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Isolation of phycoerythrin from *Kappaphycus alvarezii*: a potential natural colourant in ice cream

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Abstract

Phycoerythrin (PE) is a natural protein pigment found in the red alga *Kappaphycus alvarezii* with potential application as a natural colourant in food and cosmetics. Therefore, this study aims to extract PE by ion-exchange chromatography and then encapsulate it with kappa-carrageenan (PE-Kc) and guar-gum (PE-Gg) to measure the stability and functionality in ice cream. Results showed that PE exhibited three distinct band subunits at α-20 kDa, β-21 kDa, and γ-30 kDa in SDS-PAGE with purity index (A_{563}/A_{280}) of 2.32 and is a dark pink colour. The encapsulation efficiency (EE) and of PE-Kc was 82.56% PE load 56.78%, and PE-Gg EE and PE load were 79.47% and 51.24%, respectively. Hygroscopicity index of PE-Kc and PE-Gg was between 86.94 and 90.14%, and the particle size ranged from 10 to 80 μm. Ice cream with added microencapsulated PE showed better rheology, and the intensity of pink colour increases during 90 days of storage. PE value-added ice cream showed better scavenging action against DPPH and FRAP for PE 30% and 47%, PE-Kc 20%. and 45% and PE-Gg 18% and 56%. Therefore, this study showed the techno-economic-viability of promising pigment from *K. alvarezii* and their potential food application.

Keywords Encapsulation · Phycoerythrin · Ice cream · Rheology · Value-added products · Antioxidant

Introduction

Phycobiliproteins (PBP) are a pigment-protein complex divided into three classes of pigments such as phycocyanin (PC), allophycocyanins (APC), and phycoerythrin (PE). These proteins are found in cyanobacteria, red algae, and cryptomonads. Under physiological pH, B-PE displays pink colour. While modifying the effects of pH on PE, the colour variants could be used in cosmetic products and food (Rodriguez et al. 2016). Besides, PBPs is highly fluorescent and used as in immunolabelling for antibodies in biomedical applications (Glazer 1994; Ping et al. 2001). PBPs have been reported to have effective anti-cancer and anti-inflammatory properties (Kannaujiya and Sinha 2016). However, intrinsic and extrinsic factors such as light, temperature, pH, and protein fixation are likely to limit their application in food (Manirafasha et al. 2016). Another limitation is sensitivity against heat treatment, which results in denaturation of PE protein molecules resulting in fading of colour.

To overcome these difficulties, several techniques such as the addition of food stabilizers (Wu et al. 2016), protein structural transformation, and microencapsulation (Delia et al. 2019) have been studied to prevent protein degradation. Amongst others, benzoic acid, ascorbic acid, citric acid, $NaCl_2$, $CaCl_2$, NaN_3, dithiothreitol, and sucrose have been used to protect PBP denaturation (Mishra et al. 2010; Kannaujiya and Sinha 2016). However, some chemical preservatives might be harmful and inadmissible in some food application. Therefore, encapsulation would be a useful technique for thermally unstable compounds. It has been reported that microencapsulated phycocyanin from *Spirulina* species shows adequate stability compared with uncoated phycocyanin (Hadiyanto et al. 2019).

The process of microencapsulation is to protect the core material from unfavourable conditions to upgrade the stability and controlled the release of active compounds into the target site. There are various methods of

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encapsulation used in the food industries; amongst drying through freezing and spraying is a widely used method (Ezhilarasi et al. 2013). Freeze drying includes solidifying the material through sublimation, which converts frozen water from the solid phase to the gaseous stage by decreasing environmental pressure. This process works at a lower temperature (− 30 to − 40 °C) for an extended period (12 to 24 h), which could influence the structural arrangement of the wall and core material during freeze-drying. Moreover, the efficiency of encapsulation solely depends on the composition of wall material. Commonly used wall materials are whey protein, chitosan, carrageenan, and maltodextrin (Hadiyanto et al. 2019). However, a combination of biopolymers like proteins and carbohydrates (Young et al. 1993) has been found useful encapsulating material with emulsifying properties as well. Therefore, in this study carrageenan and guar gum were selected as a wall material to protect the PE-protein pigment against degradation.

There is increased focus on natural food colourants in recent times as synthetic colourants may cause undesirable health problems. Although many studies have applied PE for biomedical, therapeutic (Martínez et al. 2019), and functional application (Wang et al. 2019), only a few studies have used PE directly in food application (Sudhakar et al. 2015). Therefore, ice cream was selected for the incorporation of PE. As ice cream is considered as one of the frozen food that can preserve the pigment and produce vivid colour to the final product in a steady-state, as well as maintain the stability of the PE pigment in a frozen medium is an added advantage.

Therefore, the primary objective of this work was to extract R-phycoerythrin pigment from *K. alvarezii* and examine its stability under the encapsulation matrix. Secondly, the R-PE pigment was used in the ice cream as a natural food colourant to study colour intensity during storage. Hence, the outcome of this study would enhance the nutritional, rheological, and organoleptic properties of ice cream using PE from red algae.

Materials and methods

Materials

Chemicals like food-grade $(NH_4)_2SO_4$, sodium–phosphate buffer, were from LOBA Chemicals (Mumbai, India). Emulsifier and stabilizer were from Danisco Pvt Ltd. (Haryana, India). The full cream milk, MSNF, and butter were procured from Hatsun agro products (India) and sucrose from Nice Chemicals Pvt. Ltd., (India). Food-grade guar gum (E412) was from Sarda Gums & Chemicals (India); semi-refined carrageenans and kappa-carrageenan (E407a) were used from a stock of Aquagri Processing Private Limited, Manamadurai, India.

Extraction of phycoerythrin

The extraction of phycoerythrin (PE) followed the procedure of Galland-Irmouli et al. (2000). *Kappaphycus alvarezii* was collected from the coastal region of Mandapam, Rameswaram, India and then transferred to the laboratory. The fresh seaweed was crushed to a slurry and was incubated with sodium–phosphate buffer at 6.8 pH (0.02 M) overnight to extract the phycoerythrin (red colour solution). This was filtered and centrifuged at 2800×*g* for 30 min at 4 °C, and the supernatant containing the phycoerythrin was collected. The supernatant was mixed with $(NH_4)_2SO_4$ at 25% and 45% (w/v) to fractionate. This mixture was allowed to stand for 4 h and then centrifuged at 11200×*g* for 15 min at 4 °C. The red supernatant was acquired and $(NH_4)_2SO_4$ was added to get 45% saturation. After centrifugation at a similar speed then the mixture was collected and dissolved in 20 mM phosphate buffer (pH 7.0).

