and lower residues. In fact, microbial fermentations have many advantages such as cheaper production, easier extraction processes, higher yields, no lack of raw materials, no seasonal variations and more eco-friendly. Besides, pigmentation is widespread among bacteria in terrestrial and in marine heterotrophic bacteria that comprises of carotenoids, flexirubin, xanthomonadine and prodigiosin (Kim *et al*., 2007; Stafsnes *et al*., 2010; Soon-Jiun and Darah, 2011; Yong, 2012).

Prodigiosin is an alkaloid group of pigment, synthesized as secondary metabolite with unique tripyrrole chemical structure (Khanafari *et al*., 2006). Some strains of bacteria produce prodigiosin pigment such as *Serratia marcescens*, *Vibrio psychroerythrus, Streptomyces griseoviridis* and *Hahella chejuensis* where they have been shown to be associated with extracellular vesicles or present in intracellular granules (Kobayashi & Ichikawa, 1991; Teh Faridah, 2012). Prodigiosin has been reported to posses antibacterial (Samrot *et al*., 2011; Teh Faridah *et al*., 2009), antifungal (Croft *et al*., 2002), cytotoxic (Nakashima *et al*., 2005), immunological and antitumor (Perez-Tomas *et al*., 2003) activities. All these properties are coinciding with the high demand of effective and non-toxic antibacterial agent in pharmaceutical industry. The bright red colour of pigment can be used as colouring and preservative agent in food, cosmetics, textiles and aquaculture products for industrial applications. Hence, this natural red pigment is a potential product to be commercialized.

In cosmetics industry, colorant play a vital role in the world of beauty as it determines the aesthetic value of the cosmetic products (Vinensia, 2012). The development of beauty products with an attractive colour is an important goal in the cosmetics industry. However, most people are concerned about the long term effect in the use of synthetic materials, making them more alert and choose products that are natural and safe. Gradually, cosmetics producers are turning to natural cosmetic colours, since certain synthetic colour additives have proven to show negative health issues following their consumption (Bridle & Timberlake, 1997; Ali, 2011). The application of prodigiosin in cosmetic formulations not only provide an attractive colour, but can supply natural protection on lips or skins from bacterial infection, since this pigment demonstrated antibacterial activity.

1.3 Research objectives

In this research, the main objective of the study was to enhance on production of natural pigment form a marine bacterium, *S. marcescens* IBRL USM84. Besides, the pigments were evaluated on antimicrobial properties and acted as natural colorant in lipstick formulations. To complete the research study, several objectives had been planned and were listed as follows:

i. To determine the antimicrobial activity from the intracellular and extracellular pigment of *S. marcescens* IBRL USM84.

ii. To enhance the pigment production, cell growth and antibacterial activity of *S. marcescens* IBRL USM84 by physical and chemical parameters in a shake flask system.

iii. To determine the effectiveness of solvent in partitioning process and extraction of pigment.

iv. To isolate, purify and characterize the red pigment that is prodigiosin.

v. To evaluate the application of natural red pigment prodigiosin from *S. marcescens* IBRL USM84 as a natural colorant and its effectiveness as a natural antimicrobial agent in cosmetic product, (lipstick formulation).

CHAPTER 2.0: LITERATURE REVIEW

2.1 Pigment role in life and classification of pigment

The world would be dull without pigments. As the saying goes "from the green grass of home to a forest's ruddy autumn hues", we are surrounded by living colour. Life presents us with a variety of colours making our life meaningful (Ong & Tee, 1992; Omayma & Abdel Nasser, 2013). Colors affect us emotionally and psychologically since colour psychology says that the colour of the product may also influence the efficacy of therapy (Allam & Kumar, 2011).

Colors also inspire the feelings of joy, sadness and tranquillity. For example, colors in the red part of the color spectrum are known as "warm colors" and consist of red, orange and yellow (Figure 2.1). These warm colours induce emotions ranging from feelings of calm and comfort to feelings of anger and hostility. Colors on the blue part of the spectrum are known as "cool colors" and consist of blue, purple and green. These colors are often described as calm, but at different times could be described as sadness or unresponsive. The studies of psychology effects of colors are also applied to medications (Allam & Kumar, 2011).

