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# Stability of Phycobiliproteins Using Natural Preservative ε-Polylysine (ε-PL)

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## Abstract

C-Phycocyanin (PC) and C-Phycoerythrin (PE) are important phycobiliproteins (PBs) with their possible application as colorants in food industries. In the present study, effect of natural preservative, ε-polylysine and chemical preservative, citric acid on the stability of C-PC and C-PE at 4 ± 2°C was studied. Percentage loss of C-PE and C-PC content and effect of pH and fluorescence on C-PC and C-PE was studied. 0.02% ε-polylysine (w/v) was found to be optimum for storage of C-PC and C-PE at 4 ± 2°C and lesser loss of C-PC and C-PE content as compared to citric acid for its storage up to 8 days without any change in colour and pH. The amount of C-PC and C-PE left in the solution containing ε-polylysine was 90.5 and 95.24% respectively.

0.02% ε-polylysine (w/v) was found to be optimum for storage of C-PC and C-PE at 4 ± 2°C and lesser loss of C-PC and C-PE content as compared to citric acid for its storage up to 8 days without any change in colour and pH. The amount of C-PC and C-PE left in the solution containing ε-polylysine was 90.5 and 95.24% respectively. Further, there is a need to replace chemical or synthetic preservatives with natural preservative ε-polylysine as prolonged consumption of these chemical or synthetic preservatives possess health hazard. The present work provides an effective option for replacing these chemical or synthetic preservatives with ε-polylysine as natural preservative.

**Keywords:** C-phycoerythrin; C-phycocyanin; ε-polylysine; Stability; Natural preservative

**Abbreviations:** C-PE: C-Phycoerythrin; C-PC: C-Phycocyanin; APC: AlloC-PC; ε-PL: ε-polylysine

## Introduction

Phycobiliproteins are considered as a vital photosynthetic coloured pigments. Based on the light absorption potential, phycobiliproteins are classified into C-PE (PE; λmax=565-567 nm), C-PC (PC; λmax=615-620 nm) and allophycocyanin (AP; λmax=650-652 nm) which are also category of chromoproteins containing covalently bound linear tetrapyrole chromophores [1,2]. Basically, these phycobiliproteins are made up of light harvesting pigment-protein complex present in various algae (red algae, cryptophytes, glaucophytes and some pyrrophyceae) and cyanobacteria [3]. These compounds are basically an oligomeric protein having linear tetrapyrrole chromophores (bilins) covalently attached to the apoprotein by a thioether linkage [4,5].

Phycobiliproteins generally consists of chromoproteins which have various biomedical applications e.g. as anti-inflammatory agents, antioxidant potential, etc. C-PC are generally used as nutraceutical ingredient as well as natural colorant in food and natural dye in cosmetics, important therapeutic agent for oxidative stress induced diseases, fluorescent biomarker in biomedical research, etc. [6-15]. Phycobiliproteins are water soluble and are generally extracted as protein pigment complexes. However, during its extraction, control of pH and ionic strength are desired for stability of the isolated phycobiliproteins. Although, for the past few decades, researchers have developed efficient processes for these phycobiliproteins, still, recovery are relatively low due to high sensitivity of these compounds to light, oxygen, proteases, temperature and moisture, which needs to be improved while processing it along with efficient preservatives for reducing its degradation age [16].

Sodium azide ($NaN_3$) and dithiothreitol (DTT) may be used as preservatives in phycobiliproteins for analytical purpose, however, they are toxic; therefore, it needs to be replaced with food grade preservatives for edible purposes preservatives with desired properties (without affecting stability of the compound) [17-19]. Stability of purified C-PC and C-PE can also be improved by addition of edible preservatives such as benzoic acid, citric acid, sucrose, ascorbic acid, and calcium chloride up to few days both at ambient temperatures (25°C; 40°C) as well as at refrigeration conditions [20]. Addition of sucrose, citric acid and sodium chloride also effectively maintains the thermostability of phycobiliproteins [20]. However, to some extent chemical preservative possess side-effects to human health. Benzoates may cause allergies and can cause damage to human nervous system.

