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REVIEW

Recent advances in production, purification and applications of phycobiliproteins

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Abstract

An obligatory sunlight requirement for photosynthesis has exposed cyanobacteria to different quantity and quality of light. Cyanobacteria can exhibit efficient photosynthesis over broad region (450 to 650 nm) of solar spectrum with the help of brilliantly coloured pigment proteins called phycobiliproteins (PBPs). Besides light-harvesting, PBPs are found to involve in several life sustaining phenomena including photoprotection in cyanobacteria. The unique spectral features (like strong absorbance and fluorescence), proteineous nature and, some imperative properties like hepato-protective, anti-oxidants, anti-inflammatory and anti-aging activity of PBPs enable their use in food, cosmetics, pharmaceutical and biomedical industries. PBPs have been also noted to show beneficial effect in therapeutics of some disease like Alzheimer and cancer. Such large range of applications increases the demand of PBPs in commodity market. Therefore, the large-scale and coast effective production of PBPs is the real need of time. To fulfil this need, many researchers have been working to find the potential producer of PBPs for the production and purification of PBPs. Results of these efforts have caused the inventions of some novel techniques like mixotrophic and heterotrophic strategies for production and aqueous two phase separation for purification purpose. Overall, the present review summarises the recent findings and identifies gaps in the field of production, purification and applications of this biological and economically important proteins.

Key words: Phycobiliproteins; Nutraceutical; Fluorescence; Pharmaceutical; Cyanobacteria

Core tip: The present review compiles the overall research that has been done so far in the field of production, purification and application of phycobiliptoteins (PBPs). The comparative accounts for different methods for PBPs production, purification have been discussed. The potential applications of PBPs in various industries including food, cosmetics and pharmaceutical have been debated rationally. Remaining questions and research gaps in respected field have been highlighted as conclusion.

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INTRODUCTION

Cyanobacteria are considered as an earliest group of a prokaryotic organism, performing an oxygenic photosynthesis and thus accepted as an ultimate source of atmospheric oxygen[1]. They are widely distributed over aquatic and terrestrial environments including extreme habitats such as hot springs, deserts and polar region[2]. Exposure to various environmental stresses like ultraviolet radiation[3], high temperature, high/low light irradiation and many others have forced cyanobacteria towards gradual evolution to maintain the cell viability[4,5]. Especially, an obligate sunlight requirement for photosynthesis have exposed cyanobacteria to the wide range of solar radiation; which in turn, positively pressurized them to synthesize a number of photon absorbing pigment molecules[6-9]. Cyanobacteria exhibit efficient photosynthesis over the wavelength range 450 to 655 nm by absorbing light energy through a family of brilliantly colored pigment proteins called phycobiliproteins (PBPs)[10]. They stacks together in sequential fashion to form a giant mound - phycobilisome (PBS), which is stabilized and tied up by colourless linker proteins (LPs) on the outer surface of photosynthetic lamellae[11].

PBS

Principally, PBS is built up of various functional PBPs plus a few LPs in a manner to efficiently absorb and funnel photo-energy toward photosystems (Figure 1). PBPs, representing almost 80% of PBS mass[12], are pigment proteins consists of protein scaffold with covalently attached chromophore (a linear tetrapyrrole chain) (Figure 2)[13]. De Marsac and Cohen-Bazire[12] (1977) first time demonstrated the presence of 15%-20% LPs in PBS on SDS-PAGE and was suggested to have role in assembly and stabilization of PBS. Moreover, they were also found to have assisting role in optimization of absorbance characteristics and energy transfer of the PBPs to favor a unidirectional energy flow from the peripheral PBPs towards the reaction centres *via* PBS core[14]. Besides light-harvesting action, PBS also plays an important role in photo-protection mechanisms under high-irradiances[15]. Moreover, PBS may also be utilized as nutrient source under nitrogen and phosphorus starvation conditions[16].