Pigments or colorants can be from either natural or synthetic sources. Pigments from different sources and origin can be divided into three classes namely natural, synthetic and inorganic pigments. Natural pigments are extracted from living organisms such as plant, insects, algae, microorganism, etc. Some color pigments made by modification of materials from living organisms, where are considered natural though they are not available in nature. Examples are caramel, vegetable carbon and Cu-chlorophyllin (vide infra) (Ali, 2011). Natural pigments are safer, healthier and better than synthetic pigments. Scientists have to intensify research on natural pigment sources to meet market demand for the natural pigment products.

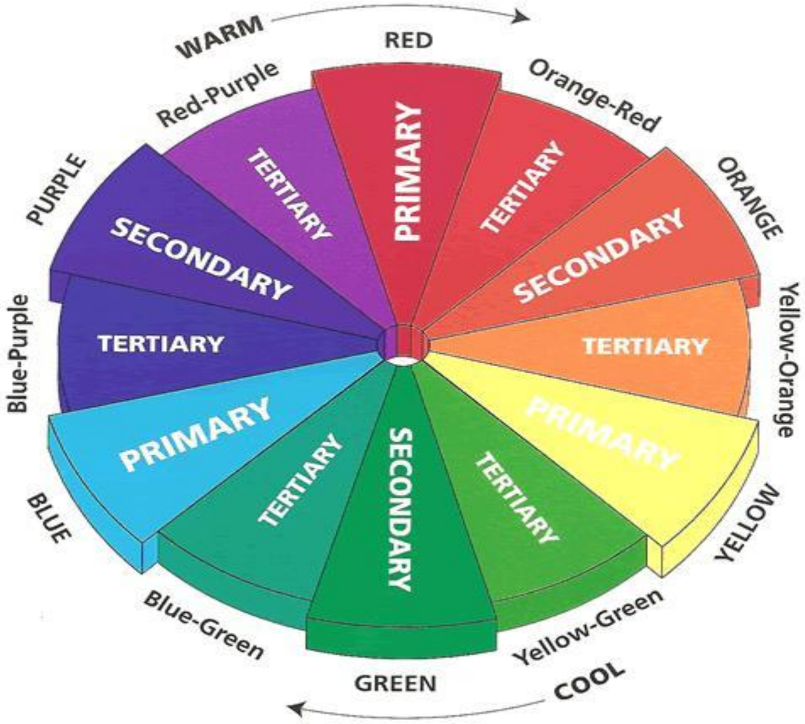

Figure 2.1: Color wheel (Allam & Kumar, 2011)

Synthetic pigments are known as artificial pigments which are manufactured chemically in laboratories. Synthetic pigments have lower toxicity effect compared to inorganic pigments. These pigments usually applied for colouring agent in many industrial applications such as cosmetic materials, textiles, foodstuffs, dyestuffs, plastics, paints, fish feeds and many more (Ni *et al.*, 2008). Examples include monoazo pigments, diazo pigments (Mortensen, 2006), Tartrazine, Erythrosine, Sunset Yellow and Patent Blue V, etc (Allam & Kumar, 2011). Chemical synthesis will cause inherent waste disposal problems like pollution of heavy metal compounds with high toxicity (Hamlyn, 1995). In fact, disposable problem could threaten human health, environmental virginity and ecosystem. Natural pigment is a good alternative to synthetic pigment for sustainable livelihood.

Inorganic pigments are known as mineral pigments are often used to color foods and drugs. Most of the pigment sources show lack of color intensity and limited colour spectrum compared to the other two classes of pigments. These pigments are not recommended due to many that have toxic effects and they are rapidly replaced by synthetic dyes (Allam & Kumar, 2011). Some examples of inorganic colours are gold, silver and titanium dioxide (Ali, 2011).

