ISOLATION AND CHARACTERISATION OF PINK-PIGMENTED FACULTATIVE METHYLOTROPHS BACTERIA ISOLATED FROM LEAFY MALAYSIAN *ULAM* AND ITS APPLICATIONS ON BEEF

By

NUR ISTI'ANAH BINTI RAMLI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

June 2022

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DEDICATION

This thesis is wholeheartedly dedicated to my beloved parents and siblings, who have been my source of inspiration and gave me strength when I thought of giving up and who continually provide their moral, spiritual, emotional, and financial support.
To my supervisor, who always guided and motivated me to do my best.
To my friends, who stood beside me for their assistance and support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Presently, developing new colourants for the food industry is challenging as colourants need to be suitable for many factors, mainly food flavours, safety, and nutritional value, with minimal impact on the price of the product. As compared to natural food colourants, synthetic food colourants are selected due to their stability in long term usage and low cost, even though their safety and nutritional values can be questioned. To tackle this issue, a safe and yet nutritious natural food colourants are the alternatives. Interestingly, pigment of microorganism known as pink-pigmented facultative methylotrophs (PPFM) bacteria have not yet been utilised as a source of natural food colourant. Hence, the aim of this study is to analyse the antioxidant activity of pigments from these bacteria isolated from leafy Malaysian *ulam*. Initially, the PPFM colonies from three types of *ulam* leaves *Cosmos caudatus* Kunth (CC), *Oenanthe javanica* (Blume) DC. (OJ), and *Melicope lunu-ankenda* (Gaertn.) T. G. Hartley (ML) were isolated, and their genus and species characteristics were identified. Then, the growth pattern of the selected isolate was studied, followed by mass cell number production, and the pigment was extracted using solvents. The compounds in the extracted pigment were characterised, and their antioxidant activities and total phenolic content were evaluated. Besides, its toxicity level was estimated and its effect on colour, pH, and lipid oxidation in raw beef at certain storage times was observed. The results show a significant difference in PPFM population between the leaf surfaces of CC, OJ, and ML. All the collected isolates belong to *Methylobacterium* sp. in general, as they demonstrate Gram-negative properties, are motile, and have a rod shape with size (µm) ranging from 7.50 to 2.00 in length and 2.25 to 1.00 in width. They show positive results for catalase activity (all), lipids (most), starch and casein hydrolysis activities (less), citrate utilisation (a few), urease test (a few), oxidase test (most), and nitrate reduction test (a few). However, none of the isolates can degrade gelatin or cellulose as well as glucose fermentation and its metabolism action, or producing indole and hydrogen sulphide gaseous. The species of selected isolates of PPFM were identified as *M. radiotolerans* strain JCM 2831 (ML8), *M. radiotolerans* strain DSM 1819 (OJ4), and *Methylorubrum salsuginis* strain MR (OJ154). The growth study of selected isolate (ML8) display obvious pigmentation occurrence at day 4^{th} and stationary phase is good time for cell harvest. About 5.30 ± 0.21% of pigments can be extracted through methanol higher than ethanol which is only 2.41 ± 0.14% yield. Moreover, a few significant peaks of ML8 pigment extract exist at 460.2, 490.8, 520.4, and 360.6 nm in the UV-visible spectrum and peaks at a range of 3462–990 cm^{-1} in the fourier-transform infrared spectroscopy (FTIR) spectrum, indicating the presence of carotenoid compounds. Through gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analysis, several bioactive compound that are responsible for antioxidant activity, including phenolic and carotenoids compounds, were identified. Ethanolic ML8 pigment extract has a higher value than methanolic ML8 pigment extract in all tested antioxidant activities (except ABTS) and phenolic compounds. In addition, both pigment extracts are not toxic. There is improvement in the quality of the colour (redness a*) value, pH value, and lipid oxidation formation of raw minced beef treated with ethanolic ML8 pigment extract, particularly the 1.0% solution. Hence, the bacteria *Methylobacterium* sp. might be a good candidate for a source of pigment that has natural, safe, and nutritious colourant characteristics for food materials such as beef.