Chemically prepared citric acid uses sulfuric acid which possesses some side effects to human health such as asthmatic attacks and allergic reactions. Citric acid can also cause damage to dental cells, necrosis in hepatocytes and may lead to chromosomal aberrations [21-24]. Natural food preservatives are, in fact, far safer to use than the harmful chemical food preservatives to avoid all kinds of side effects of chemical preservatives.

The aim of this study was to replace food grade chemical preservative to natural preservative ε-polylysine which can be used to store C-PC and PC-PE at 4°C with minimum degradation.

### Practical application

Phycobiliproteins are considered to have antimicrobial, antioxidant, anti-ageing, anti-inflammatory, neuroprotective and hepatoprotective properties. Phycobiliproteins are extensively used as natural colorants in food and cosmetics, fluorescent neoglycoproteins, probes for single particle fluorescence and imaging fluorescent applications in clinical and immunological analysis. Preservation of such high value and sensitive product with chemicals possess certain side-effects to human being. Benzoates may cause allergies and damage to human nervous system. Chemically prepared citric acid uses sulfuric acid which has side effects to human, such as asthmatic attacks with allergic reactions. Citric acid causes damage to dental cells, necrosis in hepatocytes and leading to chromosomal aberrations. Natural food preservatives e.g. ε-polylysine are safer than the synthetic food preservatives. In the present study, successful efforts have been made to replace food grade chemical preservative with natural preservative ε-polylysine which can be used to store aqueous C-PC and C-PE at 4°C with minimum degradation (10 days).

## Materials and Methods

### Materials

ε-polylysine was procured from Handary S.A. and Citric acid of analytical grade from commercial sources. Phycobiliproteins used in the present study was extracted from CSIR-Central Salt and Marine Chemicals Research Institute (CSIR-CSMCRI's) developed protocol [16].

### Organism and culture condition

Axenic cultures of *Desertifilum* sp. (CCNM 2005) and *Lyngbya* (CCNM 2053) strains, were obtained from CSMCRI's micro algal culture repository. Stock culture of CCNM 2005 was maintained in Zarrouk's medium (g l–1): NaNO3, 2.50; $K_2HPO_4$, 0.50; $NaHCO_3$, 16.8; NaCl, 1.00; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.04; $FeSO_4{\cdot}7H_2O$, 0.01; $K_2SO_4$, 1; EDTA, 0.08;A-5 and B-6 ) and of *Lyngbya* (CCNM 2053) was maintained in ASN III (g l-1): NaCl, 25.0; $MgSO_4$, 3.5; $MgCl_2{\cdot}6H_2O$, 2.0; $NaNO_3$,0.75; $K_2HPO_4{\cdot}3H_2O$, 0.75; $CaCl_2{\cdot}2H_2O$, 0.5; KCl 0.5; $NaCO_3$, 0.02; Citric acid, 3.0 mg; Ferric ammonium citrate, 3.0 mg; Mg EDTA,0.5 mg; Vitamin B12, 10.0 µg; A-5 trace minerals, 1.0 ml). The experimental conditions used were, the temperature of 25°C, photon flask density of 14 $\mu molm^{-2}s^{-1}$, and a 12/12 h of light:dark photoperiod cycle.

### Extraction and purification of phycobiliproteins

The cell biomass was harvested by centrifugation (10,000 g, 12 min, and 15°C), and Phycobiliproteins were extracted by using Phosphate buffer (pH-7) according to our earlier report [1]. Here C-PC and C-PE were extracted from CCNM 2005 and CCNM 2053 respectively. All the experiments were performed in triplicate.

The biomass was harvested using centrifugation (10000 × g, 15°C, 12 min), washed with distilled water and Phycobiliproteins were extracted by using Phosphate buffer according to our earlier report [1]. Here C-PC and C-PE were extracted from CCNM 2005 and CCNM 2053 respectively. 0.5 g of the wet biomass was suspended in 50 mM potassium phosphate buffer (pH 7.2,). The mixture was repeatedly frozen at -70°C followed by rapid thawing at 25°C; this cycle was repeated 3-4 times until the supernatant turned a bright blue (C-PC) and pink (C-PE) colour. The residual biomass was pelleted using centrifugation (10000 × g, 4°C, 12 min) and the supernatant was subjected to 25% followed by 50% ammonium sulphate precipitation. The pellet obtained after 50% precipitation was dialyzed against the extraction buffer overnight with 4 changes. Further stability studies were done using these dialyzed samples.

