Encapsulation of Phycocyanin-Alginate for High Stability and Antioxidant Activity

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Abstract. The aim of this study was to obtain optimal condition of phycocyanin-alginate encapsulation, encapsulation efficiency and phycocyanin load, physicochemical properties of beads, in vitro release study, stability and antioxidant activity. The result product with alginate content 1.5% (w/v) and 2% (w/v) produced were in spherical shape than product with alginate content 2.5% (w/v) by ratio of phycocyanin 1:1. Increasing alginate content on encapsulation process will increase of encapsulation efficiency and phycocyanin load. In vitro released study showed that phycocyanin-alginate beads were more resistant in simulated gastric fluid, while rapidly release in simulated intestinal fluid. The antioxidant activity showed that phycocyanin antioxidant activity decreased after encapsulation process due to duration of storage and the possibility of a cracking which will cause reduced stability of phycocyanin.

Keywords: encapsulation, phycocyanin, alginate, extrusion, antioxidant

1. Introduction
Phycocyanin is a blue-light pigment found in cyanobacteria and two eukaryotics algae such as Rhodophyta and Cryptophyta. Phycocyanin is soluble in water and has a strong fluorescent properties as an antioxidant and it is normally used in food, cosmetics, biotechnology and drugs. Phycocyanin is a blue phycobiliprotein composed of two homologous units: α-chain and β-chain attached to the cysteine cluster [1]. Moreover, besides its use of antioxidant, phycocyanin has also properties as an anti-cancer, anti-inflammatory [2], neuroprotection [3], and prevention of hypertension [4].

However application of phycocyanin as active compounds in functional foods is often accompanied by problems of stability caused by elevation of moisture content, light and temperature which cause degradation of phycocyanin [5]. Several researchers showed that microencapsulation is an effective and economical method to maintain the stability of phycocyanin [6]. Coating in encapsulated product will protect the active compound from external influences and also regulates the release of the compound capsules [7].

Alginate is a biodegradable polymer that is mostly used as coating material because it can easily form a gel beads in a solution without the use of organic solvents and at room temperature [4]. The current study is carried out to evaluate the use of alginate as coating material for encapsulation of...
phycocyanin under variation of concentration of alginate, while CaCl$_2$ was used as linking agent. Ca$^{2+}$ was chosen because not toxic and is frequently used as a fitting cross formation with a polymer alginate microspheres. Moreover CaCl$_2$ easily connected with alginate because Ca$^{2+}$ ions bound to guluronic acid residue which is a component of alginate[8].

2. Material and Methods

2.1. Material
Phycocyanin was extracted from microalgae *Spirulina platensis*. Alginate and CaCl$_2$ used in this study were food grade quality obtained from the local market. All other reagents used were analytical grade.

2.2. Encapsulation of phycocyanin – alginate
Alginate solution was prepared with concentration of 1.5% (w/v), 2% (w/v), 2.5% (w/v) referring to previous research of Yan et al[9]. Phycocyanin added to the alginate with the ratio of 1: 1 (w/w) and then stirred using a magnetic stirrer until the solution is homogeneous. The homogenized suspension was inserted into the syringe and injected into a solution of CaCl$_2$ 2.5% (w/v) using a 23G gauge needle and stirred by a magnetic stirrer. The beads were collected and washed using distilled water to remove Cl$^{-}$ ions. The CaCl$_2$ and distilled water were collected to calculate the encapsulation efficiency and beads were stored in the refrigerator.

