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# Purification, Spectral analysis and characterization of r-phycoerythrin pigment from *Solieria robusta* (Greville) Kylin

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**Abstract**

Principal feature underlying energy transduction by the photoautotrophs on earth is harvesting of light energy by specific pigments. The harvested energy is converted to chemical energy by photosynthesis. Photosynthetic pigments are two types - primary (Chlorophylls) and accessory (carotenes and xanthophylls). To use a greater part of the light spectrum aquatic algal members evolved a diverse array of pigment system to use various wavelengths of light efficiently. Phycobiliproteins are a family of light-harvesting pigment protein complexes widely found in the chloroplasts of red algae and cyanobacteria[20]. R-phycoerythrins (R-PEs) are the most abundant phycobiliproteins in the marine red algae. R-PE from *Solieria robusta* was purified using Sephadex G-200 column followed by spectral analysis using UV-VIS spectrophotometer. Signature absorbance pattern of r-phycoerythrin pigment were observed in active fractions which enabled fast separation and purification of the pigment. *Solieria robusta* from the Arabian Sea coast of Okha, Gujarat, India can be a potent source of R-PE.

Capture and entrapment of light by various pigments on earth is the basis of life on earth since the origin and evolution of photoautotrophs which initiated in the Precambrian era. Precambrian an age of algae with doubtful credentials[28]. The harvested energy is converted to chemical energy by photosynthesis. Except for small ecosystems around hydrothermal vents and in deep mines, photosynthesis is the basis for nearly all life on Earth, providing food, fuel, and oxygen. Aquatic photoautotroph accounts for more than 50% of the photosynthesis occurring on Earth. They additionally contain other types of accessory pigments. Phycobiliproteins are a family of light-harvesting pigment-protein complexes found widely in cyanobacteria, red algae and some cryptomonads. According to their light absorption properties, phycobiliproteins are classified into three main groups: phycoerythrin (PE; λmax = 565–567 nm), phycocyanin (PC; λmax = 615–620 nm) and

allophycocyanin (AP; λmax = 650–652 nm). In cyanobacteria and red algae, these phycobiliproteins assemble to form a supermolecular protein complex, named phycobilisome (PBS)[6]. Their special absorption spectrum in native state is a three-peak spectrum with absorption maxima at 565, 539 and 498 nm, respectively. The sun light harvested by PBSs from surroundings is transferred from such accessory pigment system with an overall quantum efficiency closed to 100% finally to reaction centres comprising chlorophylls.

R-PE is fluorescent, with high quantum efficiency, a large Stokes shift and excitation and emission bands at visible wavelengths (Agilent Products). R-PEs are used in the production of food and cosmetics, and play an important role in many biochemical techniques due to their fluorescence properties[1]. R-PE is commonly used as a fluorescent label in immunology, cell biology[18] and flow cytometry (Wilson & *et al*. 1991). R-PE possesses a high molar extinction coefficient ($\varepsilon = 2 \times 10^6\ M^{-1}\ cm^{-1}$ for 240 kDa) and a high fluorescence yield, at least 10 times more than those usually observed[18,33]. It is also applied as a natural food dye[5] and as a marker in gel electrophoresis and isoelectrofocusing. In recent years seaweeds have increasingly attracted interest in the search for new drugs and have been shown to be a primary source of bioactive natural compounds and biomaterials[9]. Among the phycobiliprotein-containing algae, eukaryotic red algae occupy a critical position in the evolution of oxygen-evolving photosynthetic organisms, for they are an inheritor of prokaryotic cyanobacteria and a predecessor of eukaryotic cryptophytes. These are organized in high molecular mass particles called phycobilisomes bound to the outer faces of photosynthetic membranes and funnel excitation energy to photosynthetic reaction centres (RCs) over long distances with >90% efficiency. Phycobiliproteins containing unicellular cyanobacteria and Rhodophycean (red algae) members originated as one of the earliest photosynthesizing entities on earth right in the Precambrian era[3]. These protein pigment system allow to efficiently exploit a wide range of light colors in aquatic algal community. This could greatly increase the efficiency of the algal communities towards light harvesting and co-existence of various members (other groups of algae) in a single habitat.