Ion-exchange chromatography

The PE-rich solution obtained from the previous step further purified in an ion-exchange fast flow column (1.6 cm × 10 cm—DEAE-Sepharose) equilibrated with phosphate buffer (20 mM) containing of NaCl (0.05 M). After washing with 30 mL of the same buffer, the column was eluted with 20 mM phosphate buffer containing 0.05 M NaCl with a gradient (pH 5.6–4.0, 2× 50 mL) at 1 mL min^{-1}. The elute measured at the absorbance of 280 nm and collected in 2 mL fractions. Then elute was taken into dialyzing tube and kept overnight in running tap water to remove salts and then blended with selected hydrocolloids and lyophilized to obtain encapsulated PE.

Determination of purification and SDS-PAGE electrophoresis

The purified PE at various concentrations of 10, 25, and 50 μg was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Liu et al. 2005). The samples (PE) were mixed with an identical amount of Tris-HCl buffer (20 mM, pH 6.8) containing SDS (0.2%) and glycerol (20%). This mixture was heated to 70 °C for 15 min and 30 μL was poured to mini-slab gel well comprised of 12% and 5% polyacrylamide in separation and upper gels. The gels were recoloured with CBB R250. The MW of purified PE was evaluated under UV-visible range between 420 and 600 nm using UV-Vis double beam spectrometer (Model 2377, Electronics India). All spectra were recorded at 25 °C in room temperature. The purity of obtained PE was evaluated using the following ratio: A_{563}/A_{280} nm.

Encapsulation of phycoerythrin

The PE was encapsulated using the food hydrocolloids kappa-carrageenan (Kc) and guar gum (Gg). About 20 g of selected encapsulation material was mixed with 60 g of PE extract (containing 23.6 g of solid-PE) blended with water (53.3 g) to achieve 30% total concentration. The final wall to core ratio would be 1:1. Later, these two samples were continuously mixed on a magnetic stirrer (Q 19A, Remi 2- MLH, India) for 30 min until solid materials were dissolved (1% PE solution). Then, the dispersions were freeze-dried to obtain the encapsulated PE powder. The microencapsulated PE powder was collected and stored in the desiccator for further analysis.

Encapsulation efficiency (%)

Encapsulation efficiency (EE) and PE load are critical indices, which used to depict the qualities of microencapsulation (McNamee et al. 2001). The EE was determined based on the PE mass before and after encapsulation.

$$\mathrm{EE}\ (\%) = \frac{\mathrm{PE_{total}} - \mathrm{PE_{unencapsulated}}}{\mathrm{PE_{total}}} \times 100 \quad (1)$$

where $\mathrm{PE_{total}}$ represents phycoerythrin added in the solution and $\mathrm{PE_{unencapsulated}}$ represents phycoerythrin powder alone.

PE load (%)

The PE load (%) within the encapsulated matrix was evaluated as per Hadiyanto et al. (2019) with few modifications. In brief, encapsulated PE (25 mg) was dissolved in 25 mL of sodium buffer at pH of 7.4 for 30 min. This mixture was permitted to stand for another 30 min to precipitate kappa-carrageenan and guar gum. This solution was filtered using a membrane filter with a molecular cutoff of 100 kDa and the collected mixture was dried to know the weight, and PE load was measured in using the following equation:

$$\mathrm{PE}\ (\%) = \frac{\mathrm{PE_{encapsulated}}}{\mathrm{PE_{feed\ liquid}}} \times 100 \quad (2)$$

where $\mathrm{PE_{encapsulated}}$ represents the weight of PE present in supernatant from the weight of dried encapsulated PE minus by total weight of Kc and Gg in the filtrate and $\mathrm{PE_{feed\ liquid}}$ is the weight of dissolved dried PE microcapsule, which the value was 25 mg.

Powder morphology

The size and morphology of the purified PE and encapsulated PE powder were evaluated by scanning electron microscopy (S-3400 N, Hitachi, Japan) at an acceleration of 15 kV. The surface area of the powder was covered with a thin layer of gold-palladium (Au–Pd) using a sputter-coater.

Hygroscopicity and water solubility

PE and microencapsulated PE were measured for its hygroscopicity as per procedure of Pieczykolan and Kurek (2019). About 1 g of the test sample (PE, PE-Kc, PE-Gg powder) was kept inside the aluminium case consisting of 75% of NaCl in saturated form. This sample mixture was placed in the desiccator at 25 °C for 48 h to maintain equilibrium. Then the obtained values were measured using the following equation:

$$\mathrm{HG} = \frac{\frac{\Delta \mathrm{m}}{M + Mi}}{1 + \Delta \mathrm{m}/_{M}} \quad (3)$$

where Δm (g) is increment in weight of sample powder after incubation, M (g) is the initial weight of sample powder, and Mi (g) is the weight of free water in sample powder before it reaches to moist air. The values obtained from the above equation were expressed in g $(100\ \mathrm{g})^{-1}$.

To determine the water dissolvability of the encapsulated material was evaluated as follows. About 1 g of test powder was dissolved in 100 mL distilled water and then placed on a magnetic stirrer for 5 min, followed by centrifugation at 3000×g for 15 min, and 25 mL of the supernatant was transferred to 50 mL beaker and then dried in a hot air oven at 105 °C for 12 h. The values obtained were calculated using the following equation:

$$\mathrm{Solubility}\ (\%) = {}^{\mathrm{Pa-Pb}}\!/_{0.25} \times 100 \quad (4)$$

where Pa (g) is the sample weight in the beaker after drying and Pb (g) is the initial mass of beaker without sample.

Incorporation of microencapsulated PE into ice cream

The production of ice cream was followed as per Ghandehari Yazdi et al. (2020) with some modifications. In the first step, milk (50%), milk cream with fat (25%), sucrose (5%) and milk solid-non-fat (5%) were mixed continuously and heated to 45 °C. In the second step, stabilizer (0.1%), emulsifier (0.08%) and sucrose (10%) were added; agitated (Remi motor-RQ 122, Remi Elektrotechnik Ltd., India) and heated at 60 °C, followed by homogenisation in two stages performed at 13,789 kPa for 5 min and 3447 kPa for 10 min. This mixture was pasteurized in a water bath for 20 min at 85 °C and then immediately cooled at 4 °C and stored for 12 h ageing. At this stage, 0.1% of purified PE, microencapsulated PE-Kc and PE-Gg powder and strawberry flavour were added in the mixture. The entire mix was homogenized for 15 min and stored at − 18 °C for 24 h and then transferred to laboratory soft-serve ice cream making machine (Techmate, Gelto,

India) to freeze at − 5 °C. Freshly prepared ice cream samples with PE were evaluated for sensory attributes and rheological parameters. For all the analyses, ice cream without PE, no colourant, and strawberry flavour served as a control.

