SYNTHESIS AND OPTIMIZATION OF MICRO-SIZED BACTERIAL-BASED VIOLET PIGMENT USING RESPONSE SURFACE METHODOLOGY

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To My Beloved Mak & Ayah

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In the name of *ALLAH* S.W.T., The Most Gracious and The Most Merciful.

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ABSTRACT

Violet pigments, extracted from a bacteria known as *Chromobacterium violaceum*, has raised the enthusiasm of researchers in conducting comprehensive studies on these pigments due to their diverse biological activities include antibacterial and antioxidant properties. There is, however, a limitation related with the solubility of the violet pigment, by which it is commonly dissolved in toxic solvents such as dimethyl sulfoxide (DMSO) and methanol instead of being soluble in biological fluids and water. This approach did not synchronized with the public demands for products that are both eco-friendly and safe towards the environment and human body. Hence, this study provides a method to synthesise the violet pigment in microscale through an encapsulation technique using chitosan-tripolyphosphate (TPP) microparticles. Owing to the exceptional properties of high surface to volume ratio of microparticles, the solubility of the violet pigment in water and biological fluid could be improved. The synthesis of microparticles in this study involved ionic gelation between chitosan and TPP, in which several parameters were taken into consideration in order to control the dispersion stability of the violet pigment in the suspension. It is well known that particles in microscale will tend to aggregate, thus causing diminution of their biological activities. Therefore, preparation parameters, including the concentration of chitosan, tripolyphosphate (TPP) and pigment as well as the mass ratio of chitosan to TPP, were optimized using Response Surface Methodology (RSM). The aim was to obtain small particles size down to microscale with low range of polydispersity index (PDI) and high zeta potential. Minimum particle size of 149.0 nm with polydispersity index of 0.367 and zeta potential of +23.40 mV was obtained at the optimal formulations of 2.33 mg/mL of Cs, 1.5 mg/mL of TPP and 1 ppm of violet pigment and at mass ratio of chitosan:TPP of 7:1

ABSTRAK

Pigmen ungu, diekstrak daripada bakteria dikenali sebagai *Chromobacterium violaceum*, telah menimbulkan semangat para penyelidik dalam menjalankan penyelidikan yang menyeluruh ke atas pigmen ini disebabkan oleh aktiviti-aktiviti biologi mereka termasuk sifat nyahbakteria dan sifat nyahoksida. Walaubagaimanapun, terdapat beberapa masalah berkait dengan kelarutan pigmen ungu di mana kebiasaannya larut di dalam pelarut yang toksik seperti dimethyl sulfoxide (DMSO) dan methanol dimana sepatutnya menjadi larut di dalam cecair biologi dan air. Pendekatan ini tidak selari dengan permintaan awam terhadap barang yang mesra alam dan selamat untuk alam persekitaran dan badan manusia. Oleh itu, kajian ini menyediakan kaedah dalam mensintesis pigmen ungu dalam skala mikro melalui teknik pengkapsulan dengan chitosan-tripolyphosphate (TPP) mikropartikel. Kelarutan pigmen ungu ke dalam air dapat diatasi disebabkan sifat luar biasanya iaitu tinggi nisbah antara permukaan dan isi padu. Sintesis mikropartikel di dalam kajian ini melibatkan penggelan ionik antara chitosan dan TPP dimana beberapa parameter telah dititikberatkan bagi mengawal kestabilan pembubaran pigmen ungu di dalam ampaian. Ianya telah diketahui ramai bahawa partikel di skala mikro cenderung untuk mengalami penggumpalan yang juga mengurangkan aktiviti-aktiviti biologinya. Oleh itu, parameter-parameter penyediaan dalam kajian ini termasuk kepekatan chitosan, tripolyphosphate (TPP) dan pigmen serta nisbah jisim chitosan kepada TPP telah dioptimumkan menggunakan Response Surface Methodology (RSM). Ini bertujuan untuk mendapatkan saiz partikel yang kecil sehingga skala mikro dengan indeks taburan (PDI) yang rendah dan tinggi nilai potensi zeta.Saiz minimum partikel, 149.0 nm bersama PDI, 0.367 dan potensi zeta, +23.40 mV telah diperolehi di formulasi optimum, 2.33 mg/mL chitosan, 1.5 mg/mL TPP dan 1 ppm pigmen ungu pada nisbah jisim chitosan: TPP 7:1.