*Aims and objective :*

*Extraction and purification of r-phycoerythrin (R-PE) pigment from red algal member Solieria robusta (Greville) Kylin :*

Finding a potential and fast method of purification using water based solvent, cold centrifugation and simultaneous analysis using UV-VIS spectrophotometer. Partial Purification of phycobilins and quantification of r-phycoerythrin and r-phycocyanin from algal sample. Finding a potential solvent for easy and fast extraction of R-PE and estimating a suitable temperature range for storage. To find major phycobilin pigments in the Red algae *Solieria robusta* (Greville) Kylin. Spectral analysis of the pigments, a potent method for fast estimation of the pigment in question from the Rhodophycean member namely - *Solieria robusta*. To find a potential source of r-phycoerythrin from marine red algae for future harvest.

*Material :*

Marine Red algae *Solieria robusta*, was collected by the author from coast of Okha, Gujarat (lat. 22°28_N and long. 69°05_E) in the month of February, 2019. Gregarious growth of the red algae was observed found associated with the substratum. Temperature ranged from 21.5°C to 30°C. Salinity of the collection site was within 35.46 to 37.32 PSU. The samples were kept frozen under -20°C until use.


Fig. 1. Western Arabian Sea coast of Okha, Gujarat, India[17]

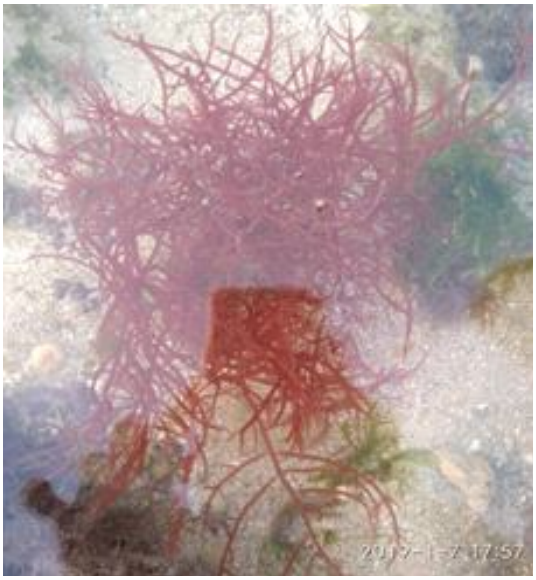


Fig. 2. *S. robusta* at the site of collection

Fig. 3. Fresh thallus after collection

Fig. 4. Thallus before homogenization in the laboratory

*Tissue maceration to obtain the homogenate:*

Plant body was washed in cold water and weighed, homogenized in pre-chilled mortar and pestle using 50mM Tris-Acetate buffer with pH 7.5 and neutral sand. The homogenate was filtered, kept apart for future assay. Remaining homogenate (35ml) was cold (at 4 $^{o}$C) centrifuged at 10,000 rpm for 20 minutes.

*Low speed supernatant and pellet :*

Both the supernatant and pellet were collected. The pellet was dissolved in 12 ml Tris-acetate buffer. Then both the supernatant and pellet were measured. 5 ml of 10k supernatant was kept for future assay and spectral analysis; remaining 10 k supernatant are kept in -21$^{o}$C.

*Ammonium sulfate fractionation :*

With the 10k supernatant ammonium sulfate (0 to 80%) salting out was performed. When fully dissolved kept in chilled condition for 1-2 hours, then cold centrifuged at 10,000 rpm for 20 minutes. After centrifugation, both the supernatant and pellet were collected for spectral analysis. The pellet was dissolved using 50MmTris-acetate buffer (pH 7.5) for dialysis.

*Gel filtration through Sephadex G-200 :*

Both fractions run to Sephadex G-200 and 10 fractions were collected from each supernatant and pellet.

Spectrometry of the collected fractions was carried out with the help of Perkin-Elmer Lambda 25 UV-VIS Spectrometer which enabled periodic data taking in the various levels of purification from homogenization, cold centrifugation, Sephadex – G 200 gel filtered fractions.