### Experiment for stability of phycobiliproteins using natural preservative ε-polylysine

The stability of C-PC (0.041 mg/ml) and C-PE (0.042 mg/ml) in aqueous form (0.1M phosphate buffer) was checked by adding ε-polylysine at various concentrations (0.005%, 0.02%, 0.08% and 0.32%), 0.4% citric acid as per optimum concentration [16]. Concentration, Colour change, and change in pH of phycobiliproteins (PBPs) was observed till 12th day of incubation at 40°C.

| Effect of C-PC (mg/ml) | Loss of C-PC (mg/ml) after 8 days | Concentration of C-PC (mg/ml) during initial day | Concentration of C-PC (mg/ml) after 8 days |
|---|---|---|---|
| C- C-PC in citric acid | 0.008 | 0.042 | 0.014 |
| C-PC in 0.02% ε-polylysine | 0.004 | 0.042 | 0.038 |
| C-PC in phosphate buffer | 0.010 | 0.042 | 0.031 |

**Table 1:** Loss of C-PC content (mg/ml) after 8 days in presence of preservative of ε-polylysine as natural preservative, citric acid as synthetic preservative and in phosphate buffer as control.

| Effect of C-PE (mg/ml) | Loss of C-PE (mg/ml) after 8 days | Concentration of C-PE (mg/ml) during initial day | Concentration of C-PE (mg/ml) after 8 days |
|---|---|---|---|
| C-PE in citric acid | 0.010 | 0.042 | 0.032 |
| C-PE in 0.02% polylysine | 0.002 | 0.042 | 0.040 |
| C-PE in phosphate buffer | 0.013 | 0.042 | 0.029 |

**Table 2:** Loss of C-PE (C-PE) content (mg/ml) after 8 days in presence of preservative of ε-polylysine as natural preservative, citric acid as synthetic preservative and in phosphate buffer as control.

### Estimation of phycobiliproteins

The absorbance of preservative containing C-PC and C-PE solution was measured every alternate day for 15 days on a CARY 500 Scan

UV-vis, NIR spectrophotometer at wavelength 250-700 nm for calculating the concentration of C-PC using the following equation [24,25].

C-PC (mg/mL)=[$A_{620}$-0.474($A_{620}$)]/5.34 → (1)

Allo PC (mg $mL^{-1}$)=[$A_{652}$-0.208($A_{620}$)]/5.09 → (2)

C-PE (mg/mL)=[$A_{568}$-2.41(PC)-0.849 (APC)]/9.62 → (3)

### Color determination using a hunter colorimeter

The studies pertaining to colour degradation are important since colour retention during storage of phycobiliproteins is one of the essential parameters of food quality. The colour degradation study of C-PC and C-PE (containing citric acid as chemical preservative, ε-PL as natural preservative and phosphate buffer as control) was performed by analysing the color parameters a*, b*, and L* using a Hunter lab spectrophotometer. To monitor color, samples were analyzed using a LabScan XE Hunter Colorimeter (Hunter LAB, Reston, Virginia) as directed by the manufacturer with a port view and area size set to 1.2 and 0.5 inches, respectively. Samples were analyzed on a weekly basis in the scintillation vials in which they were stored using Universal V3.7.1 software (Hunter LAB, Reston, Virginia). The scintillation vials were cleaned with a Kimwipe and the instrument was calibrated using white and black tiles prior to analysis. Hunter L, a, and b values were determined with results indicated as: L representing whiteness (100) and darkness (0); a indicating redness (positive values) and greenness (negative values); and b describing yellowness (positive values) and blueness (negative values). All analyses were conducted in triplicate. Results were further used to calculate the total color difference (dE) using Equation 4 [26]; Chroma value (C*).

$$\Delta E = \sqrt{(L - L_o)^2 + (a - a_o)^2 + (b - b_o)^2} \rightarrow (4)$$

$$Chroma\ value\ (C^*) = \sqrt{\left(a^*\right)^2 + \left(b^*\right)^2} \rightarrow (5)$$

Where, $L_o$, $a_o$ and $b_o$ are color values from day 0; while $L$, $a$, and $b$ values represented the data collected at various time points.