Natural pigments can show better biodegradability, eco-friendly and normally have a higher compatibility with the environment. People have been using chemicals or synthetic pigments in food and beverages, cosmetics, textiles and pharmaceutical products which are having side effects and health complication. Hence, many researchers searching for alternative pigment from natural sources like microbes and plants which can synthesize pigments (Kang *et al*., 1996). These natural pigments are safe to use and do not have side effects. There is a high demand for microbial pigments from terrestrial and marine sources due to their innate characters, medicinal properties (anti-bacterial, anti-fungi, anti-cancer, etc) and simple production (Carels & Shephard, 1997).

2.2 The risk of synthetic pigment usage

The usage of synthetic chemicals and artificial colors excessively in the last one and half century via production and application has threatened human health, environment and eco-system. Most of the countries have banned the use of some synthetic dyes as food colouring agents and allowed limited number of synthetic colours under specified maximum limits. For example, Germany and Netherlands have imposed ban on the use of specific synthetic dyes for textile dyeing in the textile industry. The 70-odd azo-dyes as colouring agent for textile and other consumer goods are banned in India meanwhile about 118 chemicals have been put up in Red-List (Kapoor, 2006).

2.2.1 Human health risks

Already centuries, textiles have been dyed with extracts from natural sources like minerals, plants, and animals. Since 1856, people started to move to synthetic dyes when scientists discovered how to make them (Zollinger, 1991). The new dyes successful changed the playing field because the production of synthetic dyes is cheaper, brighter, color-fast, and easy to apply to fabric. This phenomenon cause natural dyes to become obsolete for most applications (Lomax & Learner, 2006). Unfortunately, the chemicals used to produce dyes today are often highly toxic, carcinogenic, or even explosive alarming the world because it is very dangerous for consumers. For example, azo dyes are considered deadly poisons and dangerous to work with, also being highly flammable. In addition, other harmful chemicals used in the dying process include dioxin cause a carcinogen and possible hormone disrupter, toxic heavy metals such as chrome, copper, and zinc are known very carcinogens while formaldehyde a suspected carcinogen (Brit, 2008).

Most of the synthesized synthetic dye manufacturing stage is banned due to the carcinogenicity of the precursor or product which dangers for dye workers (Dufosse, 2009; Venil *et al.,* 2013). In the late nineteenth century, there have no guarantee of safety commensurate for dye workers. In fact, the workers who manufactured dye and who dyed garments would be facing deadly risks. In Japan, dye workers were at higher risk of cancers meanwhile in the United States, death among factory workers caused by cancers, cerebrovascular disease and lung disease are forty times higher than caused by other diseases (Brit, 2008).

Inorganic colors derived from mineral-earth pigment such as lead chromate and copper sulphate may also cause serious health problems (Hari *et. al.,* 1994). However, since about thirty years ago, synthetic colours and additives are harshly criticized and consumers show reluctance toward these products, hence they prefer to use the natural colorants (Koes *et al.,* 1994). United State in 1960s, the use of such food additives has been opposed and demonstrated by environmental activists who campaigned for the natural colorants are the best alternative to synthetic colorants. They were highlighting the good nutritional characteristics of natural colorants compared to synthetic colorants as a sales tool. This strategy is not effective at early stage. Finally, changes in social attitude indicated the health campaign was successful. As an implication, a worldwide tendency to use natural colorants is generated widely (Krishnamurthy *et al.,* 2002).

2.2.2 Environmental pollution risks

The textile industry is one among the rapidly developing and growing industries worldwide. Textile industry utilizes large amounts of synthetic dyes in manufacturing the textile products. The difficulty is the by-products during manufacturing process that is often very difficult to treat and dispose consequently become a serious threat to the environment and has become a very critical problem in environment conservation. Moreover, they also pollute the ground water resources of drinking water, fisheries activities and agriculture practices (Krishna *et al.,* 2011).

Most countries require the factories themselves to treat dye waste matter before it is dumped. Irresponsible actions of some manufacturer by dumping the chemical wastes into the river without treating them in advance are very disappointing. In fact, after dying a batch of fabric, the cost is cheaper to dump the used water rather than to clean and reuse the water in the factory. Hence, dye factories all over the world are dumping millions of tons of dye effluent into rivers. As a result, fields and rivers near jeans factories in Mexico City are turning dark blue from untreated and unregulated dye effluent. Factories dying denims for Levi and Gap dump waste-water contaminated with synthetic indigo straight into the environment without a sense of responsibility. Local folks and farmers report health problems and became unsure if the food they are obliged to grow in nearby fields is safe to eat (Brit, 2008).