Pigment content of r-phycoerythrin (R-PE) and r-phycocyanin (R-PC) were estimated from all Sephadex G-200 fractions using the equations $R\text{-}PE = 0.1247(A564 - A730) - 0.4583(A618 - A730)$ and $R\text{-}PC = 0.154\ (A618\text{-}A730)$ respectively according to o'Carra 1965; Siegelman and Kycia 1978. There was eighty fold higher r-phycoerythrin (R-PE) content was observed in comparison to r-phycocyanin (R-PC) content. Highest quantity of R-PE was observed in initial fractions which went on decreasing in a steep and linear manner. The result indicated the presence of much higher content of R-PE than R-PC in *S. robusta.*

Pigment content of R-PE versus R-PC in subsequent stages of purification was quantified since the tissue disorganization using Tris-acetate buffer (with pH 7.5) which again confirmed much higher content of R-PE than R-PC in all the stages of purification of the red pigment. 10k supernatant (10,000 rpm supernatant) contained the pigment under study in contrast to 10k pellets which was discarded. Furthermore, in order to get rid of the crude contents ammonium sulfate salting out was performed and cold centrifuged results showed supernatant contained lower R-PE content than in the pellets. Colour intensity of the red pigment in case of pellet fractions were far higher than the supernatant fractions when centrifuged after ammonium sulfate salting out was done.

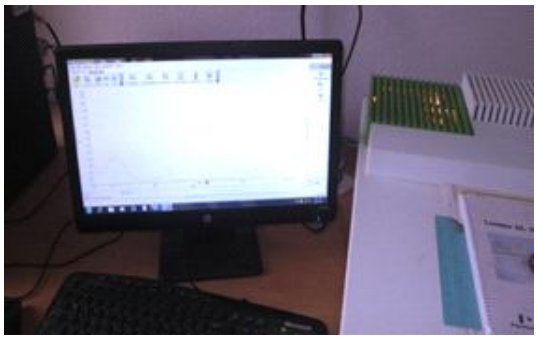


Fig. 5. Perkin-Elmer Lambda 25 UV-VIS Spectrometer  Fig. 6. Absorption spectra of a liquid sample

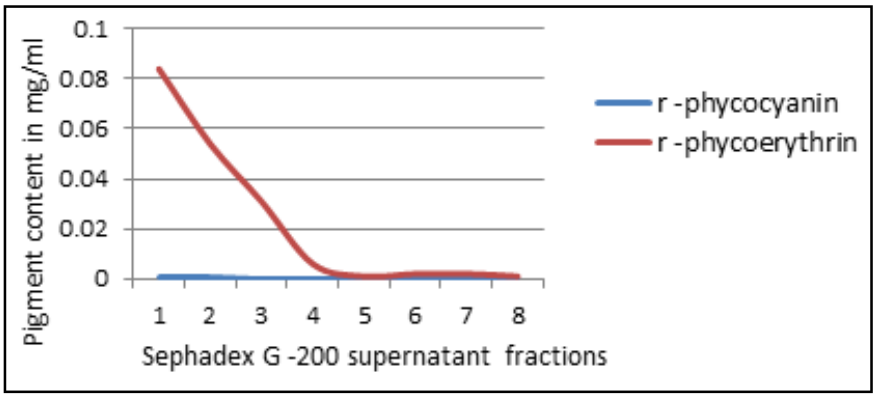
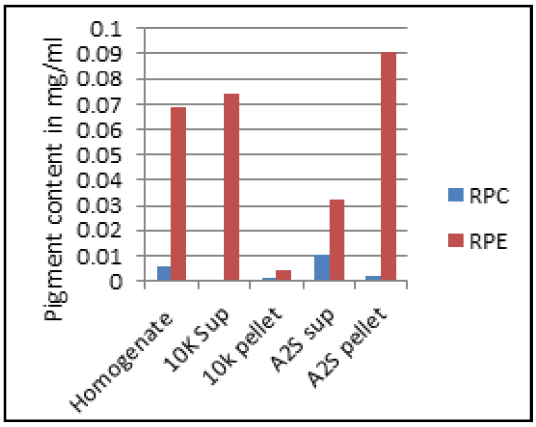