## Results and Discussion

### Effect of natural preservative ε-polylysine on C-PC

The absorption spectra were recorded with preservative and without preservative at 4°C. It was observed that lesser loss of C-PE content in aqueous solution(in phosphate buffer of pH 7.0) containing 0.02% ε-PL as compared to solution containing citric acid at 4°C as preservative. However, the present study focusses on the effect of preservatives at 4°C as loss of C-PC content is less at 4 ± 2°C than in ambient temperature 35 ± 5°C [16]. The spectrum of C-PC shows maximum λmax at 620 nm [16]. Lesser loss of C-PC was obtained after addition of 0.02% ε-PL at 4°C ranging from 4.8% (after 8 days) to 36.6% (after 12 days). However, addition of 0.4% citric acid lead to more loss of C-PC ranging from 19.2% (after 8days) to 51.22% (after 12 days).

C-PC was found to be more stable in aqueous solution (in phosphate buffer at pH 7.0) with 0.02% (w/v) ε-polylysine as there was only loss of 0.004 mg/ml of C-PC from 0.042 mg/ml C-PC after 8 days at 4°C (Table 1). No discoloration of C-PC was observed after 8 days with 0.02% (w/v) ε-polylysine. Simultaneously, 0.4% citric acid added to C-PC in aqueous solution showed slight decolouration after 8 days with 19.2% loss in C-PC content (Figures 1 and 2). There are reports wherein 4 mg/ml citric acid was found potential for C-PC stability at 35 ± 5°C and 0 ± 5°C in aqueous solution for 45 days [16].

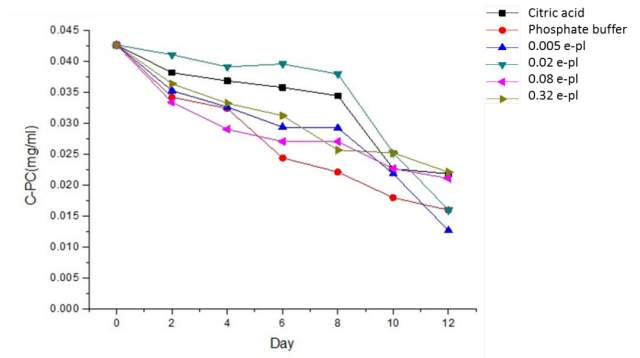


**Figure 1:** Loss of C-PC content till 12 days after addition of citric acid, 0.005% (w/v); ε-polylysine-0.02% (w/v); ε-polylysine-0.08% (w/v); ε-polylysine-0.32% (w/v) and in phosphate buffer.

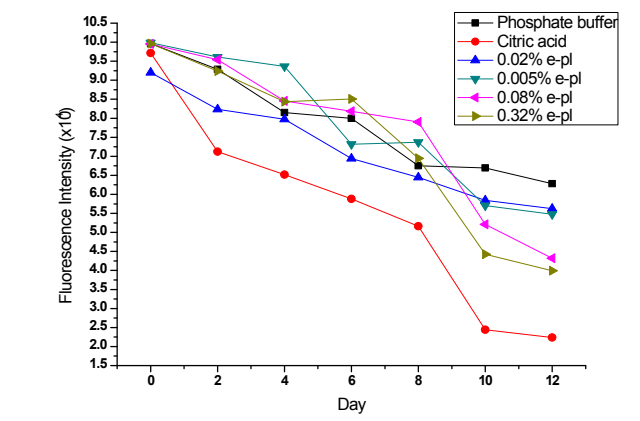


**Figure 2:** Loss of fluorescence intensity in C-PC till 12 days after addition of citric acid, 0.005% (w/v) ε-polylysine, 0.02% (w/v) ε-polylysine, 0.08%(w/v) ε-polylysine, 0.32% (w/v) and in phosphate buffer.