Quality evaluation of ice cream

Physicochemical analysis

The total protein content was measured by the Kjeldahl method using $N = 6.25$ factor. The changes in the total solids, acidity, overrun, and fat destabilization of the developed ice cream was estimated as per previous methods (Ilansuriyan and Shanmugam 2018; Singo and Beswa 2019). For viscosity 100 g of ice cream samples were directly placed in the viscometer (DV-II + pro-Brookfield, USA). The hardness of the ice creams was evaluated using a texture analyser (CT3, Brookfield, USA). To assess the melting rate, 25 g of ice cream placed in the SS wire mesh along with the beaker, this setup was kept in a controlled chamber (25 °C) until completely melts. Overrun of the ice cream was measured as per the following equation (Hart and Fisher 2012):

$$\text{overrun} = \frac{\text{Weight of unit ice cream mix} - \text{weight of equal volume of ice cream}}{\text{weight of equal volume of ice cream}} \times 100 \quad (5)$$

Rheological examination

The rheological characteristics of aged ice cream were assessed using a rotational viscometer. About 100 mL of samples were allowed to equilibrate at 5 °C for 200 s^{-1} to study shear stress using the Herschel-Bulkley (H-B) model. The apparent viscosity of ice cream was calculated at a shear rate of 50 s^{-1}, which consider as an effective oral shear rate for low viscous liquid. Shear rate was increased from 14.4 to 600 s^{-1} using the following equation (Javidi et al. 2016):

$$\mathrm{r} = \mathrm{r_o} + \mathrm{k}\ddot{Y}^{n} \quad (6)$$

where r_o = (H-B) yield stress (Pa), $\ddot{Y}$ = shear rate (s^{-1}), κ = consistency coefficient (Pa s^n), and n = flow behaviour index.

Sensory analysis

The PE incorporated ice cream was studied for sensory parameters using a 9-point hedonic rating scale (Stone et al. 2012). Twenty-five panellists belong to the age group of 20–45 years both male and female were chosen. The five attributes such as colour, taste, texture, and flavour of PE, PE-Kc, and PE-Gg against control ice cream without PE were served to the panels. About 25 g of ice cream samples were given to each panellist with water to rinse the palate before and after performing each group of ice cream. All samples were blind coded and served in the cold. The scores are recorded based on the following rating as 9 = Like remarkably, 8 = Like very much, 7 = Like moderately, 6 = Like slightly, 5 = Neither like nor a dislike, 4 = Dislike somewhat, 3 = Dislike moderately, 2 = Dislike very much, and 1 = Dislike immensely.

Instrumental colour analysis

PE and microencapsulated PE ice cream images were taken using a digital camera (Olympus OM-D E-M10, Japan), and the lighting system was adopted from the previous procedure (Mendoza et al. 2006) with few modifications. All tested samples were illuminated using two parallel fluorescent lamps with a distance of 35 cm facing above the samples at an angle of 45°. Raw images were taken without zoom or flash in JPEG format. The images were pre-processed for segmentation using ImageJ (NIH software, USA), which collected the binary values of RGB colour space for every ice cream samples. All the samples were defined for region of interest (ROI) ranging between 2200 and 3500 mm^2 area measured. The RGB values were obtained from the grayscale images, which were further analysed for $L^*a^*b^*$ values, where L^* ranges from 0 (totally black) to 100 (totally white), a^* axis represents red to green ranging from − 120 to + 120°, and b^* axis displays blue to yellow ranges between − 120 and + 120°.

Antioxidant activities of ice cream samples

In vitro antioxidant activities of PE incorporated ice cream was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging activity (Brand-Williams et al. 1995) and ferric ion reducing ability of plasma (FRAP) assay (Benzie and Strain 1996) using BHA as a standard.

Statistical analysis

All the data obtained in this study are represented as mean values ± standard deviation. Data analysis was performed using one-way ANOVA using Tukey's test, and $p < 0.05$ was considered a significant difference. The colour analysis was tested for the variation in a colour gradient using CIELAB in MATLAB; region of interest (ROI) was adopted from ImageJ. Interpretation of data was performed using MATLAB (version R2017a, Mathworks, USA).

Results

Extraction and purification of PBPs

Different concentration of the sample (10, 25, and 50 μg) was run on PAGE along with the standard MW protein markers.

The tested compound exhibited three distinct bands as α, β, and γ subunits (α-20 kDa, β-21 kDa, and γ-30 kDa) (refer Fig. 1). The alpha (α) and beta (β) bands slightly merged and showed 20 kDa, and gamma (γ) band was observed against 30 kDa protein markers (see Fig. 1). Further, the isolated PE was confirmed by the absorption spectrum ranges at 439, 538, and 565 nm, which shows the presence of PC and PE (Fig. 2). The purified compound was exposed to UV light; it showed a rich fluorescent activity, which confirms the presence of phycoerythrin in pure form in pure form with purity index 2.32 (A_{563}/A_{280} nm).

Stability of encapsulated PE

Encapsulation efficiency (EE)

The recovery percent of PE and load capacities of encapsulated PE-Kc and PE-Gg are presented in Table 1. The EE and PE of carrageenan encapsulate were 82.56% and 56.78%, respectively, whereas guar gum EE and PE load were 79.47% and 51.24%, respectively. The EE (%) was found to be more in the guar gum wall matrix compared with carrageenan on PE.

Hygroscopicity and water solubility

The utilization of carrageenan as a wall material had decreased hygroscopicity as compared with its counterpart (Table 1). Besides, 86.94% and 90.14% of PE-Kc and PE-Gg dissolved in water. This high degree of dissolvability was due to thermal degradation of core material at 102 °C occurred during the dry heating process.