Fig. 7. Pigment content in mg/ml of R-PE versus R-PC showing much higher content of R-PE in the Sephadex G-200 supernatant fractions



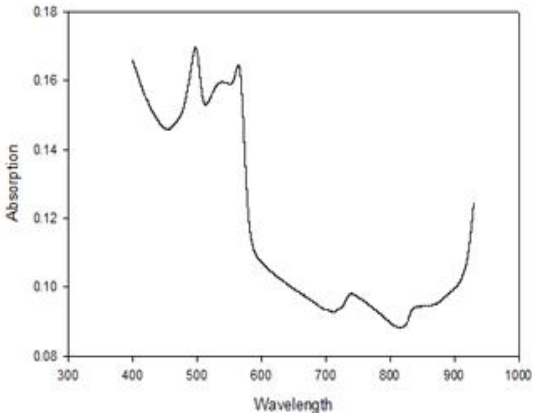




Fig. 8. Pigment conk,tent in various stages of purification (L);  Fig. 9. Absorption spectra of the Homogenate from *Solieria robusta* (R)

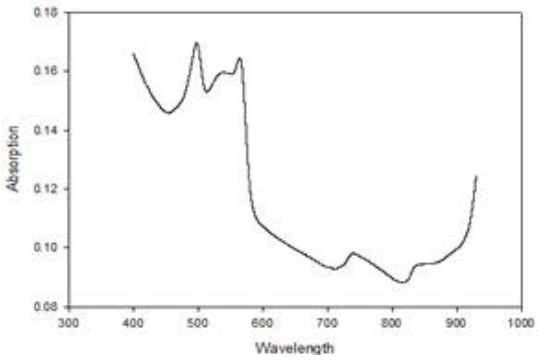



Fig. 10. Absorption spectrum of 10k centrifuged pellet

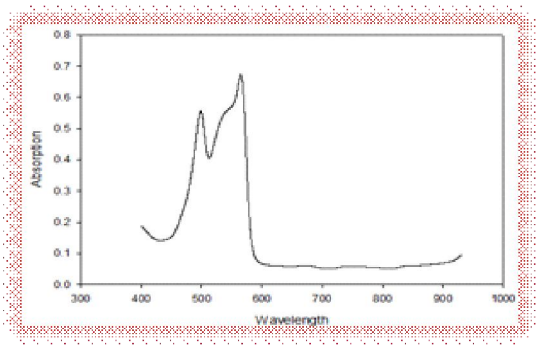



Fig. 11. Absorption spectrum of 10k Supernatant (Characteristic absorption containing plate is highlighted in red colour)