| **C-Phycocyanin containing 0.02% (w/v) ε-polylysine** | | | | | |
|---|---|---|---|---|---|
| Days | L* | a* | b* | ΔE | C* |
| 0 | 27.9 ± 0.08 | 5 ± 0.082 | -8.4 ± 0.11 | 2.52 ± 0.013 | 9.77548 ± 0.1 |
| 8 | 27 ± 0.05 | 3.6 ± 0.08 | -6.5 ± 0.12 | | 7.430343 ± 0.1 |
| **C-Phycoerythrin containing 0.02% (w/v) ε-polylysine** | | | | | |
| Days | L* | a* | b* | ΔE | C* |
| 0 | 30.5 ± 0.013 | 5.6 ± 0.6 | -1.4 ± 0.13 | 5.1 ± 0.007 | 5.772348 ± 0.37 |
| 8 | 26.8 ± 0.014 | 2.1 ± 0.15 | -1.1 ± 0.10 | | 2.370654 ± 0.13 |
| **C-Phycocyanin containing 0.4% citric acid** | | | | | |

| Days | L* | a* | b* | ΔE | C* |
|---|---|---|---|---|---|
| 0 | 28.8 ± 0.11 | 4.2 ± 0.53 | -9.3 ± 0.2 | 6.07 ± 0.115 | 10.20441 ± 0.37 |
| 8 | 28.2 ± 0.15 | 3.5 ± 0.26 | -3.3 ± 0.17 | | 4.810405 ± 0.22 |
| **C-Phycoerythrin containing 0.4% citric acid** | | | | | |
| Days | L* | a* | b* | ΔE | C* |
| 0 | 25.8 ± 0.27 | 1.4 ± 0.8 | -3.3 ± 0.2 | 2.96 ± 0.105 | 3.58469 ± 0.5 |
| 8 | 23.7 ± 0.03 | 2 ± 0.36 | -1.3 ± 0.09 | | 2.385372 ± 0.23 |
| **C-Phycocyanin in phosphate buffer** | | | | | |
| Days | L* | a* | b* | ΔE | C* |
| 0 | 31.6 ± 0.14 | 2.4 ± 0.1 | -6.8 ± 0.15 | 5.52 ± 0.055 | 7.211103 ± 0.08 |
| 8 | 28.7 ± 0.21 | 0.1 ± 0.02 | -2.7 ± 0.04 | | 2.701851 ± 0.03 |
| **C-Phycoerythrin in phosphate buffer** | | | | | |
| Days | L* | a* | b* | ΔE | C* |
| 0 | 30.8 ± 0.02 | 1.4 ± 0.12 | -4.2 ± 0.05 | 3.72 ± 0.015 | 4.427189 ± 0.09 |
| 8 | 29.2 ± 0.12 | 0.8 ± 0.14 | -0.9 ± 0.18 | | 1.204159 ± 0.16 |

**Table 3:** The values of the L*, a*, b*, C* and ΔE for C-PC and C-PE containing preservatives shows the change in colour upto 8 days.

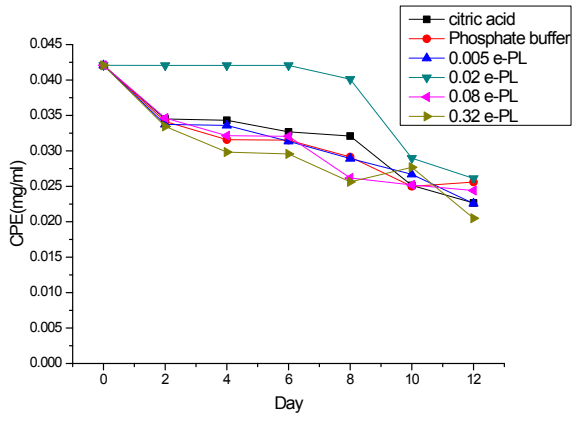


**Figure 3:** Loss of C-PE content till 12 days after addition of citric acid, 0.005% (w/v) ε-polylysine, 0.02% (w/v) ε-polylysine, 0.08% (w/v) ε-polylysine, 0.32% (w/v) and in phosphate buffer.