Keywords: antioxidant, bacterial pigment, natural colorant, pink pigmented facultative methylotrophs (PPFM), toxicity.

SDG: GOAL 2 (Zero Hunger); GOAL 3 (Good Health and Well-being); GOAL 12 (Ensure Sustainable Consumption and Production Patterns)

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PEMENCILAN DAN PENCIRIAN METILOTROF FAKULTATIF BERPIGMEN MERAH JAMBU YANG DIPENCILKAN DARIPADA *ULAM* MALAYSIA BERDAUN DAN APLIKASINYA KE ATAS DAGING LEMBU

Oleh

NUR ISTI'ANAH BINTI RAMLI

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Pada masa ini, pembangunan pewarna baharu untuk industri makanan adalah mencabar kerana pewarna perlu bersesuaian dengan banyak faktor terutamanya perasa makanan, keselamatan dan nilai nutrisi serta impak minimum ke atas harga produk. Jika dibandingkan dengan pewarna makanan semula jadi, pewarna makanan sintetik dipilih kerana kestabilannya dalam penggunaan jangka panjang dan kos yang rendah walaupun keselamatan dan nilai nutrisinya boleh dipersoalkan. Untuk menangani isu ini, pewarna makanan semulajadi yang selamat dan bernutrisi adalah alternatifnya. Menariknya, pigmen mikroorganisma yang dikenali sebagai bakteria metilotrof fakultatif berpigmen merah jambu (PPFM) masih belum digunakan sebagai sumber pewarna makanan semula jadi. Oleh itu, tujuan kajian ini adalah untuk menganalisis aktiviti antioksidan pigmen bakteria ini yang diasingkan daripada *ulam* Malaysia yang berdaun. Pada mulanya, koloni PPFM daripada tiga jenis daun *ulam* iaitu *Cosmos caudatus* Kunth (CC), *Oenanthe javanica* (Blume) DC. (OJ) dan *Melicope lunu-ankenda* (Gaertn.) T. G. Hartley (ML) telah diasingkan dan ciri-ciri genus serta spesiesnya telah dikenalpasti. Kemudian, corak pertumbuhan isolat terpilih telah dikaji diikuti dengan penghasilan sel jisim secara besar-besaran dan pigmen telah diekstrak menggunakan pelarut. Sebatian dalam pigmen yang telah diekstrak telah dicirikan dan aktiviti-aktiviti antioksidan dan kandungan fenolik keseluruhannya telah dinilai. Selain itu, tahap ketoksikannya telah dianggarkan dan kesannya terhadap warna, pH dan pengoksidaan lemak dalam daging lembu mentah pada masa penyimpanan tertentu telah diperhatikan. Keputusan menunjukkan perbezaan populasi PPFM yang ketara di antara permukaan daun CC, OJ dan ML. Kesemua isolat yang dikumpul adalah *Methylobacterium* sp. secara umum kerana ia menunjukkan sifat Gram negatif, motil dan mempunyai bentuk batang dengan saiz (µm) antara 7.50 hingga 2.00 panjang dan 2.25 hingga 1.00 lebar. Mereka menunjukkan keputusan positif untuk aktiviti katalase (semua), lipid (kebanyakan), aktiviti-aktiviti hidrolisis kanji dan kasein (sedikit), penggunaan