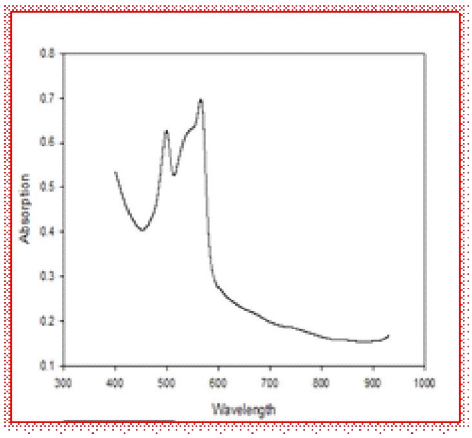



Fig. 12. Absorption spectrum of A2S pellet

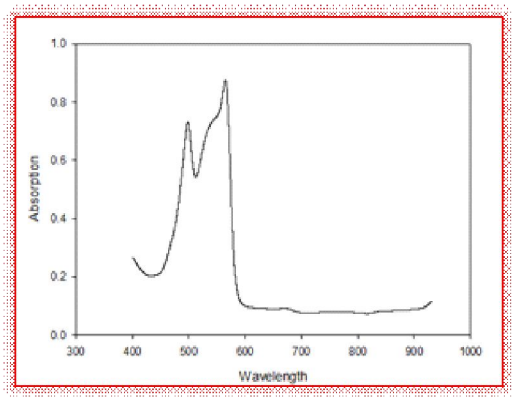



Fig. 13. Absorption spectrum of A2S supernatant

Spectral analysis was performed in all steps of purification using Perkin-Elmer Lambda 25 UV-Vis Spectrometer and the data was plotted in a 2 dimensional graph ranging from 400nm to 950nm of wavelength in the x axis. R-PE exhibited the signature absorption pattern with two peaks in between 480 to 590nm which confirmed its presence in a specific fraction. The characteristic pattern of absorption as depicted in Fig. 11 was used as a tool to confirm the presence of R-PE pigment in the sample of question.

After the ammonium sulfate ($A_2S$) salting out was done - absorption pattern of pellet as well as supernatant were recorded which depicted presence of R-PE in considerable amount in both the samples. Thus ($A_2S$) pellet and supernatant both were preserved for next step of purification which was done with Sephadex G -200 column of approximately 5.5cm length. Out of 8 fractions collected following the molecular sieve using Sephadex G-200, all the fractions were analysed to find whether the characteristic signature curve of R-PE is available or not. Thus using the characteristic curve purification process was enhanced.

Absorption characteristics of pellet versus supernatant Sephadex G 200 fractions during purification (below)

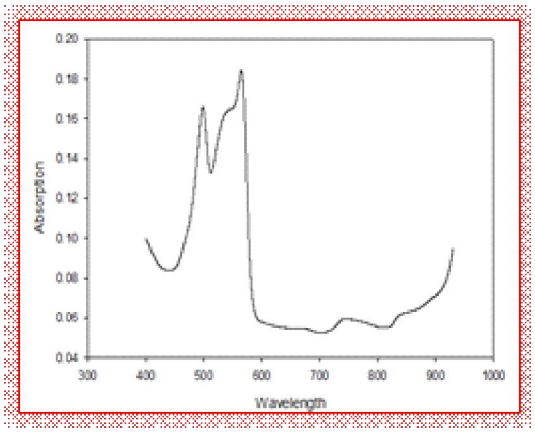



Fig. 14. Pellet Fraction 1 (L)

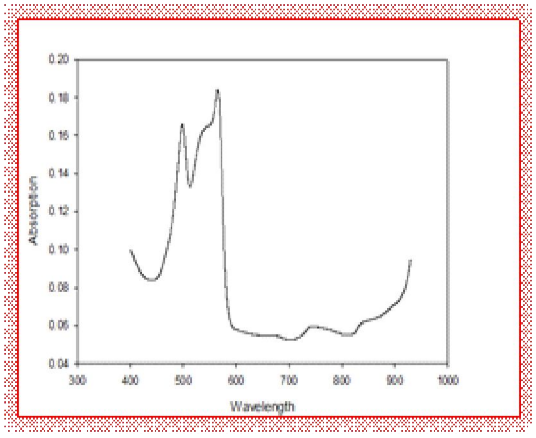



Fig. 15. Supernatant Fraction 1 (R)