Undeniable the use of synthetic dyes in textile industry massively has an undesirable effect on all forms of life. Utilization of various chemical substances such as sulphur, naphtha, vat dyes, nitrates, acetic acid, soaps, enzymes chromium compounds and heavy metals like copper, arsenic, lead, cadmium, mercury, nickel, and cobalt and certain auxiliary chemicals all collectively make the textile effluent highly toxic. Besides, formaldehyde based dye fixing agents, chlorinated stain removers and hydrocarbon based softeners are another harmful chemicals present in the water. These organic materials easily react with many disinfectants especially chlorine and form by products (DBP'S) that are often carcinogenic and show allergic reactions (Kant, 2012).

Water pollution with colloidal matter appears along with colors and oily froth gives the water a bad appearance and smelly, meanwhile increases the water turbidity. As a result, it prevents the penetration of sunlight required for the process of photosynthesis in aquatic ecosystem (Banat *et al.*, 1996). This condition hinders the oxygen transfer mechanism from the water surface into water. Reduction of dissolved oxygen in water will threaten aquatic life because the dissolved oxygen is very essential to survive. In addition, the chemicals can clog the pores of soil and then reduce the plant growth and soil productivity due to soil hardening and penetration of roots is prevented (Kant, 2012).

2.3 Pigment distribution

Terrestrial and marine environment are natural sources of abundance pigment. Pigment distribution discovery in plants and microorganisms make researchers race to find the precious pigment. Almost half of the known drugs that are used to cure illness originated from natural products produced by terrestrial organisms (Bruckner, 2002). However, high demand in natural bioactive compounds from land-based makes it more challenging to find. As an alternative, natural compounds from water-based are promising sources in many field such as pharmacology, cosmetic and textile for industrial and commercial applications (Soliev *et al.*, 2011).

In terrestrial environments, the natural pigments are synthesized by higher plants and microorganisms (fungi, yeasts and bacteria) while natural pigments from marine environments are produced by lower plants and microorganisms (Ibrahim, 2008). Humans and animals are unable to synthesize pigments *de novo* (Cvejic *et al.*, 2007). Hence, they get any essential pigments from their diet either consume directly from nature sources or formulated diet.

Pigment from plants and animals are not suitable for industrial and commercial application since the sources become very limited with high potential for environmental pollution and damage of the biodiversity (Shatila *et al.*, 2013). Besides, pigments from plant origin have drawbacks such as unstable under light exposure, heat or adverse pH, insolubility in water, seasonal and limited resources. Meanwhile, pigment produced by microbial have good stability (Raisainen *et al.*, 2002) and the availability of cultivation technology will increase its applicability for industrial production (Venil *et al.,* 2013).

2.3.1 Terrestrial environment

2.3.1 (a) Terrestrial plants

Henna is a natural red colour from plant of Middle-East. Natural color from henna has been used to decorate the hands and feet of bride. In the traditional medical system, henna has anti-cancer, anti-inflammatory and anti-pyretic properties (Kingston *et al.,* 2009). *Indigofera tinctoria* also called true indigo is a species of plant that was used in eye shadow and body painting. Natural dye from *I. tinctoria* also used as coloring agent where it is known as tarum in Indonesia and nila in Malaysia (Tantituvanont *et al.,* 2008).

Pandanus amaryllfolius (pandan leaves) a well known as a food colorant among South-East Asian countries and is found across Malaysia, Sri Lanka, India and Hawaii. The green color from the pandan leaves is due to the synthesis of chlorophyll which is important for photosynthesis process (Stone, 1978). Butterfly pea (*Clitoria ternatea*) is a native plant origin found in equatorial Asia regions which contribute a deep blue color of the flowers with high amount of anthocyanins (Tantituvanont *et al.,* 2008). Malaysian people used the flower extract to make “nasi kerabu” which is well known as a special meal in Kelantan.