2.3. Determination of encapsulation efficiency and phycocyanin load
Encapsulation efficiency (EE) was calculated from the mass phycocyanin, which was not encapsulated compared to the mass of phycocyanin added at the beginning of process [10]:

$$EE(\%) = \frac{\text{initial mass of phycocyanin} - \text{mass of uncoated phycocyanin}}{\text{Initial mass of phycocyanin}} \times 100$$ (1)

Phycocyanin load was calculated by dissolving 0.025 gr dried encapsulated products in Na phosphate buffer pH 7.4. After 20 min then it was diluted using 25 mL flask and then allowed to stand for 30 minutes in order to precipitate alginate. The filtrate was separated from the solution and absorbance was measured. The phycocyanin load was calculated using equation 2:

$$\text{Phycocyanin load(\%)} = \frac{\text{mass of phycocyanin in beads}}{\text{mass of coating material in beads}} \times 100$$ (2)

2.4. Characterization of encapsulation
Beads were characterized using SEM instrument to determine the morphology and FT-IR was used to determine the chemical interactions that occur after the encapsulation.

2.5. In vitro release study
Dissolution test was conducted by weighting 253.8 mg beads under concentration variation of 2.5% (equivalent to 116.5125 mg pure phycocyanin). The beads then was put into HCl buffer at pH 1.2 as simulated gastric fluid(SGF) medium after 2 hours and then transferred to Na phosphate buffer pH 7.4 as simulated intestinal fluid(SIF) medium for 6 hours. A 5 mL sample was measured every 60 minutes and was measured for its absorbance by using Uv-Vis spectrophotometer.
2.6. Stability and antioxidant activity assay
Antioxidant test using DPPH method was referred to Molyneux[6]. A 9 mg phycocyanin was weighed for a control and 25 mg of the encapsulated samples (equivalent to 11.5 mg of pure phycocyanin) were then dissolved in Na phosphate buffer pH 7.4 using 25 mL volumetric flask to obtain a concentration of 360 ppm for control and 460 ppm for sample encapsulation. The samples were prepared by varying concentration of 18 ppm, 36 ppm, 54 ppm, 72 ppm and 90 ppm for control and 23 ppm, 46 ppm, 69 ppm, 92 ppm and 115 ppm for encapsulation. A 2 mL of sample was added to 2 mL of 50 μM DPPH (in methanol) and the mixture was shaken and then incubated in the dark for 30 minutes. Blank solution consisted of 2 ml Na phosphate buffer pH 7.4 and 2 mL of DPPH 50 μM. The solution was measured for its absorbance at the maximum λ. The percentage of inhibition was calculated by equation 3:

\[
\% \text{ Inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \quad (3)
\]

Storage stability was performed under temperature variation of 35, 45, 55°C for 3 days. Samples were measured for their antioxidant activities after incubation for 3 days.

3. Result and Discussion
3.1. Preparation of phycocyanin – alginate encapsulation
Figure 1 shows the product of encapsulation with variation of concentration. Most of the products showed the homogenous sizes however, at concentration of 2.5%, the shape of encapsulated products was tailed beads. This is because at concentration of 2.5%, the alginate solution has a high viscosity which affecting the extrusion.

![Figure 1. Morphology of encapsulated phycocyanin at various concentrations. Note: Concentration of (a) 1,5%; (b) 2%; (c) 2,5% and (1) Before dried; (2) After dried](image-url)
At a concentration of 1.5% the beads produced are more lenient than the concentration of 2% and 2.5% where the beads produced are harder. Increase the levels of alginate used in the producing beads increased the numbers of polymer chains that bind to the cross-linking material [11]. An increase amount of polymer chains bound to the coating material lead to harder beads produced at a concentration of 2% and 2.5% than the concentration of 1.5%. The drying process have shrunk the bead sizes due to loss of water evaporation [9]. The diameter of beads at a concentration of 1.5% is 0.756 ± 0.0532 mm, 2% concentration is 0.872 ± 0.0622 mm and concentration of 2.5% is 1.032 ± 0.0795 mm.

In this study the ionic cross-linking agent used is Ca$^{2+}$ derived from CaCl$_2$. Ca$^{2+}$ has been selected because it is not toxic and is frequently used as cross-linking of microsphere formation with Na alginate polymer [12]. Alginate can form a formation of egg-box by using ionic cross-linking agents, such as divalent cations (i.e., Ca$^{2+}$). The divalent cation binding group or guluronic blocks of alginate chains, as the structure of guluronic blocks that enable high-level coordination from the divalent ions. Guluronic blocks of one polymer then forms a bond with guluronic blocks of other adjacent polymer forming egg-box model of cross-linking, producing a gel structure [12].

