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PRODUCTION AND CHARACTERIZATION OF CRUDE AND ENCAPSULATED PRODIGIOSIN PIGMENT

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ABSTRACT:

Pigments from microorganisms can serve as an alternative source to replace synthetic pigments used in various industries. Natural pigments have some limitations including solubility, sensitivity and short stability upon exposure to light, pH and high temperature. Thus, employing methods such as encapsulating can be a good alternative to enhance the pigment's properties. The present work is a comparison of encapsulated and non-encapsulated (crude) prodigiosin extracted from *Serratia marcescens* in terms of stability and solubility. The results show that the pigment is more stable and soluble in water in encapsulated form when stored in the absence of light and thus has superior stability compared to pigment in its crude form. The results suggest that the encapsulated prodigiosin can be a more stable pigment under the above optimum conditions.

KEYWORDS: *Serratia marcescens*, Prodigiosin, Encapsulation, Pigment, Spray Drying

[I] INTRODUCTION

Natural pigments have been used to replace synthetic dyes in recent decades due to potential hazard to human health and environment [1-3]. Pigments from organisms have been shown to have biological activities and potential health benefits as well as biodegradability [4]. Prodigiosin is a natural pigment responsible for the red colour in *Serratia marcescens* [5-8]. Characteristics such as antimicrobial, antitumor and antibiotic of prodigiosin make this natural pigment appropriate for medical applications [9, 10]. Besides unique characteristics of prodigiosin, this pigment is applicable as a colorant in textile and foodstuffs [11].

However, undesirable properties of this natural pigment such as low solubility and rapid colour fading upon exposure to sunlight, high temperature and pH limit their application [12].

In order to make natural pigments widely applicable and comparable to synthetic pigments, some properties of natural colorants need to be modified. This is done *via* techniques such as encapsulation which will improve the properties of substances. Encapsulation is a

technique which offers preservation of natural pigments by entrapping them within the coating materials [13-20].

Among encapsulation methods, spray drying is the most common process employed in the industries especially food industry due to feasible and economic operation. By employing the spray drying method the replacement of natural pigments with synthetic colorants is possible in the colour related industries such as textile, food and beverages [21-31].

Encapsulation of prodigiosin and its comparison with crude form has not been study so far. Thus, we studied the benefits of encapsulated prodigiosin in terms of stability by means spray drying method.

[II] MATERIALS AND METHODS

In this study, the coloured bacterium, *Serratia marcescens*, was obtained from the oxidation pond at Universiti Teknologi Malaysia, Skudai. The kappa-carrageenan (Fluka) was obtained commercially. All other reagents and chemicals used were of analytical grade (AnalaR).

2.1. Growth Media

Growth media used in this study are nutrient broth (NB), nutrient agar (NA) and brown sugar (BS) medium. Nutrient broth (NB) was prepared by dissolving 8 g of NB powder (Merck) in 1 L of DDW followed by autoclaving at 121ºC, 101.3 kPa for 15 minutes. Nutrient agar (NA) was prepared by dissolving 20 g of nutrient agar (Merck) in 1 L of DDW. Then, the solution was sterilized by autoclaving at 121ºC, 101.3 kPa for 15 minutes. When the solution cooled down around 50ºC, the solution was poured into sterile Petri dishes. The agar was left to harden and was incubated in the incubator (Memmert) for 24 hours at 30ºC. The brown sugar (BS) solution was prepared by dissolving 40 g of BS in 1 L DDW. The solution was stirred and filtered before subjected to autoclaving at 121ºC, 101.3 kPa for 15 minutes.

2.2. Active Culture

The active culture of *S. marcescens* was prepared by inoculating a loopful of bacterial cells from NA plate into NB solution (25 mL) followed by incubating the cultures for 12 hours with agitation speed at 200 rpm using an orbital shaker (Certomar®R, B. Braun). The temperature for incubation of *S. marcescens was* 30°C.

2.3. Production of Prodigiosin

S. marcescens was cultivated by inoculating the active culture (2.5 mL) into an Erlenmeyer flask (250 mL) containing a combination of sterile DDW (20 mL) and 2.5 mL of BS solution (40 g/L). The culture was shaken at 200 rpm at 25°C for 24 hours.