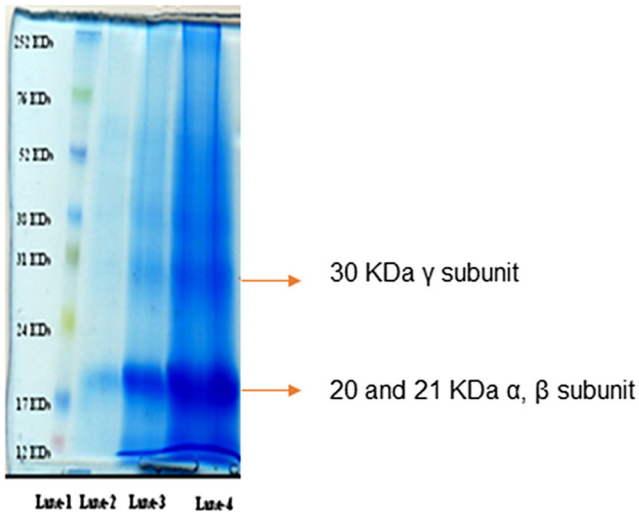

Fig. 1 SDS-PAGE of protein bands absorbed at 30, 20, and 21 kDa representing α and β subunits

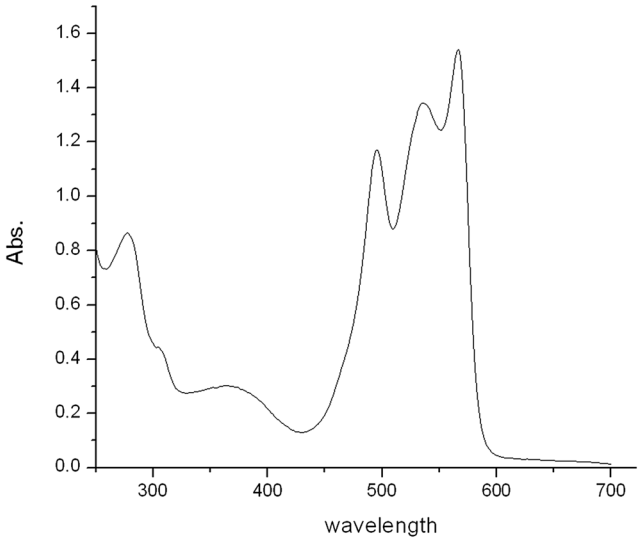

Fig. 2 UV-Vis spectra of R-Phycoerythrin at 498, 539, and 565 nm absorption range

Morphological changes of encapsulated PE

The structural morphology of PE and microencapsulated PE (PE-Kc and PE-Gg) were examined by scanning electron microscopy (SEM; supplementary figure 1a–1c). The utilization of wall material had created a significant impact on the size of the particle encapsulated. Sample PE-Kc was 10–80 µm and PE-Gg had 25–60 µm particle size, showing that guar gum has more stickiness to the particles and aggregated on PE-protein pigment. In contrast, carrageenan as a wall material showed intact appearance on PE. It could be the parental bond between the polysaccharide and pigment-protein from *K. alvarzeii*. However, there were more crystals on PE-Kc possibly due to sudden cooling and rapid change in moisture content. As seen in the SEM images, the particles were showed the bigger size and fragmented pieces like structure (supplementary figure 1a,b, c).

Physiochemical properties of ice cream

The physicochemical properties of PE and microencapsulated PE in ice cream are given in Table 2. The protein content of ice cream ranged from 4.2 to 6.2%, and after 90-day period it ranges between 4.0 and 7.2%. There were no significant differences found in titratable acidity on PE pigment incorporated ice cream, which ranged between 0.17 and 0.22%, initially. At the end of the storage period, it increased from 0.18 to 0.26%, with the significant difference ($p < 0.05$) found in the PE and PE-Gg ice cream. The total solids were in the range of 39.98 and

Table 1 Encapsulation efficiency (EE) and phycoerythrin load (PE-load)

Encapsulated matrix	EE (%)	PE load (%)	Hygroscopicity index (g (100 g)$^{-1}$)	Water solubility (%)
PE-Kc	82.56 ± 0.18*	56.78 ± 0.96*	0.25 ± 0.09*	86.94 ± 1.02*
PE-Gg	79.47 ± 1.02*	51.24 ± 0.42*	0.32 ± 0.03*	90.14 ± 0.18*

Mean ± SD, $n = 4$, *$p < 0.05$; microencapsulated PE in kappa-carrageenan (PE-Kc) and guar-gum (PE-Gg)

40.52% during the initial days, and finally 38–47.21%. There were no significant differences between the groups at the time of storing but varied ($p < 0.05$) after 90 days of storage.

The HLB reflected in the destabilization index of ice cream, which could be influenced by the presence of PE. Similarly, the control group (49%) had the highest fat destabilization, while microencapsulated PE showed similar values (45%) compared with PE alone (37%). The effect of PE in the overrun percent of ice cream is shown in Table 2. Overrun values showed a difference ($p < 0.05$) between fat destabilization and viscosity of the ice cream samples. Further, all the ice cream samples were entirely melted within 70 min, up to 2 min; the dripped amount of ice cream remained zero for all the samples. However, PE-Gg ice cream had the lowest melting rate of 9.5% in 10 min and 59% in 30 min ($p < 0.05$), whereas it was 60 and 61% of melting rate in the PE-Kc and PE added ice cream, respectively.

Rheological properties

As given in Table 3, the Herschel–Bulkley model represents the shear thinning characteristics of all ice cream samples. The flow behaviour index (n) value of samples was ranging between 0.518 and 0.721. Low-flow behaviour index recorded in PE added ice cream before storage (0.518) and high n values seen in PE and control ice cream, which was more viscous compared with PE-Kc and PE-Gg ice creams. They had maximum K values of 2.342 and 2.301, respectively. The flow behaviour and apparent viscosity showed inversely proportional values in our study.