2.3.1 (b) Terrestrial animal

Very limited research on natural organic pigments from terrestrial animals due to it has low potential to use as coloring agent in industrial application. Delgado-Vargas *et al.,* (2002) reported the pigment from cochineal insect (*Dactylopius coccus*) has been used as a dye clothes for centuries especially in India, Europe and Persia. Cleopatra also used the red colour extracted from crushed carmine beetles and ants to make the red color lips to look beautiful (Garner, 2008; Azwanida *et al.,* 2015).

2.3.1 (c) Terrestrial microorganism

Soil fungi are known as pigment producer that may be actively growing or closely associated with other organisms. Soil fungi prefer to produce many substances like bioactive compounds and pigments in acidic condition (Calestino *et al.,* 2014; Akilandeswari & Pradeep, 2016). Plants, animals, bacteria, fungi, and microalgae have capability to synthesis pigments. Based on previous study report, fungi have high potential to produce great amounts of pigments (Mortensen, 2006; Kirti *et al,.* 2014). Some of the fungal species produce many pigment colours with high stability (Nagia & El-Mohamedy 2007). Submerged fermentation is a common technique for quantitative yield of pigments isolated from few soil fungi as listed in Table 2.1. The newest alternative to produce pigment in a large scale industry is use of filamentous fungi cultured on different agro-industrial by-products (Lopes *et al.,* 2013).

Fungi are considered as a potential source of pigments with a broad range of biological activities (Zhang *et al.,* 2004). The most dominant genera of fungi in the soil are species of *Trichoderma, Penicillium, Paecilomyces, Aspergillus,* and *Fusarium* (Celestino *et al.,* 2014). Almost all pigment produced by fungi belong to aromatic polyketide groups which is melanins, quinines (Dufosse *et al.,* 2005; *Caro et al.,* 2012), falvins, ankaflavin, naphthoquinone, and anthraquinone (Dufosse, 2006). The fungal pigments would be useful in various industrial applications due to their simple and fast growth in cheap culture medium which is made from waste product. The pigments also have different colour shades and being independent of unpredicted weather conditions (Venil & Lakshmanaperumalsamy 2009). Various species of fungi have been isolated from soil that has the ability to produce natural pigments. Table 2.2 shows some of the fungi isolated from soil and their potential applications.

Table 2.1: Quantitative yield of pigments isolated from soil fungi by submerged fermentation technique

Fungi	pH	Colour	Yield (g/L)	References
Monascus purpureus	5	Red	0.07	Mukherjee & Singh, (2011)
Paecilomyces sinclairii	6	Red	4.40	Cho *et al.*,(2002)
Penicillium funiculosum IBT3954	8	Red	0.13	Jens *et al.*, (2012)
Penicillium sp.	5	Violet	0.20	Ogihara *et al.*, (2000)
Penicillium sclerotiorum	5	Orange	0.31	Lucas *et al.*, (2010)

Yeast is known as carotenoids pigment producer and a total of 600-700 g/mL of carotenoids has been reported to be produced by yeast species such as *Cystofilobasidium capitatum, Rhodosporidium diobovatum, R. sphaerocarpum, Rhodotorula glutinis, Rh. minuta,* and *Sporobolomyces roseus* (Yurkov *et al.*, 2008). Apart from pigmented fungus, yeast also have spectrum that is wide, can grow well in temperature that is high and produce huge amounts of biomass (Kvasnikov *et al.*, 1978).

Table 2.2: Examples of fungal pigments from soil and their suggested application

Fungi	Colour	Pigment	Molecular formula	Application	References
Aspergillus niger	Black	Aspergilin	$C_{24}H_{35}NO_4$	Antimicrobial activity	Ray & Eakin, (1975)
Aspergillus niger	Brown	-	-	Textile dyeing Antibacterial activity	Atalla *et al.*, (2011); Aishwarya, (2014)
Aspergillus sclerotiorum	Yellow	Neoaspergilic acid	-	Antibacterial activity	Micetich & Macdonald, (1965); Teixeria *et al.*, (2012)
Aspergillus versicolor	Yellow	Asperversin	$C_{47}H_{58}O_{10}$	Antifungal activity	Miao *et al.*, (2012)
Fusarium oxysporum	Pink/ violet	Anthraquinone	$C_{14}H_8O_2$	Textile dyeing Antibacterial activity	Glessler *et al.*, (2013)
Fusarium verticillioides	Yellow	Naphthoquinone	$C_{10}H_6O_2$	Antibacterial activity	Boonyapranai *et al.*, (2008); Kurobane *et al.*, (1986)