2.4. Extraction of Prodigiosin

Prodigiosin pigment was extracted from cell suspension of *S. marcescens* after centrifugation of culture for 20 min (7,500 rpm) followed by separation of the bacterial cell pellets and supernatant. The supernatant obtained from the centrifugation was extracted for prodigiosin using ethyl acetate (4:1) where the organic layer obtained was collected while the aqueous layer discarded. Prodigiosin content in the organic layer was concentrated using rotary evaporator (BÜCHI R-210, Switzerland) at 50 °C determined for dry weight for 3 days at 60°C prior to use for spray drying procedure.

2.5. Procedure for Spray drying of Prodigiosin

The feed solution of the spray dryer prepared by dissolving κ -carrageenan (1% w/v) as an encapsulation agent in 100 mL of distilled water and mixing with 100 mL of diluted prodigiosin (150 ppm) as a core material. The mixture was stirred at 70°C until the ethyl acetate was completely evaporated. The suspension (1:1 ratio of prodigiosin/ κ-carrageenan) was homogenized using a sonicator (Elmasonic, S 100/ H) for 10 minutes and then subjected to a pilot scale spray dryer (LU-222, SD-05, Labultima, Japan) for further processing. The spray drying parameters adjust based on previous studies on technical operation of spray drying [27-31]. The airflow was set to 60 m^3/h, the atomizing air and temperature feed rate were kept constant at 1.5 bar and at 30°C. The feed rates, inlet and outlet temperature were kept constant at 3 mL/min, 200°C and 100°C, respectively [27]. The spray-dried prodigiosin was kept in a sealed container in dark for future stability studies.

2.6. Characterization of Crude and Encapsulated Prodigiosin

The characterization of extracted prodigiosin (crude) carried out using UV–Vis (HACH DR-5000). Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer) in the range of 4000 - 450 cm^{-1}.These methods were also used to characterize encapsulated prodigiosin after dissolving in water.

2.7. The Stability of Crude and Encapsulated Pigment

The studies on crude and encapsulated prodigiosin fading mechanism and degradation i.e. pH, light, temperature and its solubility has been monitored using several tests for the period of known time.

To evaluation of pigment solubility, the following solvent were used; water, HCl (0.1M), NaOH (0.1M), acetone (QReC^{TM}), *n*-hexane (QReC^{TM}), ethyl acetate (J.T.BAKER) and methanol (QReC^{TM}). Into a series of test tubes containing 100 mg/L of crude pigment in ethyl acetate (4.2 g/L dry wt.), five milliliters of the solvents was added. The same process was repeated for encapsulated pigment (0.05 g) prior to mixing at 200 rpm for 12 h at room temperature. The λ_{max} and absorbance were recorded using a HACH DR-5000 UV–Vis spectrophotometer.

The simultaneous effect of light and pH on crude pigment (100 mg/L in ethyl acetate) was recorded by varying the pH between 2 and 11, using either 1 M of NaOH or 1 M of HCl. A low-pressured Hg discharge Phillips fluorescent light (18 W; 1 m distance) was used to irradiate the crude pigment solutions. The evaluation of crude pigment's stability was done immediately after the pH changing by means of spectrophotometer at regular time intervals. The same process was repeated for studying the effect of pH on encapsulated pigment (0.05 g). The control sets were kept in dark for both crude and encapsulated pigments.

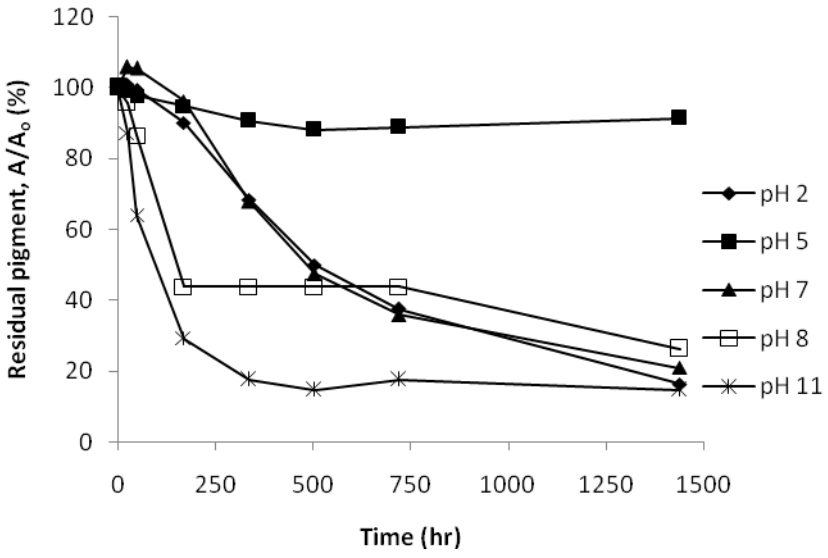
To study the effects of various temperatures on crude and encapsulated pigment, the samples were kept in water bath at 20 °C, 40 °C, 60 °C, 80 °C, and 100 °C for 15, 30, 60, 90, and 120 min [32].