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LIST OF ABBREVIATIONS

BBD	Box- Behnken design
Cs	Chitosan
^{o}C	Degree Celcius
DMSO	Dimethyl sulfoxide
DLS	Dynamic Light Scattering
FESEM	Field Emission Scanning Electron Microscope
h	hours
L	Liter
μL	Microliter
μm	Micrometer
mg	Milligram
min	minutes
mL	Milliliter
mV	Millivolt
M	Molar
nm	Micrometer
ppm	Part per million
PDI	Polydispersity index
RSM	Response surface methodology
R^2	R squared
rpm	Revolutions per minute
TPP	Tripolyphosphate
% v/v	Volume/volume percent

CHAPTER 1

INTRODUCTION

1.1 Background of study

Natural and synthetic pigments have been applied in food, clothes, cosmetics, inks, papers and many other materials whereby they imparted beauty in all the materials by transmitting color. Nowadays, people around the world have begun to reset and change their perspectives and concerns toward health issues and environmental conservation. This resulted into an upsurge in demand for natural pigments from the industries due to their non-toxicity, non-carcinogenicity and biodegradable properties (Aruldass, 2015). Pigments have become essential and although they are present in all organisms due to their primary function as a colorant, only natural pigments have specific functions. Some of those functions include photosynthesis in plants, oxygen and carbon dioxide transportation in organisms and as a protective screen in humans and other vertebrates. Moreover, there are a many pigments that possess biological activities such as antibacterial, antioxidant, anticancer and many others (Delgado Vargas *et al.*, 2000; Kumar *et al.*, 2015). Microorganism is recognized as a potential source of natural pigment over plants due to their advantages of high yield, stability, cost efficiency, accessibility and easy scale-up production of pigment (Tuli *et al.*, 2014).

In nature, numerous microorganisms, including yeast, fungi, algae and bacteria, have been known to produce pigments. However, bacteria offers several exceptional advantages owing to their short half life cycle, invulnerable toward season and climate, ability to create various pigments with different colors and shades as well as having an easier scale-up production process and many others (Sutthiwong *et al.*, 2014). In this study, violet pigment produced by *Chromobacterium violaceum* UTM5 was utilized due to its interesting pharmacological properties including antibacterial activity and their facile growth in common laboratory media such as nutrient agar (Aruldass, 2015).

1.2 Problem Statement

Demands on natural pigment, primarily on bacterial pigment, have been rising for decades due to its peculiar features that are independent toward environmental changes to provide various shades and colors of pigment, as well as exhibiting an easy scale-up production of pigment. Violet pigment produced from *Chromobacterium violaceum* UTM5 has great potential in pharmaceutical applications due to its many properties such as antimicrobial, antiviral, antiprotozoal and antioxidant properties. Violet pigment is composed of two derivatives which are violacein and deoxyviolacein. However, violacein derivatives of the violet pigment have a limitation, in where they can only dissolved in toxic solvents such as dimethyl sulfoxide (DMSO) and methanol. Hence, encapsulation of this bacterial-based violet pigments with the polymeric chitosan-tripolyphosphate (chitosan-TPP) microparticles is expected to improve its solubility owing to the distinct features of moderately large activated surface area to volume ratio. This allows the interaction between the pigment and water molecules to occur more easily. Thus, this could improve the dissolving process of the violet pigment without the use of toxic solvents. Nevertheless, chitosan-tripolyphosphate (Cs-TPP) microparticles prepared by ionotropic gelation method have been known to aggregate or fuse directly after preparation and has a limited stability during storage. Therefore, optimization of preparation parameters such as Cs, TPP and pigment concentration as well as mass ratio of Cs to TPP was carried out using Response Surface Methodology. The purpose of the optimization was to acquire the optimum conditions for attaining micro-sized violet pigment with low polydispersity index and high zeta potential.

1.3 Objectives of study

The objectives of this study are:

i. To synthesize micro-sized bacterial-based violet pigment *via* encapsulation technique.
ii. To optimize the preparation and stabilization parameters using Response Surface Methodology (RSM)
iii. To characterize the optimized formulated micro-sized violet pigment

1.4 Scope of study

Micro-sized violet pigment was synthesized *via* encapsulation technique with chitosan-tripolyphosphate (Cs-TPP) microparticles. Ionic gelation method was applied as method to prepare Cs-TPP microparticles. The synthesized micro-sized violet pigment were characterized for their physico-chemical properties which included average particle size distribution, polydispersity index (PDI) and zeta potential using Zetasizer Nano (Malvern, UK.). Optimization of the preparation parameters of micro-sized violet pigment was analysed using Response Surface Methodology (RSM) using the Box-Behnken design. Then, the optimized micro-sized violet pigment obtained using the optimal RSM formulation was characterized for their morphology using Field Emission Scanning Electron Microscope (FESEM).

1.5 Significance of study

The development of micro-sized violet pigment from this study could improve the solubility of violet pigment which is known as poorly water-soluble pigment. Additionally, the reduction in size of violet pigment into micro-scale could minimize its toxicity towards human in order to be applied as an additive and a colorant in various materials such as foods, cosmetics, clothes, ink, paper and so forth. The synthesized micro-sized violet pigment can also possibly act as therapeutic agents in the pharmaceutical fields. Moreover, the optimization studies could give ideas on the important preparation parameters to be considered when synthesizing violet pigment in the micro-scale.

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