## Effect of natural preservative ε-polylysine on C-PE

C-PE (C-PE) was found to be more stable in aqueous solution (in phosphate buffer at pH 7.0) with 0.02% (w/v) ε-polylysine as there was only loss of 0.002 mg/ml of C-PE from 0.042 mg/ml C-PC after 8 days at 4°C (Table 2). No discoloration of C-PE was observed after 8 days 0.02% (w/v) ε-polylysine. It showed only 4.8% loss of C-PE after 8 days. C-PE in aqueous solution (without any preservative) showed not decolouration after 8 days. Simultaneously, 0.4% citric acid added to C-PE in aqueous solution showed slight decolouration after 8 days with 23.81% loss in C-PE content (Figure 3). Fluorescence intensity data also shows the similar result which gives the confirmation. In the present case, fluorescence intensity decreases after 8$^{th}$ day (Figure 4).

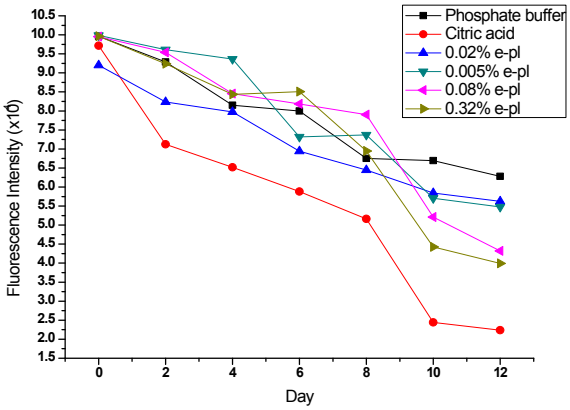


**Figure 4:** Loss of fluorescence content in C-PE till 12 days after addition of citric acid, 0.005% (w/v) ε-polylysine, 0.02% (w/v) ε-polylysine, 0.08%(w/v) ε-polylysine, 0.32% (w/v) and in phosphate buffer.

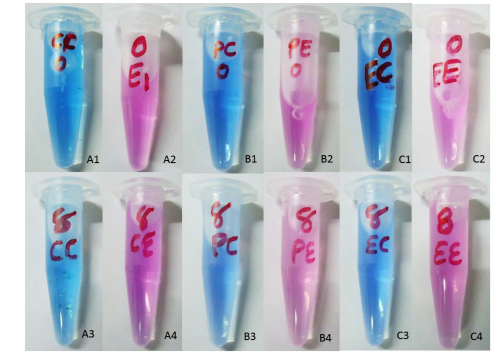


**Figure 5:** Colour change after 8 Day of preservation; (Colour at 0 Day, C-PC with Citric acid-A1, Phosphate buffer-B1 and 0.2% Ɛ-PL-C1 ; C-PE with Citric acid-A2, Phosphate buffer-B2 and 0.2% ε-PL-C2; Colour after 8th day preservation of C-PC with Citric acid-A3, Phosphate buffer- B3 and 0.2% ε-PL-C3; C-PE with Citric acid-A4, Phosphate buffer-B4, and 0.2% Ɛ-PL-C4.).

There was no pH change in phosphate buffer in phycobiliproteins (PBPs). However, PBPs in ε-polylysine and citric acid was stable till 8$^{th}$ day having neutral pH 7.0. Similarly, there was no change in colour of phycobiliproteins till 8$^{th}$ day after addition of 0.02% w/v ε-polylysine (Figure 5). There are reports wherein 4 mg/ml citric acid was found potential for C-PE stability at 35 ± 5°C and 0 ± 5°C in aqueous solution for 45 days. The probable reason for stability of phycobiliproteins is the property for inhibiting the growth of bacteria, yeasts and moulds

bacteria, especially to the Gram +ve bacteria which can't easily inhibit by others which are responsible for increasing or decreasing the pH [17].