[III] RESULTS DISCUSSION

3.1. Stability of Crude and Encapsulated Prodigiosin towards pH and Light

The stability study shows that the crude pigment is more prone to changes compared to encapsulated pigment at different pH values in either light or without light. The colour of the crude pigment changes from pink to red, orange and yellow as the pH increases while encapsulated pigment colour change from pink to orange at high pH **[Figure 1 and 2].**

a)



b)

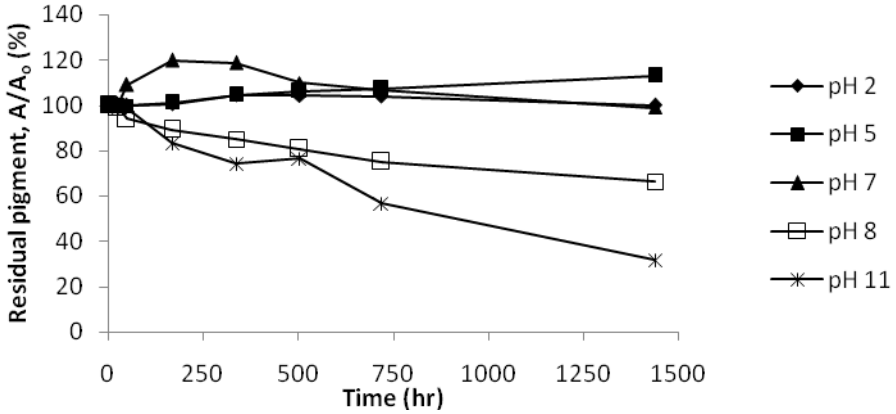


Figure 1: Stability of crude pigment at various pH upon exposure to fluorescent light (a) and kept in the dark (b) for 30 days