Table 2.2: continued

Fungi	Colour	Pigment	Molecular formula	Application	References
Monascus sp.	Yellow	Monascin	$C_{21}H_{26}O_5$	Food colorant	Juzlova &Martinkova, (1996);
		Ankaflavin	$C_{23}H_{30}O_5$	Pharmaceuticals	Mostafa &Abbady, (2014);
	Orange	Monascorubrin	$C_{23}H_{26}O_5$	Antibacterial activity	Babitha *et al.*, (2008);
		Rubropuntatin	$C_{21}H_{22}O_5$	Anticancer activity	Moharram *et al.*, (2012);
	Red	Monascorubramine	$C_{23}H_{27}O_4$	Antioxidant	Babula *et al.*,(2009);
		Rubropuntamine	$C_{21}H_{23}O_4$		Yang *et al.*, (2014)
Penicillium herquei	Yellow	Atronenetin	-	Food additive Antioxidant	Takahashi & Carvalho, (2010)
Penicillium oxalicum	Red	Anthraquinone	$C_{14}H_8O_2$	Anticancer effectin food and pharmaceuticals Textile dyeing	Dufosse, (2006); Atalla *et al.*,(2011)

Table 2.2: continued

Fungi	Colour	Pigment	Molecular formula	Application	References
Penicillium purpurogenum	Orange to Yellow	Purpurogenone	$C_{14}H_{12}O_5$	Dyeing of cotton fabrics	Buchi *et al.*, (1965); Martinkova *et al.*, (1995); Mapari *et al.*,(2005); Takahashi & Carvalho, (2010); Teixeria *et al.*, (2012); Santos-Ebinuma *et al.*, (2013)
	Yellow to Orange	Mitorubrin	$C_{21}H_{18}O_7$	Food, pharmaceuticals, and cosmetics	
	Red	Mitorubrinol	$C_{21}H_{18}O_8$		
Penicillium sclerotiorum	Yellow to Orange	Pencolide	$C_9H_9NO_4$	Antibacterial activity	Brikinshaw *et al.*, (1963);
		Sclerotiorin	$C_{21}H_{23}ClO_5$	Antibacterial activity	Chidananda & Sattur, (2007);

Table 2.2: continued

Fungi	Colour	Pigment	Molecular formula	Application	References
		Isochromophilone VI		Antibacterial activity	Lucas *et al.*, (2007);
			-	Antifungal activity	Lucas *et al.*, (2010)
Trichoderma viride	Yellow Green Brown	Viridin -	$C_{20}H_{16}O_6$ -	Textile dyeing Antifungal activity Food industry	Chitale *et al.*,(2012); Neethu *et al.*, (2012); Gupta *et al.*,(2013)
Trichoderma virens	Yellow	Viridol Virone	$C_{20}H_{18}O_6$ $C_{22}H_{24}O_4$	Textile dyeing Antifungal activity	Mukherjee & Kenerley, (2010); Sharma *et al.*, (2012); Kamal *et al.*, (2015)

There are various *Rhodotorula* type species that are being isolated from plant leaves, flowers, fruits, slime fluxes (or exudates) of deciduous trees, soil, refinery waste water, air and yoghurt (Phaff *et al.,* 1972; Sakaki *et al.,* 2000; Aksu & Eren, 2007). Carotenoid production by *Rhodotorula* sp. is usually influenced by the species, media content and environmental circumstances. Carotenoid total production category produced by this species can be classified as low (less than 100 μg/g), medium (101 to 500 μg/g) and high (more than 500 μg/g) as reported by earlier researcher (Costa *et al.,* 1987; Squina *et al.,* 2002; Aksu & Eren, 2007; Maldonade *et al.,* 2008; Luna-Flores *et al.,* 2010; Saenge *et al.,* 2011).