sitrat (beberapa), ujian urease (beberapa), ujian oksidase (kebanyakan) dan ujian pengurangan nitrat (beberapa). Walau bagaimanapun, tiada satu pun daripada isolat boleh menguraikan gelatin atau selulosa serta penapaian glukosa dan tindakan metabolismanya, atau menghasilkan gas-gas indole dan hidrogen sulfida. Spesies isolat terpilih PPFM telah dikenal pasti sebagai *M. radiotolerans* jenis JCM 2831 (ML8), *M. radiotolerans* jenis DSM 1819 (OJ4) dan *Methylorubrum salsuginis* jenis MR (OJ154). Kajian pertumbuhan bagi isolat terpilih (ML8) memaparkan pembentukan pigmentasi yang jelas pada hari ke-4 dan fasa pegun adalah masa yang baik untuk penuaian sel. Kira-kira 5.30 ± 0.21% pigmen boleh diekstrak melalui metanol lebih tinggi daripada etanol iaitu hanya 2.41 ± 0.14% hasil. Lebih-lebih lagi, beberapa puncak ketara ekstrak pigmen ML8 wujud pada 460.2, 490.8, 520.4, dan 360.6 nm dalam UV-visible spektrum dan puncak-puncak pada julat 3462–990 cm^{-1} dalam spektrum FTIR menunjukkan kehadiran sebatian-sebatian karotenoid. Melalui analisis GC-MS dan LC-MS, beberapa sebatian bioaktif yang bertanggungjawab untuk aktiviti antioksidan termasuk sebatian-sebatian fenolik dan karotenoid telah dikenalpasti. Ekstrak etanolik pigmen ML8 mempunyai nilai yang lebih tinggi daripada ekstrak metanol pigmen ML8 dalam semua aktiviti-aktiviti antioksidan yang diuji (kecuali ABTS) dan sebatian-sebatian fenolik. Sebagai tambahan, kedua-dua ekstrak pigmen adalah tidak toksik. Terdapat penambahbaikan pada kualiti warna (kemerahan a*), nilai pH dan pembentukan pengoksidaan lemak daging lembu cincang mentah yang dirawat dengan ekstrak etanol pigmen ML8 terutamanya larutan 1.0%. Oleh itu, bakteria *Methylobacterium* sp. berkemungkinan merupakan calon yang baik sebagai sumber pigmen yang mempunyai ciri-ciri pewarna semula jadi, selamat dan berkhasiat untuk bahan makanan seperti daging lembu.

Kata kunci: antioksidan, pigmen bakteria, pewarna semulajadi, metilotrof fakultatif berpigmen merah jambu (PPFM), ketoksikan.

SDG: MATLAMAT 2 (Sifar Kelaparan); MATLAMAT 3 (Kesihatan dan Kesejahteraan yang Baik); MATLAMAT 12 (Memastikan Corak Penggunaan dan Pengeluaran Mampan)

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LIST OF ABREVIATIONS

a*	Red/greenness
ABTS	2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AHLs	N-acylhomoserine lactones
ANOVA	Analysis of variance
AOD	O_2-dependent alcohol oxidase
ATR	Attenuated total reflectance
b*	Yellow/blueness
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
ca.	Circa (approximately)
CC	*Cosmos caudatus* Kunth
CFU	Colony forming unit
Cm	Centimeter
CMC	Carboxymethyl cellulose
CTAB	N-cetyl-N,N,N,trimethylammoniumbromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides triphosphates
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DW	Distilled water
EC_{50}	Half maximal effective concentration
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
Eq.	Equivalent
FASTA	Fast-all" or "fastA"

FDA	U.S. Food and Drug Administration
FRAP	Ferric ion reducing antioxidant power
FTIR	Fourier-transform infrared
GAE	Gallic acid equivalent
GC-MS	Gas chromatography–mass spectrometry
gDNA	Genomic deoxyribonucleic acid
GP	Glycerol-peptone
HPLC	High-performance liquid chromatography
IC_{50}	Half-maximal inhibitory concentration
ID	Inner diameter
ISR	Induced systemic resistance
Kb	Kilobase
KI	Kovalt retention index
LC_{50}	Median lethal concentration
LC-MS	Liquid chromatography–mass spectrometry
m/z	Mass-to-charge ratio
Mb	Myoglobin
MDA	Malondialdehyde
MDHs	Methanol dehydrogenases
MetMb	Metylmyoglobin
MF	Molecular formula
ML	*Melicope lunu-ankenda* (Gaertn.) T. G. Hartley
ML8	PPFM bacteria isolate of *M. Lunu-ankenda* (Gaertn.) T. G. Hartley leaf
MMO	Methane monooxygenase
MMS	Methanol mineral salts
MPA	Modified pseudomonas agar