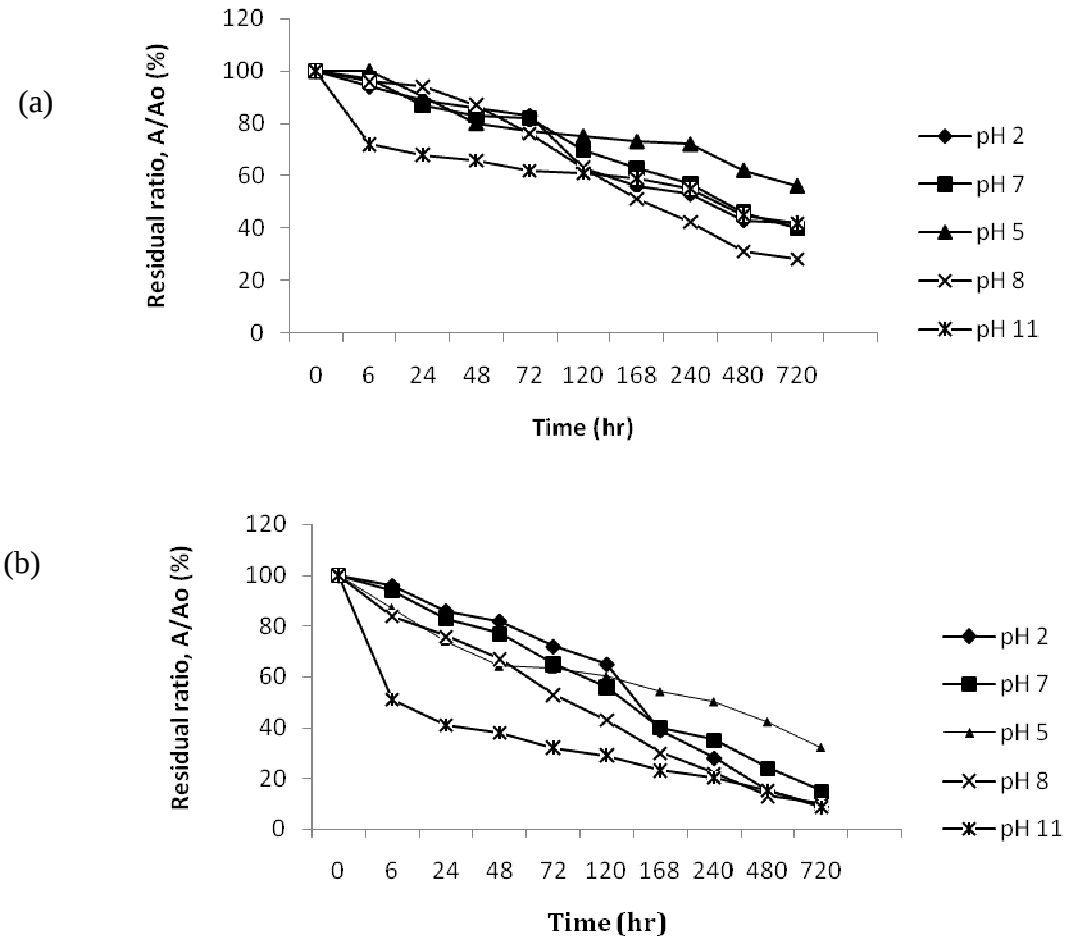


Figure 2: The effect of pH on the stability of encapsulated prodigiosin (a) in the presence of light; (b) in the absence of light.

The residual pigment is the absorbance percentage of encapsulated prodigiosin at specified time intervals and the initial residual absorbance percentage. It can be seen that fast degradation took place when the prodigiosin was exposed to light in either crude or encapsulated form. The stability decreases with the increasing of pH as can be observed from the residual pigment percentage. However, pH 5 is the most stable condition observed in the absence and presence of light **[Figure 2].** The remaining residual of crude pigment at pH 5 exposed and unexposed to indoor light are 90% and 100% respectively. The rate of pigment degradation is faster in alkaline condition and upon exposure to light.

In an acidic medium the dissolved encapsulated pigment is pink while in an alkaline medium the solution changes to orange-yellow. This is due to protonation of pyrrole group in prodigiosin structure. In extreme acidic pH, the protonation of pyrrole group occurs on one of the carbon atoms of the second position in the ring and not on nitrogen atom and therefore become non-aromatic [33]. This protonation causes fading in prodigiosin colour. In extreme alkaline pH, the OH^- group deprotonated amine group in the structure forming anion. Both conditions lead to

the destruction of the highly conjugated system of double bonds and therefore, responsible for the degradation of the pigment, especially in alkaline pH where the rate of reduction is faster. Therefore, exposure of the pigment to light accelerate their colour destruction due to charge delocalization of the pyrrole group which is responsible for the redish colour in either crude or encapsulated form. The rate of decolourization of the encapsulated prodigiosin is much lower than crude form due to protection by the encapsulating agent.

3.2. Stability of Crude and Encapsulated Pigment towards Temperature

The residual pigment was calculated after treating for a maximum of 2 hours in controlled water bath at temperatures of 40, 60, 80 and 100ºC **[Figure 3]**.