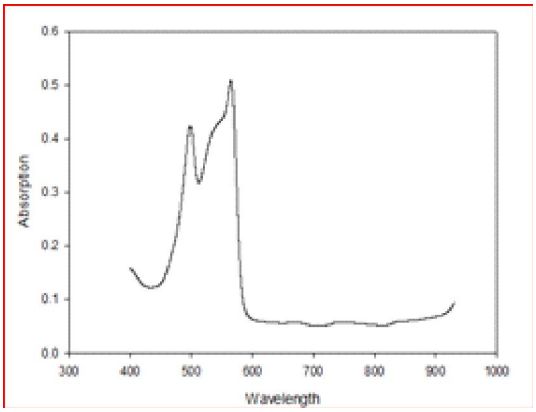



Fig. 16. Pellet Fraction 2

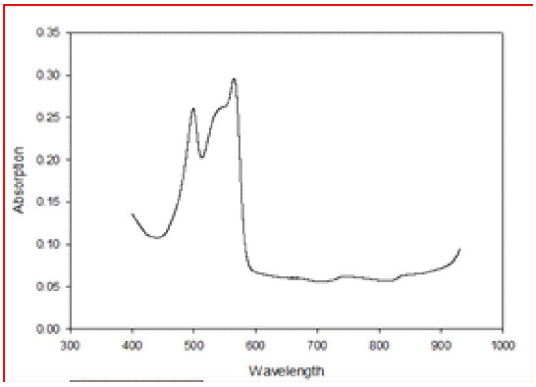



Fig. 17. Supernatant Fraction 2

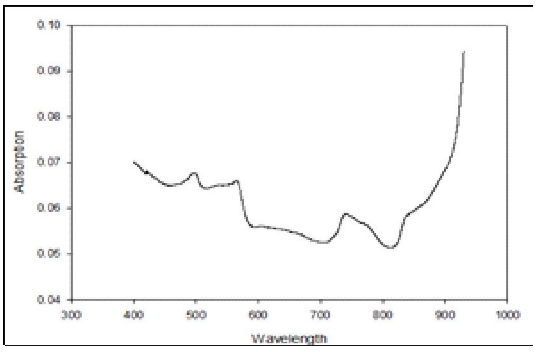



Fig. 18. Pellet Fraction 5

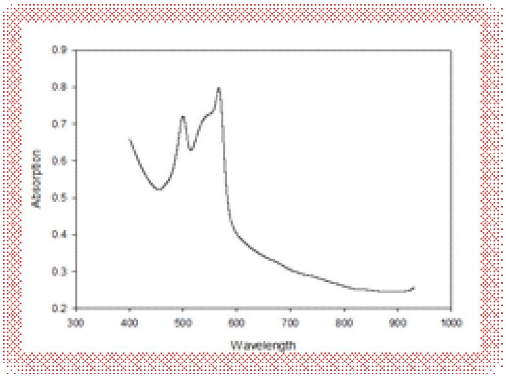



Fig. 19. Supernatant Fraction 5

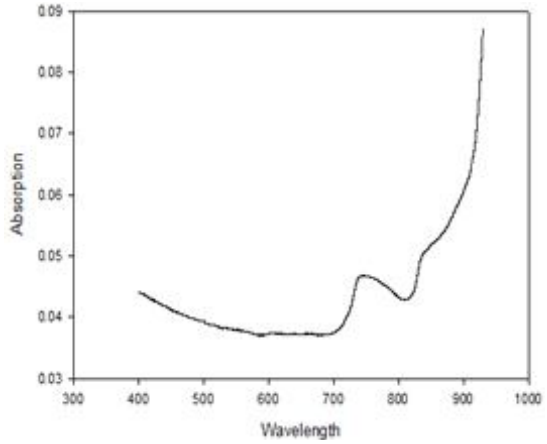



Fig. 20. Pellet Fraction 7

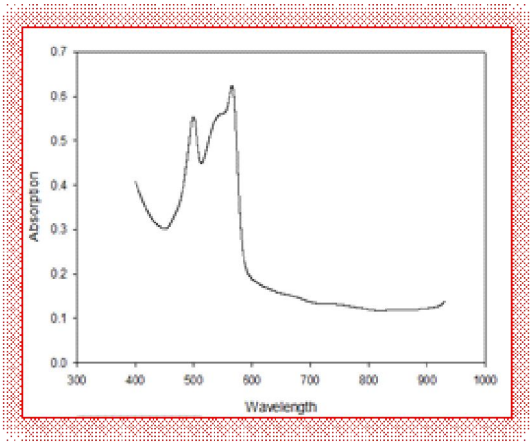



Fig. 21. Supernatant Fraction 7

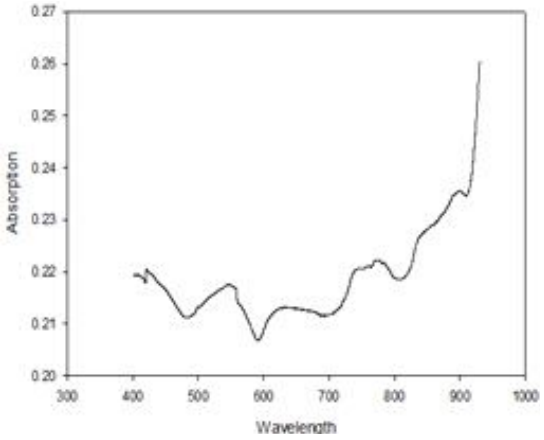



Fig. 22. Pellet Fraction 8

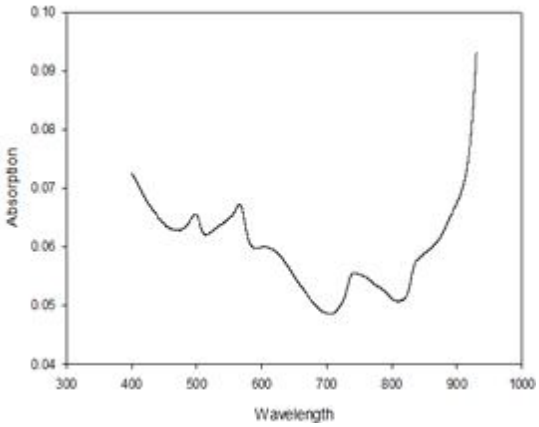



Fig. 23. Supernatant Fraction 8

Spectral analysis of the pellet, supernatant fractions were performed and absorption through different wavelength of the visible spectrum were plotted subsequently as shown in the Figures 14 to 23 and presence of R-PE was observed with the help of characteristic absorption pattern. R-PE content in the supernatant fractions were comparatively higher in contrast to the pellet fractions as it was thoroughly observed up to fraction 7 whereas in supernatant fraction R-PE ceased to be present after Sephadex G-200 fraction no. 2. R-PE pigment was present up to pellet fraction no. 2 after which subsequent fractions of the pellets did not contain appreciable amount of R-PE. This is confirmed by the absorbance pattern of the pellet fractions beyond fraction no. 2 as they lacked the characteristic absorbance pattern for R-PE pigments.