MR	Methyl red
MRVP	Methyl red Voges Proskauer
MTBE	Methyl-tert-butyl ether
mV	Millivolts
MW	Molecular weight
mxaF	Methanol dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NCDs	Non-communicable diseases
NIST	National Institute of Standards and Technology
OD	Optical density
OJ	*Oenanthe javanica* (Blume) DC.
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyric acid
PI	Polarity index
PPFM	Pink-pigmented facultative methylotrophs
PQQ	Pyrroloquinoline quinone
QS	Quorum sense
rDNA	16s ribosomal RNA (rRNA)
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute

RuMP	Ribulose monophosphate pathway
SCA	Simmons citrate agar
SI	Similarity index
SIM	Sulphide indole motility
TBARS	Thiobarbituric acid
TCE	Trichloroethylene
TE	Trolox equivalent
Tlx	Trolox
TNT	2,4,6-trinitrotoluene
TPC	Total phenolic content
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
TSA	Trypticase soy agar
VP	Voges-Proskauer

CHAPTER 1

INTRODUCTION

1.1 Background

Food properties, specifically colours, a visual feature associated with the spectral distribution of light resulting from the interaction with matter, mainly determine the consumer's satisfaction and expectations, affecting their choice and eating desires. Food colourants affect product recognition and acceptability (spoiled food indicator sign), as they are a basic part of sensory perceptions such as sight, texture, smell, and taste (Martins et al., 2016). Still, the degradation of existing natural pigments can prevail during food processing, which in most cases requires the addition of colourants to restore or enhance colour. Thus, a large number of food products incorporate colours in order to obtain a pleasing appearance or dye feature. For example, in order to improve consumers' preferences, food colourants are commonly used in edible ices, desserts, pastry and fine bakery products, decorations and the coatings of pastry, confectionary products, sauces, fruit juices, snacks and soft drinks, and alcoholic beverages (Silva et al., 2022; Dey and Nagababu, 2022).

According to the FDA (2010), a food colourant can be defined as "*any dye, pigment, or substance which, when added or applied to a food, drug, cosmetic, or the human body, is capable (alone or through reactions with other substances) of imparting colour*". In the European Union, the use of food colourants—similarly to the use of other food additives—is regulated by specific laws, with these being the reported categories of food products, maximum usable quantities, chemical characterisation and purity. Accordingly, colourants can only be used if they are integrated into one of three categories: having a defined acceptable daily intake, or being permitted to be used only in special or specific cases. Also, each food additive has a code in the EU, which includes the letter E (for Europe), followed by three or four digits. The numbering scheme follows that of the International Numbering System (INS), as determined by the Codex Alimentarius Committee ("Guidance on Food Additives," 2015; Silva et al., 2022).

Food colourants can be synthetic (synthesised equally to the natural) or naturally derived. Even though most of the natural food colourants are derived from plants, some others are obtained from animals or even ores. Most natural colourants have some disadvantages, such as reactivity towards other ingredients in foods or in the presence of aromas or odours, as well as instability in water or when exposed to light and heat. Moreover, the consumption of natural food colourants can have significant benefits; they are in demand for their reliability, functionality, biological potential, and health benefits as compared to synthetic dye (Mohamad et al., 2019; Luzardo-Ocampo et al., 2021).

In fact, natural colourants are obtained from nature, and can be isolated by more or less complicated extraction processes, whereas synthetic dyes are chemically synthesised. Nevertheless, sometimes it is not easy to classify them into synthetic or natural colourants, as food colour processing can become very complicated because, although they may start with natural substances, a set of chemical processes involving extraction or modification procedures of the initial substance might be required (Silva et al., 2022). The synthetic food colourants do not occur in nature due to chemical structures, but have the advantage of prevailing in the form of powders, pastes, or granulates and are soluble in water (Arocas et al., 2013).