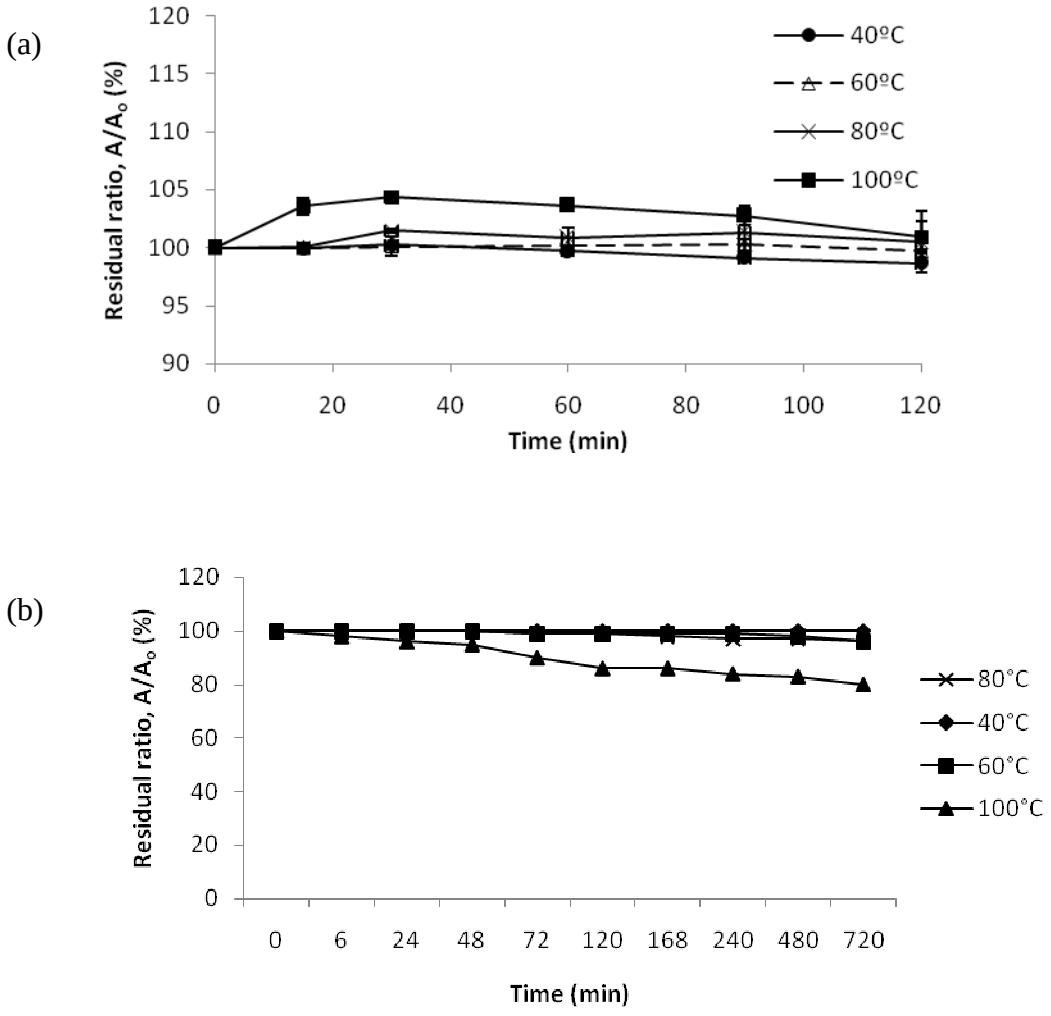


Figure 3: Stability of prodigiosin (a) crude (in ethyl acetate) (b) encapsulated (dissolved in water) at different temperatures

The crude pigment was stable at the temperatures studied as there was little change in the residual percentage (5%) of pigment and colour of the solution. However, the changes in residual percentage of encapsulated pigment at the different temperatures showed a better stability with the increasing of the temperature. More pigments are protected when encapsulated and therefore, intensity of the colour is maintained. The decrease in colour intensity in crude pigment can be attributed to the destruction of pyrrole group presence in prodigiosin structure. The results are in agreement with the work done by Rocha *et al*. (2012) for the application of lycopene pigment as a food colorant. Thus, the encapsulated pigment was stable up to 100ºC and therefore suitable for processing in various applications [28].

3.3. Degradation Rate of Crude Pigment

The rate of pigment degradation upon exposure to light can be calculated by assuming first order kinetic as follows:

$$\ln\left(\frac{R_i}{R_O}\right) = -kt \qquad \text{(Eq.1)}$$

Where R_i is the residual pigment at specific time interval, R_o is residual percentage of pigment at time = 0 and the degradation rate constant (k) can be obtained from the slopes of regression lines by plotting ln (R_i/R_o) as a function of time. The half life ($t_{1/2}$) of the pigment was calculated using the first order Arrhenius equation (Equation 2).

$$t_{1/2} = \frac{0.693}{k} \qquad \text{(Eq.2)}$$

[Table 1] summarized the degradation constant, k and half life, $t_{1/2}$ crude pigment at different pH exposed to light. At pH 11, the rate of crude pigment degradation was very fast. Most of the pigment was degraded after 24hours. Crude prodigiosin was very stable at pH 5 where the pigment content remains unchanged for 60 days. At pH 8 and 11, the pigment was gradually degraded and became constant after a week therefore does not obey first-order kinetics. Crude pigment unexposed to light at pH range (2 – 7) is more stable and remain unchanged.