Melanin is a special dark coloured pigment that could be generated by halophilic black yeast, *Hortaea werneckii*. Melanins can be used widely in various fields like agriculture, cosmetic, and pharmaceutical industries. Survey results also showed melanin of *Hortaea werneckii* has inhibition activity on pathogenic bacteria namely *Salmonella typhi* and *Vibrio parahaemolyticus*. This could be due to the melanin of *Hortaea werneckii* isolated from solar salterns is new discovery of natural product that pose an antimicrobial agent (Kalaiselvam *et al.*, 2013).

Besides, bacteria are also great contributor to natural pigment production. The application of pigment produced by bacteria as natural colorants has been investigated by many scientists (Joshi *et al.,* 2003; Venil & Lakshmanaperumalsamy, 2009). The hazardous effects from synthetic color usage (Venil *et al.,* 2013) caused an increasing demand for natural colors from industries. Bacteria provided large scope for commercial production of biological pigments such as carotenoids, anthraquinone, cholorophyll, melanin, flavins, quinones, and violacein (Keneni & Gupta 2011).

Duc *et al.*, (2006) reported some pigmented spore-forming bacterial isolates were found from human faeces from subjects in Vietnam. Two different pigments found in vegetative cells are yellow pigment and orange pigments found in spores were identified as carotenoids pigment. All these colonies were identified with close relatives of *Bacillus cibi*, *Bacillus jeotgali* and *Bacillus indicus*. (Duc *et al.*, 2006). This finding is very closely related with fermented seafood condiment known as uoc Mam or Vietnamese fish sauce in Vietnamese diet. A novel strain has identified as *Bacillus vietnamensis* sp. nov. in Nuoc Mam (Noguchi *et al.*, 2004).

From 216 bacterial species that were isolated from five different samples types namely vermicompost soil, cattleshed soil, garden soil, rhizosphere banana and rhizosphere papaya, only 13 isolates produced diverse colours of pigment. Five selected bacteria with different pigment color which is orange, red, bluish, lemon yellow and golden yellow were selected and identified as *Micrococcus nishinomiyaensis, Serratia marcescens, Psedomonas aeruginosa, Micrococccus luteus,* and *Staphylococcus aureus* (Rajguru *et al.*, 2016).

Some of the natural pigments produced by bacteria as listed in Table 2.3 (Malik *et al.,* 2012). Both red and yellow pigments become the main focus in development of pigment production study. For example, monascus produced by *Monascus* sp. (Yongsmith*et al.*, 1998), carotenoid from *Phaffia rhodozyma* (Vazquez *et al.*, 1997), *Micrococcus roseus* (Chattopadhyay*et al.*, 1997), *Brevibacterium linens* (Guyomarc'h *et al.*, 2000) and *Bradyrhizobium* sp. (Lorquin *et al.*, 1997) and xanthomonadin from *Xanthomonas campestrispv* (Poplawsky*et al.*, 2000). However, only a few bacterial isolates are able to produce blue pigment.

Table 2.3: Natural pigments produced by bacteria (Malik *et al.*, 2012)

Bacteria	Pigments/ Molecule	Colour	Applications
Agrobacterium aurantiacum, *Paracoccus carotinifaciens, Xanthophyllomyces dendrorhous.*	Astaxanthin	Pink-red	Feed supplement
Rhodococcus maris	Beta-carotene	Bluish-red	Used to treat various disorders such as erythropoietic protoporphyria, reduces the risk of breast cancer
Bradyrhizobium sp., *Haloferax alexandrines*	Canthaxanthin	Dark-red	Colorant in food, beverage and pharmaceutical preparations
Corynebacterium insidiosum	Indigoidine	Blue	Protection from oxidative stress
Rugamonas rubra, Streptoverticillium rubrireticuli, *Vibrio gaogenes,* *Alteromonas rubra,* *Serratia marcescens,* *Serratia rubidaea*	Prodigiosin	Red	Anticancer,immunosuppressant, antifungal, algicidal; dyeing (textile, candles, paper, ink)
Pseudomonas aeruginosa	Pyocyanin	Blue-green	Oxidative metabolism, reducing local inflammation