In contrast, most of the supernatant

fractions after passing through the molecular sieve of Sephadex G-200 had the characteristic absorption pattern of R-PE pigments and this was available from fraction no. 1 to 7 beyond which pigments were absent and thus discarded. This is adequately depicted in the plotted absorption pattern in the figures given above.

The experiment thus gives an idea regarding a simple and fast method of purifying red pigment r-phycoerythrin (R-PE) based on both chemical and physical method of spectrometry. It also throws light on the fact that marine macro red algae can be a potent source of R-PE pigment which is of immense economic value. The pigment content in *S. robusta* was also as high as 0.08 mg/ml in the first Sephadex G-200 fraction which went on diminishing (Fig. 7) in the subsequent fractions.

The partially purified R-PE pigment thus obtained were kept in the open in laboratory to ascertain the stability against time on succeeding days with daily temperature range of 27ºC to 36ºC. Stability was found to deteriorate over time owing to exposure of light and high temperature. R-PE pigment collected from *S. robusta* was considerably stable for more than 30 days (Fig. 23) after which the absorbance in its characteristic 565nm wavelength gave a lower value. The pigments isolated were kept in pH range from 5.5 to 8.5 and the best pH to preserve the partially pigments ranged from 7.0 to 7.5 as shown in Fig. 25.