Condition	pH	k (h^{-1})	R^2	$t_{1/2}$ (h)
With light	2	1.3×10^{-3}	0.9928	533
	7	1.2×10^{-3}	0.9561	578
Without light	8	3×10^{-4}	0.9409	2310
	11	8×10^{-4}	0.9801	866

Table 1: First order kinetic degradation constants of red pigment under various conditions

The effect of pH on prodigiosin has also been reported and discussed previously [32,34]. In an acidic medium the pigment in ethyl acetate is red and exhibits a sharp spectral peak at 535nm and a persistent shoulder at about 510 nm. In an alkaline medium, the pigment is orange-yellow and possesses a broader spectral curve at 470 nm. The spectral properties reported are in accordance with the result obtained in this study **[Figure 4]**.

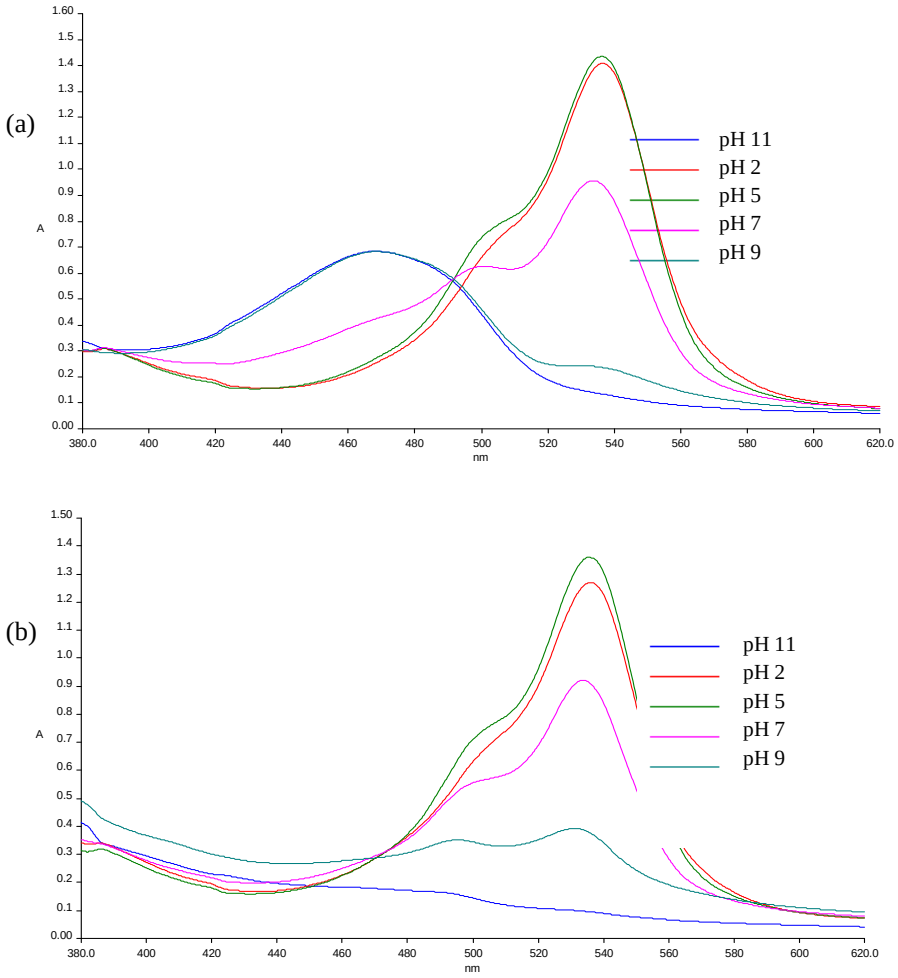


Figure 4: Absorption spectra of crude pigment at different pH; (a) 0 hours and (b) 24 hours

3.4. Degradation Rate of Encapsulated Red Pigment

Assuming a zero order kinetic, the encapsulated prodigiosin degradation rate upon exposure to light, extreme pH and the temperature can be calculated. Based on zero order reaction, the degradation rate is equal to the rate constant and is independent of the reactant concentration (absorbance in this study). Integrated zero-order rate law equation is shown below (Eq. 3):

$$[A]_t = -kt + [A]_0 \quad \text{(Eq. 3)}$$

Where, $[A]_t$ and $[A]_0$ represent the residual absorbance percentage of encapsulated prodigiosin at specified time intervals and the initial residual absorbance percentage respectively. The degradation rate (k) is obtained

from regression line slopes by plotting [A] versus time. The pigment half-life ($t_{1/2}$) was obtained using the zero order reaction half-life equation (Eq. 4):

$$t_{1/2} = [A]_0 / 2k \qquad \text{(Eq. 4)}$$

[Table 2] summarizes the degradation constant and half-life for encapsulated pigment at different conditions.