Table 2 Physiochemical properties of PE incorporated ice creams during initial and final day of storage

Physiochemical attributes	Control	PE	PE-Kc	PE-Gg
Initial				
Protein	4.2 ± 0.08^{NS}	6.2 ± 0.06^{b}	4.9 ± 0.02^{a}	5.1 ± 0.02^{c}
pH	6.1 ± 0.06^{NS}	6.1 ± 0.06 ^{NS}	6.0 ± 0.06 ^{NS}	6.4 ± 0.06^{a}
Acidity (%)	0.22 ± 0.006^{NS}	0.21 ± 0.003^{NS}	0.17 ± 0.007^{a}	0.20 ± 0.004^{NS}
Total solids (%)	40.52 ± 0.44 ^{NS}	39.98 ± 0.51^{NS}	40.14 ± 0.10^{NS}	40.21 ± 0.21^{NS}
Hardness (g cm^{-2})	4354 ± 261^{a}	3192 ± 66^{b}	5001 ± 60^{c}	5238 ± 31^{d}
Fat destabilization (%)	49.3 ± 5.92^{a,b}	37.07 ± 6.0^{b}	45.82 ± 3.45^{NS}	45.78 ± 5.6 ^{NS}
Overrun	32 ± 2.42^{a}	68 ± 3.64^{x}	69 ± 2.88^{a}	75 ± 1.92^{b}
Melting rate (g min^{-1})	0.52 ± 0.32^{c,d}	0.56 ± 0.18^{c,d}	0.46 ± 0.22 ^{axe}	0.36 ± 0.12^{by}
Final				
Protein	4.0 ± 0.02^{NS}	5.8 ± 0.12^{b}	6.8 ± 0.03^{a,y}	7.2 ± 0.06^{a,x}
pH	6.1 ± 0.06^{NS}	6.5 ± 0.06^{a}	6.2 ± 0.06 ^{NS}	5.8 ± 0.06^{ab}
Acidity (%)	0.20 ± 0.006^{NS}	0.18 ± 0.008^{y}	0.23 ± 0.007^{b}	0.26 ± 0.001^{ax}
Total solids (%)	38.52 ± 0.44^{a}	40.98 ± 0.51^{b}	44.41 ± 0.10^{a}	47.21 ± 0.21^{by}
Hardness (g cm^{-2})	42,041 ± 126^{a}	4208 ± 36^{bx}	4826 ± 42^{cy}	4658 ± 28^{ab}
Fat destabilization (%)	48.1 ± 2.56^{a}	34.24 ± 2.48^{b}	40.26 ± 1.64^{ax}	42.76 ± 2.78^{by}
Overrun	66 ± 2.79^{a}	50 ± 1.89^{b}	58 ± 2.56^{ax}	63 ± 2.42^{by}
Melting rate (g min^{-1})	0.56 ± 0.23^{NS}	0.48 ± 0.21^{ax}	0.51 ± 0.26^{NS}	0.46 ± 0.14^{by}

Mean ± SD, $n = 3$. Values with a different superscript are significantly different ($p < 0.05$) between the rows (a–d), and x and y are different between the initial and final day of storage. Microencapsulated PE in kappa-carrageenan (PE-Kc) and guar-gum (PE-Gg)

NS not significant, *PE* phycoerythrin

Table 3 Rheological parameters of PE incorporated ice creams during initial and final day of storage

Samples	Consistency coefficient K (Pa s^n)	Flow behaviour index *n* (−)	*o* (Pa)	Apparent viscosity at 50 s^{-1} (Pa s)
Initial control	2.301 ± 0.462^{NS}	0.544 ± 0.132^{NS}	3.762 ± 0.578^{NS}	0.092 ± 0.003^{NS}
PE	2.342 ± 0.084^{NS}	0.518 ± 0.124^{NS}	$3.78 \pm 0.346^{a,b}$	0.094 ± 0.003^{NS}
PE-Kc	1.743 ± 0.142^{b}	0.624 ± 0.168^{c}	2.682 ± 0.412^{NS}	0.146 ± 0.002^{a}
PE-Gg	2.242 ± 0.187^{NS}	0.507 ± 0.123^{a}	$2.522 \pm 0.486^{a,b}$	0.076 ± 0.004^{b}
Final control	2.149 ± 0.266^{d}	0.568 ± 0.108^{NS}	3.076 ± 0.504^{NS}	0.098 ± 0.004^{a}
PE	1.843 ± 0.212^{a}	0.642 ± 0.148^{b}	$2.862 \pm 0.433^{a,b}$	0.158 ± 0.003^{c}
PE-Kc	0.984 ± 0.274^{c}	0.721 ± 0.164^{c}	2.273 ± 0.128^{NS}	0.132 ± 0.003^{b}
PE-Gg	0.742 ± 0.142^{b}	0.704 ± 0.122^{a}	2.424 ± 0.402^{NS}	0.164 ± 0.002^{c}

Mean ± SD, $n = 3$. Values with a different superscript are significantly different ($p < 0.05$) between the initial and final day of storage (a–d). Microencapsulated PE in kappa-carrageenan (PE-Kc) and guar-gum (PE-Gg)

NS not significant, *PE* phycoerythrin

Similarly, the apparent viscosity and consistency index showed inverse values, i.e. 0.092, 0.098, and 0.094 of control and PE ice cream, respectively. The H-B model showed values between 0.97 and 0.99 correlation coefficients. The obtained values fitting to this model are significant for yield stress. Further, "*n*" values were less than 1, which confirms the pseudoplastic property was unchanged in the entire study period.

Sensory evaluation

Ice cream products prepared with 3 variations of PE were evaluated for organoleptic properties against control ice cream. Panellists preferred and chose PE incorporated ice cream in all sensory attributes. The score of sensory characteristics for ice cream included with PE in blend shown in Table 4. The sample PE-Kc yielded lower values in colour, taste, texture, and flavour initially as 7.6, 7.1, and 7.9, respectively, at the initial day of storage. In contrast, PE-Gg showed 7.0 overall score with no significant between two formulations. Similarly, the final day of storage obtained various scores in the sensory attributes. In this sensory study, it was observed PE and control accounted for the highest score of 7.2 amongst six variants made ($p = 0.02$; $r = -0.865$),

Colour analysis

The CIELAB colour space of PE and microencapsulated PE incorporated ice cream samples was evaluated for 90 days of storage, and the values are given in Table 5. The CIE L^*, a^*, and b^* values of a purified PE powder were 40.69, 56.69, and − 1.96, respectively. The L^* and b^* values of the samples were decreased as the storage period increases when irrespective of microencapsulated wall material (PE-Kc and PE-Gg) used. In contrast, a^* value (redness) of the PE increased during storage. However, ice cream with PE alone showed intense pink colour with the low luminosity L^* values, and PE-Kc L^* (62.45), 41.46(a^*), and − 2.47(b^*); the negative value

Table 4 Sensory evaluation of PE-incorporated ice cream during initial and final day of storage