PHYCOBILIPROTEINS

PBP is the family of brilliantly colored water-soluble pigment proteins. PBPs are classified based on their spectral characteristics. Phycoerythrin (PE, $\lambda_{A\ max}$ = 540-570 nm; $\lambda_{F\ max}$ = 575-590 nm), phycocyanin (PC, $\lambda_{A\ max}$= 610-620 nm; $\lambda_{F\ max}$: 645-653 nm) and allophycocyanin (APC, $\lambda_{A\ max}$ = 650-655 nm; $\lambda_{F\ max}$ = 657-660 nm) are the majorly found PBPs (Figure 3)[17-19]. The bright color, non-toxic protein nature and antioxidant virtues of PBPs open up the doorway for their potential application in various industries. To fulfil the increased demand of PBPs in the market, many scientists have been trying to improve the production and purification of PBPs.

PRODUCTION OF PBPs

Photoautotrophic production

Generally, PBPs are produced photo-autotrophically by two ways: By growing the red algae and cyanobacteria at large scale in open man-maid ponds or in closed bioreactor under the natural sunlight. *Spirulina platensis* is majorly used host for the PBPs production because it can grow in open ponds without being out-competed by contaminating organisms and even in alkaline conditions (pH 10.5)[20,21]. Approximately, > 3000 tons of *S. plentesis* cell mass are produced worldwide predominantly at tropical and subtropical locations around the Pacific Ocean[22,23]. In open pond, the poor availability of light below the surface level is the major problem that hampers the cell mass production. Enclosed photobioreactor (Figure 4) is used as the better option for PBPs production at large scale, which facilitates the equal light provision, CO_2 distribution, temperature control over the outdoor culturing.

Mixotrophic production

Provision of any carbon source enhances the cell mass concentrations as compared to photoautotrophic cultures[24,25]. Vonshak *et al*[24] (2000) have been observed that the maximal photosynthetic rate and the light saturation value of mixotrophically growing culture are higher than that in autotrophic condition. Feeding of glucose to *S. plentesis* was found to increase the biomass production up to 10 g/L[26]. The enhanced tolerance against high light stress was noticed in mixotrophic condition as compared to autotrophic condition[24]. Mixotrophic production is carried out in the presence of some carbon source along with the sunlight in specially designed bioreactors, which helps the photoautotrophs to compete against other contaminating microbes. *S. plentesis* was shown increased PC production in indoor mixotrophic conditions than that in outdoor photoautotrophic cultures[26,27].

Heterotrophic production

Cyanobacteria and red algae living in extreme habitat acclimatized themselves to utilize the carbon source from their surrounding rather than being photo-autotrophic. To produce the PBPs from this extremophiles, the

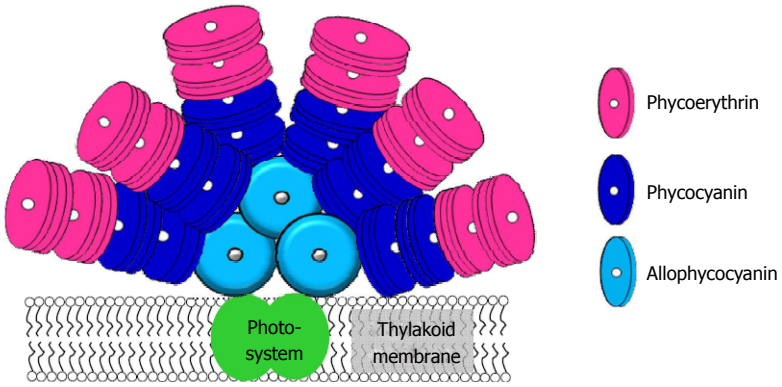

Figure 1 Schematic diagram of phycobilisome situated on the thylakoid membrane.