Table 2.3: continued

Bacteria	Pigments/ Molecule	Colour	Applications
Chromobacterium violaceum, Janthinobacterium lividum	Violacein	Purple	Pharmaceutical (antioxidant, immunomodulatory, antitumoral, antiparasitic activities); dyeing (textiles), cosmetics (lotion)
Flavobacterium sp., *Paracoccus zeaxanthinifaciens,* *Staphylococcus aureus*	Zeaxanthin	Yellow	Used to treat different disorders, mainly with affecting the eyes
Xanthomonas oryzae	Xanthomonadin	Yellow	Chemotaxonomic and diagnostic markers

2.3.2 Marine environment

2.3.2 (a) Marine plants

Natural pigment from marine algae sources are widely used in food, cosmetic and pharmacology (Pangestuti and Kim, 2011). Seaweeds are one of the marine plants and it is definitely algae because they lack vascular tissue. Macroalgae species classified into three different groups based on their photosynthetic pigment and chemical composition namely browns pigment is Phaeophyta, red pigment from Rhodophyta and green pigment is Chlorophyta (Gupta and Abu-Ghannam, 2011).

Chinnadurai *et al.,* (2013) reported six seaweeds namely *Cheatomorpha antennina, Enteromorpha intestinalis,Grateloupia lithophila, Hypnea valentiae, Padina gymnospora, Ulva fasciata* collected from Pondicherry coast were identified and analysed for the estimation of photosynthetic pigment content. The maximum total chlorophyll content of 0.68±0.01 mgg^{-1} produced by seaweed *Cheatomorpha antennina* and the maximum carotenoid content 0.63±0.02 mgg^{-1} produced by *Padina gymnospora*. The rich pigment content can be formulated in supplementary diet. The estimation of total chlorophyll and carotenoid content of six seaweeds collected from Pondicherry coast as listed in Table 2.4 **(**Chinnadurai *et al.,* 2013).

2.3.2 (b) Marine animal

The wonderful colours ranging between yellow, green, blue, brown, orange, red, purple and black also can be seen in marine animals. The various colourations in sea fan and sea feathers (Gorgonacea), blue coral (Coenothecalia), and organ pipe coral (Stolonifera) is due to rich of carotenoids or carotene proteins in marine invertebrates skeleton (Goodwin, 1968; Bandaranayake, 2006).

Table 2.4: Total chlorophyll and carotenoid content of six seaweeds (Chinnadurai *et al.*, 2013).

Seaweeds	Total chlorophyll (a&b) (mgg^{-1})	Carotenoids (mgg^{-1})
Cheatomorpha antennina	0.68±0.01	0.26±0.03
Enteromorpha intestinalis	0.54±0.02	0.38±0.01
Ulva fasciata	0.60±0.02	0.37±0.01
Padina gymnospora	0.66±0.01	0.63±0.02
Grateloupia lithophila	0.51±0.03	0.61±0.03
Hypnea valentiae	0.63±0.03	0.49±0.02

Sea coral have no ability in photosynthesis process like plant. Elde *et al.*, (2012) reported three different deep-water coral species contained carotenoids pigments which are astaxanthin and canthaxanthin-like carotenoid. There are two morphologies basic color for *Lophelia pertusa* namely orange and white (Waller & Tyler, 2005). *Paragorgia arborea* shows color pigment is white and range color pigment from deep red to white-pink. Besides, *Primnoa resedaeformis* shows common color pigment is orange-yellow (Mortensen & Buhl-Mortensen, 2005). Basically, the variation of pigment color among these species depends on probability of carotenoids bound to specific proteins (Elde *et al.*, 2012).

Sponges have existed since more than 800 million years (Radjasa *et al*., 2007) and are known to produce bioactive compounds including pigments (Donia & Hamann, 2003; Hamid *et al.*, 2013). These secondary metabolites play important role for protecting them from predators or other competitors (Pawlik *et al.*, 2002). Almost all sponges appear in bright and conspicuous colors whether cryptic, hiding in secluded caves or exposed. But the research about the bioactive compounds from pigmented sponges (Porifera) and other marine invertebrates include anemones, corals,