Condition	pH	*k* (h^{-1})	R^2	$t_{1/2}$ (h)
With light	2	0.31	0.9605	4530
	5	0.27	0.9897	5100
	7	0.184	0.9452	7600
	8	0.28	0.9744	5000
	11	0.17	0.9455	973
Without light	2	0.26	0.960	7100
	5	0.199	0.9545	7370
	7	0.13	0.956	1110
	8	0.24	0.9438	5910
	11	0.144	0.9505	1200

Table 2: Half-life profiles, regression and degradation constant of encapsulated prodigiosin degradation using zero order reaction.

Destructive effects of light, high temperature and pH on the prodigiosin is due to the electrons on the pyrrole group, therefore, decrease the colour intensity [35]. Based on encapsulation principle, which is protection of sensitive substances, prodigiosin in its encapsulated form maintain the stability longer that its crude form [13]. However, the initial colour intensity is lower in encapsulated pigment due to encapsulated agent which is added prior to spar drying process. **[Figure 5]** shows the spectral properties of crude and encapsulated prodigiosin which is in accordance with the result obtained by Song *et al*. (2000) for the crude prodigiosin [34].

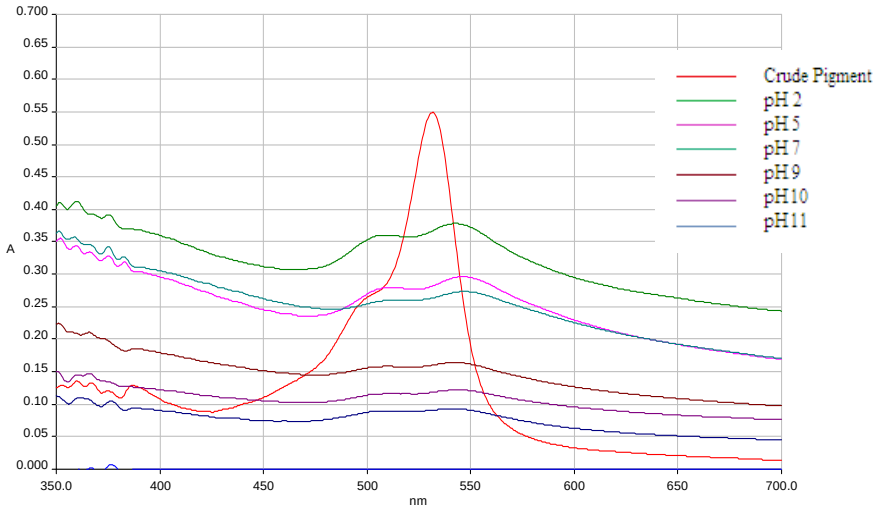


Figure 5: Absorption spectra of encapsulated pigment at different pH

3.5. Solubility of Encapsulated Prodigiosin

The encapsulated prodigiosin is not soluble in organic solvents i.e. acetone, n- hexane, ethyl acetate, methanol but it is soluble (homogenously dispersible) in water, acidic (HCl, 0.1 M) and basic (NaOH, 0.1 M) solutions. Contradictory, crude prodigiosin is insoluble in water **[Table 3]**.

Solvent at25°C	Resound of Crude Pigment	Resound of Encapsulated pigment	Resultant colour of Crude Pigment	Resultant colour of Encapsulated pigment
Water	-	+++	Colourless	Pink
HCl (0.1 M)	-	+++	Colourless	Pink
NaOH (0.1 M)	-	+++	Colourless	Orange
Acetone	+++	-	Pink	Colourless
Methanol	+++	-	Pink-Orange	Colourless
Ethyl acetate	+++	-	Pink	Colourless
n-Hexane	+	-	Pink	Colourless
Chloroform	-	-	Pink	Colourless

+ slightly soluble, +++ highly soluble, − not soluble

Table 3: The solubility of crude and encapsulated prodigiosin in different types of solvent

Increasing the water solubility of the insoluble material is one of the advantages of encapsulation technique by entrapping of hydrophobic materials with hydrophilic [13, 28]. The results show insoluble prodigiosin turns to a soluble (homogenously dispersed) compound after the spray drying process **[Figure 6].**

This is because the coating material (κ-carrageenan) has hydrophilic nature and the hydrophilic groups of κ-carrageenan are more exposed to the solvent than hydrophobic groups of prodigiosin.