Samples	Colour	Taste	Texture	Flavour	Overall acceptability
Initial control	$8.2 \pm 1.02^{b,c,d}$	$8.0 \pm 1.42^{b,d}$	$8.0 \pm 1.09^{b,c}$	$8.6 \pm 1.08^{b,c}$	$8.5 \pm 1.28^{b,c}$
PE	$7.6 \pm 1.64^{b,c}$	$7.1 \pm 1.32^{a,d}$	$7.9 \pm 1.10^{c,d}$	$7.0 \pm 1.40^{b,c}$	7.2 ± 2.13^{a}
PE-Kc	$7.2 \pm 1.20^{a,b}$	$7.0 \pm 1.08^{a,b}$	$7.2 \pm 1.20^{a,b}$	$7.10 \pm 1.12^{a,d}$	$7.1 \pm 1.06^{a,b}$
PE-Gg	6.6 ± 1.12^{a}	6.1 ± 1.24^{c}	6.4 ± 1.04^{c}	6.2 ± 1.08^{d}	7.0 ± 1.87^{a}
Final control	$7.2 \pm 1.36^{c,d}$	8.0 ± 1.12^{c}	7.2 ± 0.93^{c}	$7.2 \pm 1.36^{c,d}$	$8.0 \pm 1.15^{c,d}$
PE	$6.8 \pm 0.97^{a,b}$	$6.9 \pm 0.94^{a,b}$	$6.8 \pm 1.02^{b,c}$	$7.00 \pm 0.79^{b,d}$	$7.00 \pm 0.92^{c,d}$
PE-Kc	7.9 ± 0.09^{a}	7.5 ± 0.83^{a}	7.9 ± 0.85^{a}	$7.70 \pm 0.92^{a,b,c}$	7.75 ± 0.91^{a}
PE-Gg	$7.5 \pm 2.02^{b,c}$	$7.6 \pm 0.47^{b,c}$	$7.6 \pm 1.12^{b,c}$	$7.50 \pm 1.11^{b,c}$	$7.70 \pm 1.26^{b,c}$

Mean ± SD ($n = 25$). Values with a different superscript in the columns (a–d) are significantly different ($p < 0.05$); Microencapsulated PE in kappa-carrageenan (PE-Kc) and guar-gum (PE-Gg)

PE phycoerythrin

Table 5 Pigment analysis on PE incorporated ice creams

Sample	Storage days	L*	a*	b*	Color gradient
Purified PE		**51.41±1.28**	**58.58±2.78**	**-16.08±0.26**	
Control group	1	97.52±2.44[a]	0.64±0.23[a]	2.38±0.06[a]	
	30	94.85±1.23[b]	0.82±0.23[b]	3.02±0.23[b]	
	60	94.16±0.23[b]	0.82±0.23[b]	3.12±0.23[b]	
	90	92.15±0.23[b,c]	0.68±0.23[a]	2.03±0.23[b,c]	
PE	1	67.05±3.40[a]	50.02±0.06[a]	4.26±0.12[a]	
	30	73.01±2.46[b]	36.48±2.78[b]	0.48±0.02[b]	
	60	83.64±3.56[c]	33.01±1.48[c]	0.34±0.02[c]	
	90	84.02±3.56 [a,b,c,d]	24.32±1.48 [a,b,c,d]	-2.36±0.02 [a,b,c,d]	
PE-Kc	1	66.26±2.75[a]	35.78±0.02[a]	7.46±0.44[a]	
	30	66.05±1.89[c]	44.35±1.02[c]	2.48±0.03[c]	
	60	66.26±2.46[b]	55.23±1.12[b]	0.18±0.09[b]	
	90	58.05±0.12 [a,b,c,d]	58.09±0.19 [a,b,c,d]	-2.54±0.67 [a,b,c,d]	
PE-Gg	1	65.26±1.05[a]	32.65±0.01[a]	8.49±0.44[a]	
	30	67.45±3.12[b,d]	36.42±1.65[b]	4.49±0.48[a,b]	
	60	63.45±2.78[c]	38.26±2.23[c,d]	0.06±0.02[b,c]	
	90	62.45±2.46 [a,b,c,d]	41.46±2.48 [a,b,c,d]	-2.47±0.02[a,b,c,d]	

Mean ± SD, $n = 3$. (a–d) Different lowercase letters in the same column are statistically different ($p < 0.05$) for the same formulation on different storage days. Microencapsulated PE in kappa-carrageenan (PE-Kc), and guar-gum (PE-Gg) *PE* phycoerythrin

found in b^* refers to the blue gradient increases in the pigment over a period. However, all these changes were not identical to the purified PE colour space values. The ice cream in the control group without food colour shows positive values for a^* and b^*, which decreased L^* over 90 days of storage (refer Table 4).

Antioxidant activity of PE and microencapsulated PE ice cream on DPPH and FRAP scavenging activity

The antioxidant activity of PE and microencapsulated PE were evaluated using DPPH against BHA as the standard reference. Fig. 3 represents the scavenging action of PE, PE-Kc, and PE-Gg at a concentration of 100 mg mL^{-1} compared with control ice cream without any PE for the initial and final day of storage. The DPPH radical scavenging actions of PE, PE-Kc, and PE-Gg were 30.47%, 20.45%, and 18.56%, respectively, but lower than BHA (82.5%). There was a direct and positive correlation between the purified PE and microencapsulated PE in ice cream on DPPH radical scavenging action at the initial day (0 day). After the storage period (90th day) an inverse reaction was noticed; i.e. microencapsulated PE had exhibited greater DPPH radical action. All these changes were significantly different between the tested samples (see **F**ig. 3). The FRAP reducing ability significantly

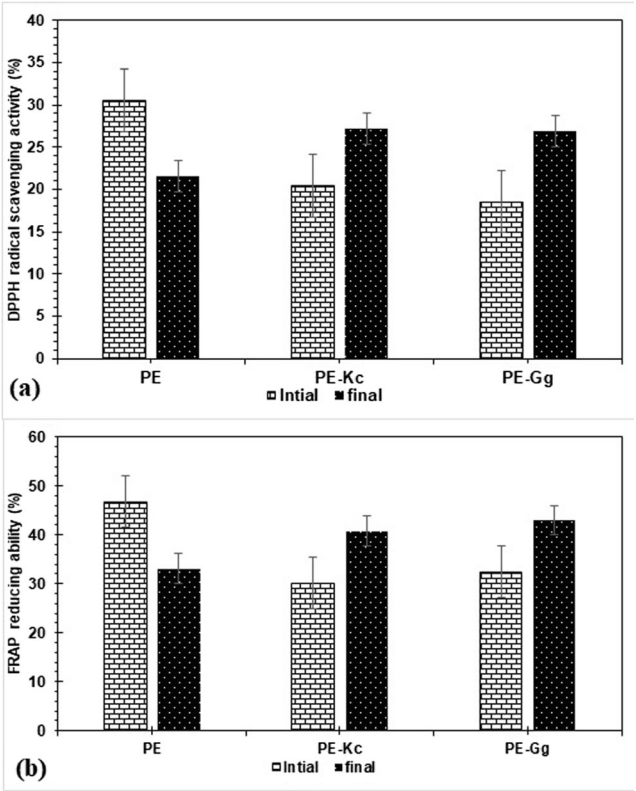

Fig. 3 **a** DPPH scavenging activity (%) of PE and microencapsulated PE ice cream samples. **b** FRAP reducing ability (%) of PE and microencapsulated PE ice cream samples