3.2. Encapsulation efficiency and phycocyanin load

The encapsulation efficiency was calculated from the free phycocyanin and from the mass of initial phycocyanin added to encapsulation process [10]. The result of encapsulation efficiency and phycocyanin load presented in Table 1.

| Alginate Concentration | Encapsulation Efficiency (%) | Phycocyanin Load (%) |
|------------------------|------------------------------|----------------------|
| 1.5%                   | 53.533 ± 0.6132              | 38.7856 ± 0.6589     |
| 2%                     | 65.7334 ± 2.7106             | 42.3856 ± 1.7379     |
| 2.5%                   | 71.7599 ± 0.1319             | 45.9095 ± 1.1788     |

The results of encapsulation efficiency and phycocyanin load increase by increasing concentrations of alginate. The results obtained are in agreement with Yan et al., [9] where both the encapsulation efficiency and phycocyanin load increase with increased concentrations of coating material. However, the research conducted by Yan et al., [9] resulted lower encapsulation efficiency (53.84 ± 0.81%) than our experiments (71.7599 ± 0.1319%).

Increased levels of alginate used in the production of microspheres causing the increase number of cross-linking material bonded to the polymer chains that lead to increasing the amount of trapped protein. CaCl$_2$ concentration did not have a significant effect on the encapsulation efficiency and the phycocyanin load due to phycocyanin has a high molecular weight such that it cannot emanated from alginate gel [13].

3.3. Beads characterization using FT-IR

FTIR was used to detect of new interactions marked by the advent of a new wave number shift (Figure 2). Peak which appears at wave numbers of 3400.04 cm$^{-1}$ (1.5%), 3401.09 cm$^{-1}$ (2%) and 3421.41 cm$^{-1}$ (2.5%) caused by stretching O-H group, while wave numbers of 1624.13 cm$^{-1}$ (1.5%), 1616.33 cm$^{-1}$ (2%) and 1630.34 cm$^{-1}$ (2.5%) caused by stretching -COO group, peak which appears at wave numbers 1424.69 cm$^{-1}$ (1.5%), 1419.84 cm$^{-1}$ (2%) and 1412.58 cm$^{-1}$ (2.5%) caused by bending C-H group. In addition, peak appears at wave numbers of 1032.25 cm$^{-1}$ (1.5%), 1032.5 cm$^{-1}$ (2%) and 1033 cm$^{-1}$ (2.5%) caused by C-O alkoxyl group and peak at wave numbers 671.04 cm$^{-1}$ (1.5%), 689.96 cm$^{-1}$ (2%) and 687.9 cm$^{-1}$ (2.5%) caused by bending C-H groups [14].

The spectra of FT-IR of phycocyanin encapsulated with alginate showed that the spectra of phycocyanin-alginate have similarity to the spectra of alginate, some peaks were not shifted in wave
numbers significantly indicating only occurs electrostatic interaction between phycocyanin with alginate in the beads [9].

![Figure 2. FT-IR analysis of phycocyanin and coating materials](image)

3.4. Beads characterization using scanning electron microscopy
Characterization using scanning electron microscopy was aimed to determine the morphology of the beads resulting from the encapsulation process. The results of morphological analysis encapsulation phycocyanin-alginate at a concentration of 2% and 2.5% shown in Figure 3.