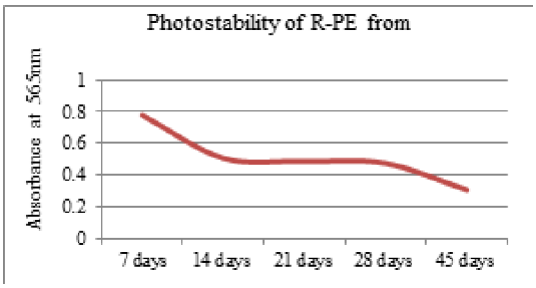



Fig. 24. Photostability of R-PE over time

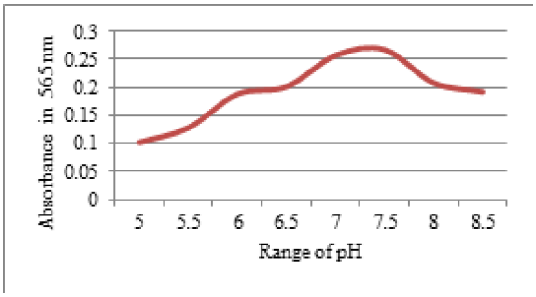
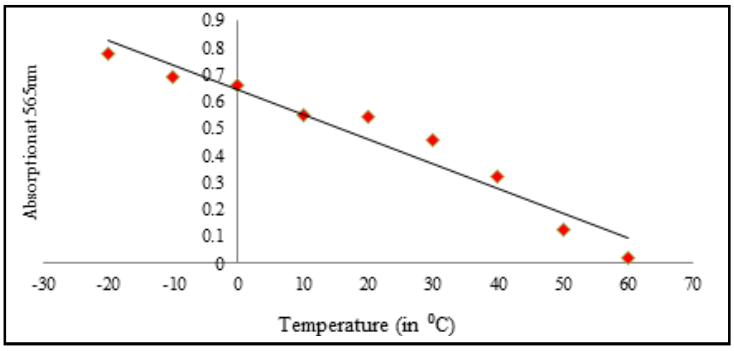



Fig. 25. pH range for R-PE preservation



The isolated pigments after Sephadex G -200 enabled molecular separation were kept in different temperature range from -30! to 60! to estimate the best temperature range which could most appropriately preserve the partially purified R-PE pigments. Temperature below zero degree Celsius and darkness was found to be most suitable for preserving the purified pigments from *S. robusta.*

Higher temperature range was seen to degrade the purified R-PE. This may be ascribed to the protein association of such pigments. R-PE pigments are associated with proteins unlike chlorophylls, carotenes, xanthophylls, anthocyanins etc. therefore suitable buffer was used throughout the experimental set. R-PE was readily soluble in water based solvents and less soluble in the organic solvents like petroleum bezene, cyclohexane, acetone etc. This property can be used for cheap extraction of phycoerythro-biliproteins from Red algal plant specimen. The biliproteins isolated were stable in -20 $^{o}$C, -10 $^{o}$C, 0 $^{o}$C etc. temperature range. Sparingly stable up to 30 $^{o}$C, but was not enough stable in the temperature well above 40 $^{o}$C. Photostability was measured by keeping in the illuminated place for 30-to 45 days. Precipitation of the protein appeared, turning the solution turbid after 30-35 days. Also in 20 $^{o}$C range when the pigments were kept in light, the absorption declined considerably.

Tapping of natural R-PE can be done from macro algae with high concentration of the pigment using spectrometric techniques. As the pigment is associated with proteins suitable buffer was used for harvesting of the pigments. Calculating R-phycoerythrin yield from the crude extract's absorption values has been the quickest and most widely used technique but at the same time, often misleading approach when impurities are present[26]. As already mentioned R-PEs are used in the production of food and cosmetics, and play an important role in many biochemical techniques due to their fluorescence properties[1]. R-PE is used as a fluorescent label in immunology, cell biology[18] and flow cytometry (Wilson & al. 1991). It is also applied as a natural food dye[5] and as a marker in gel electrophoresis and isoelectrofocusing. Systematic tapping of the pigments from suitable red algae with high content as in the case of *S. robusta* can be of great economic value. Western Arabian Sea coast of Okha, Gujarat in India is an important site of algal population with great diversity green, red and brown algal population supporting a diverse fish population. Red macro-algal members like *Gracilaria, Halymenia, Kappaphycus, Solieria, Gelidium, Corallina, Gigartina, Champia, Botrycladia* etc are available in abundance[17]. Experimental Genus *S. robusta* can be used for commercial source of R-PE to meet various biochemical, cytological, genetic need after suitable purification and preservation. It can thus also meet the demand of cosmetic based products. Marine macro red algal members from the western coast of Gujarat can be a natural source of production.

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