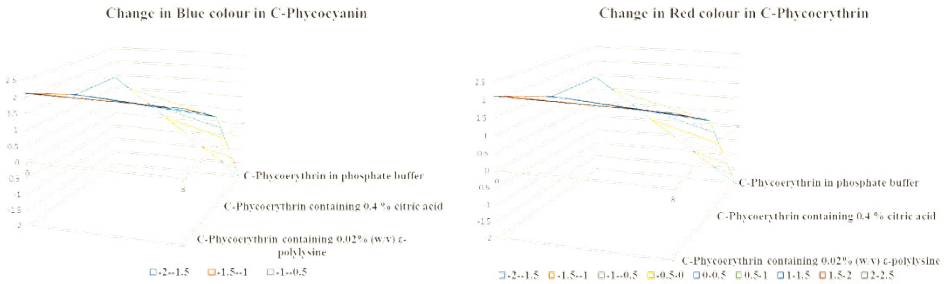


**Figure 6:** Change in colour in phycobiliproteins after addition of natural and synthetic preservatives.

## Color

Table 3 shows the parameters of L* (lightness), a* (redness: green to red), b* (yellowness: blue to yellow), ΔE (total color change, Eq. (4)) and C* (chroma value, color saturation, Eq. (5)) as a function of the effect of natural preservative. The values of the L*, a*, b*, C* and ΔE for C-PC and C-PE containing preservatives shows the change in colour upto 8 days. It can be clearly visible from the table 3 that the values for ΔE increased with increasing the number of days showing the samples are more clearer and more saturated in red and blue and with a more intense colour. However, from table 3 and figure 6 of the L, a*, b*, C* and ΔE score, it was also found that there is negligible degradation of red and blue colour in case of C-PC and C-PE containing 0.02% ε-polylysine as found in negligible decrease from the initial day. On the other hand, with increasing number of days, there was decrease on 8th day of L*, a*, b*, C* and ΔE score in case of C-PC and C-PE in phosphate buffer. However, ΔE score of 5.98 was found in case of C-PC containing 0.02% (w/v) ε-PL and 5.1 ΔE score in case of C-PE containing 0.02% (w/v) ε-PL which was in comparable range with respect to C-PC and C-PE containing citric acid as synthetic preservative. The studies being performed in the present research also lead a way for replacing synthetic preservative such as citric acid with natural preservative ε-polylysine without affecting the colour stability of phycobiliproteins as desired for its application in food products.

## Conclusion

In the present study, efforts have been made for replacing synthetic preservatives with natural preservative ε-polylysine whose 0.02% (w/v) was found to be optimum for storage of C-PC and C-PE at 4 ± 2°C with lesser loss of C-PC and C-PE content as compared to citric acid for its storage up to 8 days. C-PC was found to be more stable in aqueous solution (in phosphate buffer at pH 7.0) with 0.02% (w/v) ε-polylysine as there was only loss of 0.004 mg/ml of C-PC from 0.042 mg/ml C-PC after 8 days at 4°C. Simultaneously, C-PE was found to be more stable in aqueous solution (in phosphate buffer at pH 7.0) with 0.02% (w/v) ε-polylysine as there was only loss of 0.002 mg/ml of C-PE from 0.042 mg/ml C-PC after 8 days at 4°C. There was no change in pH and no discolouration being observed till 8 days after addition of 0.02% (w/v) ε-polylysine in aqueous solution. The probable reason for stability of phycobiliproteins is the property for inhibiting the growth of bacteria, yeasts and moulds bacteria, especially to the Gram+ bacteria which can't easily inhibit by others which are responsible for increasing or decreasing the pH. Till date, food grade chemical preservatives are being used for storage of phycobiliproteins, however, these chemical preservatives such as citric acid have certain side effects to human health if taken in more concentration. Hence, chemical preservatives needs to be replaced with natural preservatives for storage of phycobiliproteins without affecting its concentration.

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