1.2 Problem Statements

In meat products, especially processed meat, food colourants were used to enhance their aesthetic appearance and promote sales, as described by Pagáčiková and Lehotay (2015) and Hisano (2016). Studies done by Pagáčiková and Lehotay (2015) and Iammarino et al. (2020) reported the application of several types of synthetic food colourants, including Ponceau 4R (E124) and Red 2G (E128) compounds in meat products. In fact, Malaysian food regulation in "Malaysian Regulation 1985" (2016) also permitted some of their types to be used in the food industry, such as Allura Red AC (16035), Erythrosine BS (45430), and Carmoisine (14720).

However, some synthetic food colourants may present health problems, namely allergenic problems, which in children can cause hyperactivity and even mutagenic and/or carcinogenic pathologies (Silva et al., 2022). For instance, Amin (2018) has discovered that Carmoisine/Azorubine (E122) is able to interfere with female hormones, leading to an infertility condition in female rats, while Allura Red (E129) can cause allergies, food intolerance, cancer, and brain damage (Rovina et al., 2016) at high dose consumption, and Erythrosine (E127) may affect neural tube development in early-stage chicken embryos at half of the approved dose (Ovalioglu et al., 2020).

As a result, some countries, including the United States, the European Union, Australia, and New Zealand, have banned the use of certain synthetic food colourants such as Red 2G (E128) (Mota et al., 2021). Hence, in order to tackle the health side effects of the synthetic food colourants' issues, their replacement with natural food colourants is a better choice. For instance, the use of the red food colouring cochineal (E120), which is prepared from an insect (cochineal), beetroot red (E162), and the green food colouring chlorophyll (E140) were increased (Silva et al., 2022), as well as from microorganisms such as the purple pigment known as violacein from *Chromobacterium violaceum* and the red-orange pigment named beta-carotene from *Rhodotorula glutinis* (Sen et al., 2019; Rana et al., 2021).

Fascinatingly, pink-pigmented facultative methylotrophs (PPFM) bacteria with a pink appearance have yet to be used as a natural food colourant. These bacteria are a type of microorganism that can utilise one-carbon compounds as a sole source of carbon and energy as well as other multicarbon substrates present ubiquitously in nature, such as plant leaves in symbiotic relationships (Balachandar et al., 2008). To date, numerous studies have been conducted to identify the benefits of the pigments extracted from this bacteria species, but none of them focused on the same bacteria that originated from Malaysian *ulam* leaf. Hence, this study emphasised the PPFM properties together with their extracted pigments for bioactivity (antioxidant) performance evaluation from a few selections of *ulam* leaves.

1.3 Objectives

The general objective of this study is to analyse the bioactivity (antioxidant activity) of pigments from selected PPFM bacteria isolated from leafy Malaysian *ulam*. To be specific, the objectives of this study are:

1. To isolate, characterise, and identify the PPFM bacteria isolated from *Cosmos caudatus* Kunth, *Oenanthe javanica* (Blume) DC., and *Melicope lunu-ankenda* (Gaertn.) T. G. Hartley leaves.
2. To produce, extract, and characterise the pigment of isolated PPFM bacteria from selected *ulam*.
3. To determine the phenolic compound, antioxidant activity, and toxicity level of the extracted pigment.
4. To evaluate the colour change and lipid oxidation of pigment-treated raw minced beef.

1.4 Scope of Study

The scope of this study is depicted as a flowchart (Figure 1.1), which covers the isolation of pink-pigmented facultative methylotroph (PPFM) from the leaves of *ulam raja* (*C. caudatus* Kunth) (labelled as CC), *selom* [*O. javanica* (Blume) DC.] (labelled as OJ), and *tenggek burung* [*M. lunu-ankenda* (Gaertn.) T. G. Hartley] (labelled as ML) using selective modified Pseudomonas agar based on their pink pigment colony appearance. The biochemical characteristics of the selected PPFM isolates (CC128, OJ154, ML8) were examined, including Gram staining, motility, cell shape, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, cellulose degradation, citrate utilization, lipolytic activity, urease test, methyl red (MR) test, Voges-Proskauer (VP) test, nitrate reduction, catalase activity, indole production, hydrogen sulphide production, and oxidase test. The isolated PPFM was also molecularly identified through 16S rDNA gene amplification and DNA sequencing analysis.