contributes to an antioxidant potential of the bioactive compound was evaluated at 593 nm absorbance (see Fig. 3b). At a concentration of 100 mg mL^{-1} the microencapsulated ice cream showed low FRAP inhibition during the initial day of storage. However, FRAP action was increased by 40.78% (PE-Kc), 43.04% (PE-Gg) at the 90th day with a significant difference ($p < 0.05$). The antioxidant action (DPPH and FRAP) correlated with CIELAB colour gradient values, higher intensity of a^* values at 90th day of storage, was positively associated with more antioxidant activity of PE present in ice cream.

Discussion

Extraction and purification of PE

The α, β, and γ oligomers in the present result are in agreement with previous literature (Galland-Irmouli et al. 2000; Liu et al. 2005). It has been reported that α subunit contains two covalently bonded phycoerythrobilins (PEB) to cysteine residues 82 and 139. In contrast, the β subunit holds three PEBs bonded to Cys82, Cys158, Cys50, and Cys6 (Leney et al. 2018). The purity index (PI) of PE from *K. alvarezii* purified through ion-exchange chromatography in the present investigation was PI- 2.32, Nguyen et al. (2018) reported 2.89 of PI for PE from *Mastocarpus stellatus*, and Munier et al. (2015) reported PI- 1.91 for *Grateloupia turuturu*.

Encapsulation efficiency

Phycocyanin coated with carrageenan exhibited 68.66% EE and total phycocyanin load was 45.28% (Hadiyanto et al. 2019). As compared with these values, a PE load in the embodiment material was found to be high in the carrageenan matrix in the present study. Further, PE starts to degrade at 40 °C and the concentration of PE declines from 0.77 at 4 °C onwards (Kannaujiya and Sinha 2016). Therefore, the freeze-drying method is found to be efficient in protecting PE from thermal degradation. This might be due to the application of freezing (− 40 °C), while encapsulation is expelled water from a solidified form by sublimation and desorption (Ezhilarasi et al. 2013). Other literature proved that oil-based encapsulated material, walnut oil coated on thymol and carvacrol, showed had more EE, i.e. 56 to 82.3%. This study also used freeze-drying method for encapsulation. Still, they obtained more EE (82.3%) when increasing the homogenisation cycle while processing (Gursul et al. 2019).

Hygroscopicity and water solubility

Hygroscopicity study is essential to know the stability of encapsulated material. This parameter is critical for evaluating

the interaction of core and wall material during storage. Besides, the water solubility depends on the OH bonding and particle size of the compounds. However, particle size would influence the solubility; for instance, anthocyanin powder having smaller particles exhibited higher water solubility (Pieczykolan and Kurek 2019). Further, they stated that less absorption capacity of wall material leads to less solubility of microencapsulated anthocyanin powder.

Similarly, organic powder material consists of lower H (hydrogen) molecules that secure the core material and absorb less external humidity (Silva et al. 2013). They reported 18% hygroscopicity on microencapsulated *Jaboticaba* peel extract covered by maltodextrin as a wall material. Similar low hygroscopicity was recorded in microencapsulated anthocyanins coated with maltodextrin + arabic gum 0.13 g $(100\ g)^{-1}$ and maltodextrin + inulin 0.14 g $(100\ g)^{-1}$ (Pieczykolan and Kurek 2019). However, these researchers used the spray-drying method of encapsulation.

Morphology

Freeze drying would influence the morphology of core material. It happens at a low temperature (− 40 °C) from the solidified state, keeping away from any water stage responses and oxidation on account of the vacuum. This shift of liquid to the solid-state is standard when food bioactives are freeze-dried, which change the surface morphology. Mainly flake-like structures were noticed when *Garcinia* fruit extract was freeze-dried (Ezhilarasi et al. 2013) and agar and gellan gel microstructure (Tiwari et al. 2015). They stated that freeze-dried powders displayed a slight difference in their surface morphology due to the properties of embodying material while in microencapsulation. It has been reported that phycocyanin encapsulated with carrageenan was 2 μm in size and had few pores on its surface (Hadiyanto et al. 2019).

Physiochemical properties

Beta-carotene encapsulated with solid-lipid-microparticles in ice cream had 2.65% of protein (LIMA et al. 2016), and 5% carotenoid (lycopene-red pigment) extracted from tomato peel included ice cream showed 4.62% of protein (Rizk et al. 2014). These protein vales are low compared with the present results. Hence, the PE protein pigment enhances the total protein content of ice cream. Titratable acidity shows the quality and associated with milk-solid-non-fat (MSNF) content of ice cream. It has been reported that ice cream consisting of 11.7% MSNF had acidity in the range of 0.19–0.22% (Choo et al. 2010) and roselle-extract incorporated ice cream had 0.15–0.22% acidity (Singo and Beswa 2019). Thus, the values obtained in our study were within the normal range. Furthermore, other pigments like lycopene and beta-carotene incorporated ice cream showed 30.91% and 30.50% of total solids (Rizk et al. 2014; LIMA et al. 2016) as compared with our results these two studies exhibited lower total solids.

The hydrophilic and lipophilic balance (HLB) acts as an interpreter for fat destabilization index. In our results, fat destabilization was not significant between PE-Kc and PE-Gg ice cream, but changes were noticed in PE ice cream ($p < 0.05$). Overrun values showed a difference ($p < 0.05$) between fat destabilization and viscosity of the ice cream samples. Similar results have been found in grape pulp and skin incorporated ice cream (Tsevdou et al. 2019) and grape raisin and sugarcane molasses added ice cream (Soukoulis and Tzia 2018). However, ice cream incorporating beta carotene microparticles showed overrun in the range of 209–222% (LIMA et al. 2016). Ice cream with higher overrun would have fewer ice crystals (Flores and Goff 1999) and increases the volume of air increases after addition of solid-lipid microparticles in the ice cream.