![Figure 3. SEM images of phycocyanin–alginate beads (a) concentration of 2%, (b) concentration of 2.5%](image)

Figure 3 shows that the morphology of beads at concentration of 2% was more spherical than beads concentration of 2.5% which have a "tail" (tailings) due to greater viscosity alginate [14]. The surface morphology of phycocyanin-alginate beads was not smooth due process of rapid evaporation of water at cold temperatures [14]. The particles tend to agglomerate which is used by a particular space of the polymer and the electrostatic force [14].
3.5. *In vitro release study*

One objective of the phycocyanin encapsulation is to protect the phycocyanin from acidic gastric fluid. Phycocyanin was stable at pH 5 – 7.5 at room temperature (25 ± 2°C) but easily decolorized at pH above or below 5 – 7.5[15]. Phycocyanin release test results shown in Figure 4.

![Figure 4. In vitro release study](image)

After 2 hr of incubation, there was no release observed in the simulated gastric fluid medium but it released rapidly in the simulated intestinal fluid medium. The test of phycocyanin release shows that the alginate can hold phycocyanin release rate in simulated gastric fluid medium (acidic condition) and expected to avoid phycocyanin damage at pH below and above 5 - 7.5[15]. At pH 1.2, swelling of alginate beads was longer due to the strong hydrogen bonding that occurs between -COOH and -OH of the polymer chain [16] and increasing the attraction between the amine group on phycocyanin protonated and the carboxyl group of the alginate[17]. At pH 7.4, swelling of alginate beads more rapidly due to the counter ion is neutralized by the ionized carboxyl group at phycocyanin-alginate beads and the electrostatic repulsion between the carboxylate groups are ionized[18]. This can be explained by the presence of ion exchange between Ca$^{2+}$ ion in the hydrogel and Na$^+$ ions in phosphate buffer[19].

3.6. *Antioxidant activity assay*

The principle of antioxidant activity assay is by measuring the antioxidant activity quantitatively through the DPPH radical arrest by a compound as free radical activity value reduction (IC$_{50}$ value). IC$_{50}$ (Inhibitory Concentration) is defined as the concentration of test compounds that can reduce free radicals by 50%. The smaller IC$_{50}$ value, activity of reduction of free radicals are higher. Antioxidant activity assay is presented in Table 2.

| Sample                  | IC$_{50}$(ppm)          |
|-------------------------|-------------------------|
| Control                 | 197,6738 ± 9,3296       |
| Concentration of 2.5%   | 280,2862 ± 0,7481       |
| Concentration of 2%     | 382,1489 ± 132,2927     |

IC$_{50}$ results in this study was smaller than the previous study conducted by Thangam et al.[2], however they used different source of phycocyanin. The results of IC$_{50}$ in the level of 200 ppm was due to the possibility of Na phosphate buffer that has a high polarity causing interference on the antioxidant test [3]. The encapsulated phycocyanin showed greater antioxidant activity than the controls. This is
possibly due to the storage time of the making encapsulation to test the antioxidant activity long enough to affect the value of IC$_{50}$.

3.7. Antioxidant stability assay

Antioxidant stability test performed by incubating the dried beads at a temperature of 35°C, 45°C and 55°C for three days. The antioxidant activity was calculated on the third day by using DPPH assay. The results of stability testing of antioxidant show in Figure 5.

![Figure 5. Antioxidant stability (a) Control; (b) Encapsulation at alginate concentration of 2.5%](image)

The results show that encapsulation phycocyanin-alginate has a margin increase of IC$_{50}$ less than the control. This indicates that encapsulated phycocyanin is more stable [20]. The increase of temperature has increased the antioxidant activity [21].

4. Conclusion

The encapsulation at a concentration of 2.5% has highest efficiency, phycocyanin load and antioxidant activity than the other concentrations. FTIR characterization results showed that there was the emergence of a new wave number, but there was a shift wave number are small due to the electrostatic interaction after encapsulation. In the results obtained morphology using SEM surface morphology showed physical cracking after being dried at a temperature of 4°C. From the in vitro release study using encapsulation concentration of 2.5% showed that encapsulation phycocyanin - alginate can hold phycocyanin release rate in simulated gastric fluid medium and release rapidly in the small intestine fluid medium. Encapsulation phycocyanin - alginate is more stable at higher temperatures compared to phycocyanin without encapsulation.

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