Next, the growth rate of each was examined, and the isolate with the highest number of cells was selected. The selected isolate (ML8) cell growth was studied and produced massively, followed by pigment extraction using methanol and characterisation through UV-visible spectrophotometers, fourier-transform infrared spectroscopy (FTIR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS).

The total antioxidant capacity of extracted ML8 pigment was determined, which includes 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric ion reducing antioxidant power (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging and total phenolic content (TPC). Using brine shrimp assay, the toxicity level of the same extract was evaluated. Finally, a different concentration of ML8 pigment was applied to the fresh, raw minced beef. The colour change, pH, and lipid oxidation evaluations using thiobarbituric acid analyses (TBARS) of treated raw minced beef were analysed. All the data was recorded and analysed statistically.

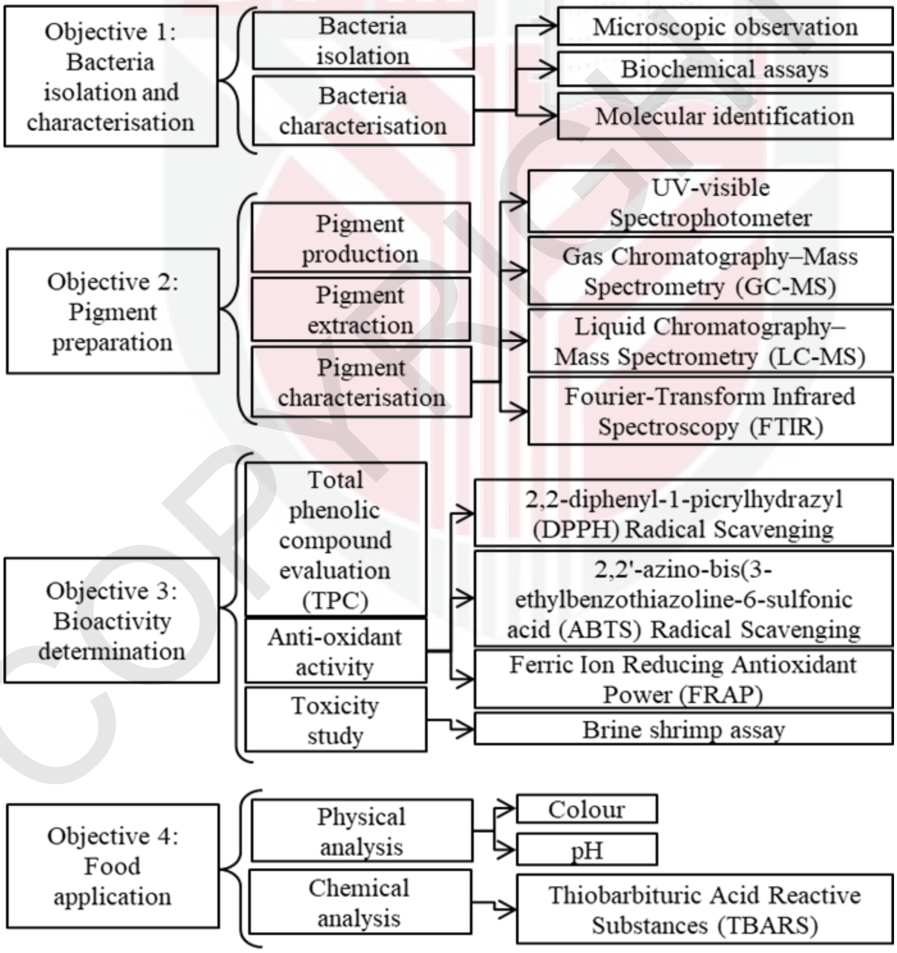

Figure 1.1: Flowchart of the study

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