Further, they reported that fat and protein content relatively influence ice cream overrun and contribute air interfaces stabilization. Melting rate is a useful indicator of structural development and resistance to collapse in ice cream (Chen et al. 2019) as a time against average dripped volume. Velásquez-Cock et al. (2019) reported the meltdown process depends on fat destabilization and viscosity. This characteristic feature is associated with the water holding capacity and retention of hydroxyl and amino group (Sun et al. 2015), which is consistent with our study.

Rheological properties

The addition of prebiotic oligosaccharides in ice cream showed maximum K values of 31.07 and 55.27, and apparent viscosity of 0.95–0.68 (Panwar and Kapoor 2019). The low n value obtained in the present study (PE-Kc, PE-Gg) shows increase entanglement of polysaccharide in the encapsulated matrix influencing the shear rate of ice cream. However, this characteristic feature enhances the fluid pumping process and smooth texture while consuming, which more pronounced in PE-Gg samples. Furthermore, basil seed gum and guar gum had better pseudoplastic property compared with carrageenan (Javidi et al. 2016). Minor changes in the "n" value by shear force influence the flow rate to a greater extent, and these two factors are essential for pseudoplastic effect. However, the pseudoplastic property was not modified throughout the storage (0–90 days). This property shows resistance to deformation when an external force is applied to it. However, the type of emulsifiers and stabilizers used in ice cream can influence the flow behaviour of ice cream. In the present investigation, the encapsulated matrix itself is a stabilizer (carrageenan/ guar gum). This might be due to the shifting of a bond between carrageenan/guar gum with PE protein, which expanded the association between neighbouring atoms due to exposure of

hydrophobic and hydrophilic locales, presenting more resistance to flow (Chen et al. 2019).

Sensory evaluation

The sensory parameters reflect the overall acceptability score of new products (Moskowitz et al. 2012). High acceptability scores received in PE and control ($p = 0.02$; $r = -0.865$) show that the consumers were attracted to the intense pink colour of ice cream. Further, mouthfeel attributes, including creaminess and iciness, are characteristics note of hardness, which reflects in texture parameter. The texture of food analysis is complicated. Food texture perception characterized by rheology and character notes (Kemp et al. 2011).

Colour analysis

The colour change was consistent with the shift in oligomeric species. For instance, pH between 8 and 6 had no changes in oligomeric species of B-PE protein complex (Leney et al. 2018). This report is on par with our results, as the pH of PE-Gg was 5.8 in the final day storage (refer Table 4), which influences the colour change of ice cream. Besides, they observed $\alpha\beta$ dimer as a predominant species at pH ≤ 5, but pH between 5 and ≥ 6–8 consists of $\alpha 6\beta 6$ complex, modifying the colour from pink to dark pink. It was demonstrated that $\alpha\beta$ oligomers in B-PE assemble reversibly at pH 4–7 to its environmental condition. Therefore, masking PE through encapsulation would be beneficial to the biotechnology industry, whereby a change in colour reports precisely on the neighbourhood condition encompassing B-PE.

Similarly, anthocyanin pigment (violet colour) from *Brassica oleracea* encapsulated by CAPSUL (modified starch) showed bathochromic shift. This resulted in the change of pink colour to an intense blue colour of anthocyanins to precarious chinoidal structures (Zanoni et al. 2020). They identified that the encapsulation process did not affect the light-assimilation properties of anthocyanins in the visible range.

Further, there was a significant difference found in the colour of all samples in the present study. It was clear that the pigment percolates through the wall material (carrageenan and guar gum) from the obtained values. However, purified PE colour analysis revealed that b^* gradients have an intense blue colour (− 11.08) on its surface compared with microencapsulated PE. The b^* values represent that microencapsulation has advantageous to promote the stability of PE pigment.

Further, the computer vision system (CVS) and hunter lab colourimeter have excellent correlation reported in an earlier study (Goñi and Salvadori 2017). Hence, the image analysis was performed in the high configuration images with preprocessing that was done to eliminate the background noise of the images using image segmentation (ImageJ NIH software).

Antioxidant activity of PE and microencapsulated PE ice cream on DPPH and FRAP scavenging activity

Seaweed *dulse* protein PE and recombinant β-subunit of dulse PE against DPPH radicals (Sato et al. 2019) showed 21.3% and 3.2% scavenging action, respectively, at 5.0 and 10.0 mg mL^{-1} concentration. Similarly, PE obtained from *Lyngbya* sp. showed 84.29% scavenging action, while PA showed 72.26% scavenging action at 150 mg concentration (Sonani et al. 2014). Also, phycocyanin from *Arthrospira platensis* displayed the highest DPPH scavenging action in freeze-dried biomass (Pan-utai and Iamtham 2019), which is comparable to our results. However, food-grade PBPs were less active against DPPH radicals in contrast with purified PBPs, and their IC_{50} were 84 and 166 μg mL^{-1} (Chen et al. 2017). These researchers reported that almost 11.02% of amino acids present in PBP are negatively charged, which donates a proton to the single pair of an electron in the DPPH radical reaction. This mechanism could inactivate the free radicals. Further, the highest (71.73 μg mL^{-1}) DPPH radical action was recorded in UV treated phycoerythrin (PE) from *Leptolyngbya fragilis* in ethyl acetate (Kokabi et al. 2019). In comparison with our result, the PE from *Halomicronema* sp. showed a better reaction against ferric-ion at 100 μg PE mL^{-1} (Patel et al. 2018). Further, these researchers reported that 17% of negatively charged and 16% positively charged amino acids might be responsible for antioxidant action.

Conclusion

Microencapsulation using kappa-carrageenan and guar gum as a wall matrix had confirmed the stability of PE in ice cream at a various time intervals. The colour intensity of the microencapsulated-PE ice cream showed vivid colour with variation in release of pigment (PE) during the 90-day storage period. After the storage period, PE-Gg shows better stability and colour in ice cream compared with PE-Kc. These changes also influence the rheological and sensory parameters of ice cream. Finally, in vitro antioxidant activity confirmed the functional property of the formulated ice cream, which added-value to the ice cream. Therefore, this study should be of interest to the commercial dairy industry in the use of natural pigment to replace synthetic pigments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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