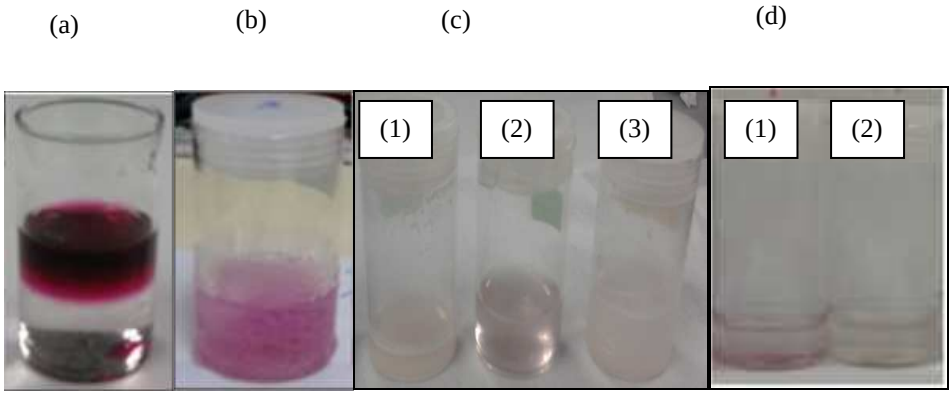


Figure 6: Effect of different solvents on crude and encapsulated prodigiosin (a) crude pigment in water, (b) encapsulated prodigiosin in water, (c) encapsulated prodigiosin in (1) acetone (2) n- hexane (3) ethyl acetate ,(d) encapsulated prodigiosin in (1) HCl (2) NaOH

3.6. Characterization of Encapsulated and Crude Prodigiosin

3.6.1. FTIR Spectroscopy

In this study, FTIR spectroscopy provided information for comparing the spectra of functional groups of prodigiosin before and after of being encapsulated using κ-carrageenan. The FTIR spectrum for the crude prodigiosin showed bands at 2909 cm^{-1}(methylene group), 1565 cm^{-1} (pyrrole group) and 3463.65 cm^{-1} (amide group). Peaks at 3400-3445 cm^{-1} are assigned for aliphatic alcohols, primary amines and amides while absorption band at 1655 cm^{-1} corresponded to (C=C) stretching vibrations. From the spectrum, the main functional groups of crude prodigiosin were pyrrole, methylene, alkane and alkene. The spectrum of pure κ-carrageenan showed absorption bands at 847, 928, 1050, 1263 and 3446 cm^{-1} which attributed to D-galactose-4-sulfate, 3, 6-anhydro-D-galactose, glycoside linkage, ester sulphate and O-H stretching of κ-carrageenan, respectively. The characteristic peak at 1642 cm^{-1} was due to C=O stretching and the peak at 1418 cm^{-1} is responsible for α-CH_2 bending. Peak at 1370 cm^{-1} is assigned for O-H bending and the peaks at 1154 and 1079 cm^{-1} were attributed to C-O stretching. CH and CH_2 bending occurred at 931 cm^{-1}, C-H bending and ring puckering caused a spectra at 856 and 763 cm^{-1}. All FTIR bands corresponded to pure κ-carrageenan was observed in encapsulated prodigiosin spectra, which indicate sufficient coating with corresponding coating material during the spray drying process. The characteristic peaks of crude prodigiosin disappeared in encapsulated spectra due to encapsulation process. A similar observation was previously reported by Krishnaiah *et al.* (2011) for the encapsulation of *Morinda citrifolia* using κ-carrageenan as coating agent.

[IV] CONCLUSION

Crude prodigiosin has less stability upon exposure to light, high pH and temperature compared to encapsulated form. The investigation of difference on crude and encapsulated pigments fading mechanism and degradation influenced by various factors i.e. pH, light, temperature are important to improve its stability and solubility as an alternative to existing synthetic colorant for various applications. Prodigiosin maintain its stability at room temperature and at pH 5 in encapsulated form. By converting the prodigiosin into encapsulated form, some properties such as solubility and stability are improved and therefore, it is suggested that the encapsulated prodigiosin has privilege to be used as a colorant.

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