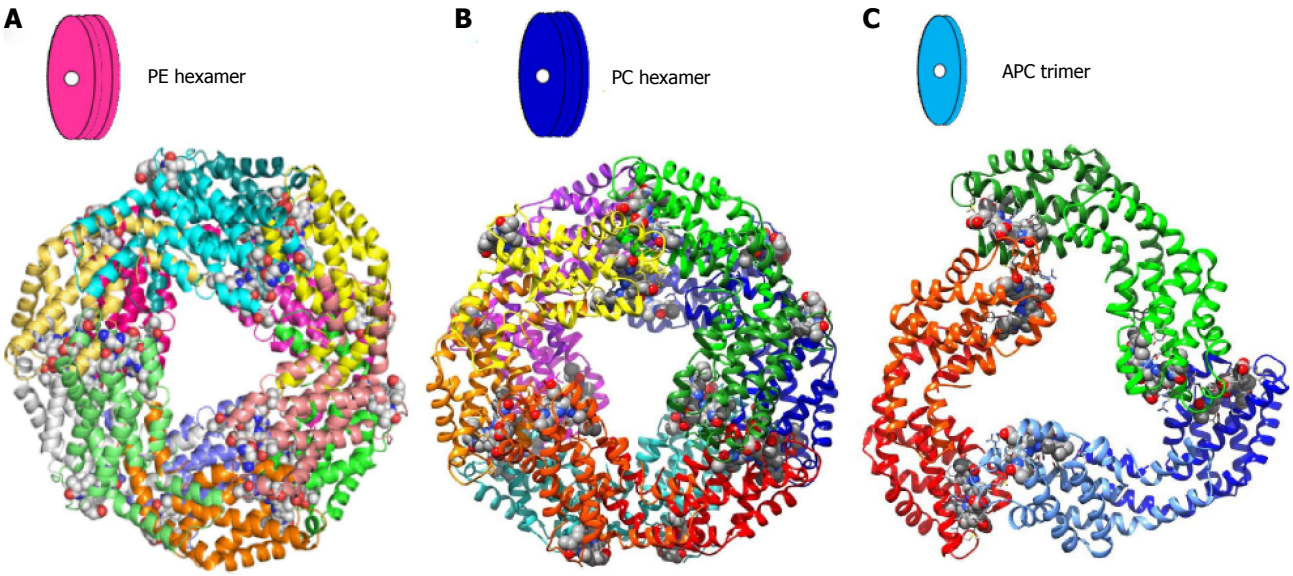

Figure 2 The phycobiliptotein is mainly made up of hexameric and trimeric disks of phycobiliptoteins. Schematic diagram and ribbon model of the structure of A: Phycoerythrin-hexamer; B: Phycocyanin-hexamer; C: Allophycocyanin-trimer. The apoprotein and chromophores are shown by ribbon and ball-stick model, respectively. PBPs: Phycobiliptoteins; PE: Phycoerythrin; PC: Phycocyanin; APC: Allophycocyanin.

heterotrophic strategies are being employed. *G. sulphuraria,* generally inhabiting in hot and acidic niche, is widely used as host for the heterotrophic production of PC[28]. The acidic pH and high temperature of culture decreases the risk of contamination and thus, permits the heterotrophic culture to be an axenic even for long time. The properties of heterotrophic PC resemble those of photoautotrophic origin.

Recombinant production

Although being very difficult, recombinant production is only alternative for the heterotrophic synthesis of PBPs. The co-expression of the numerous gene (for apoprotein synthesis, chromophore synthesis and chromophore attachment) is required for the production of recombinant PBPs. Recently, Overkamp *et al*[29] (2014) have demonstrated the recombinant PBPs production using the eukaryotic PBP lyses. Cherdkiatikul *et al*[30] (2014) cloned and overexpressed the *S. plentesis* allophycocyanin and phycocyanin apo-proteins in *E. coli BL21* through pETDuet-1 vector. Several other reports have also demonstrated the recombinant production of PBPs apoproteins (alpha- and beta-subunits)[31,32], however, the recombinant production of holoproteins is poorly reported[33,34].

PURIFICATION OF PBPs

Due to owing diverse applications in various fields, it becomes essential to develop an easy and cost-effective method for PBPs purification from cyanobacteria to meet its increasing demand. Researchers have made many efforts to purify PBPs with maximum purity and yield. Bermejo Román *et al*[35] (2002) described a fast two-step chromatographic method for the purification of PE from *Porphyridium cruentum, i.e.*, expended-bed adsorption chromatography using streamline DEAE followed by conventional ion-exchange chromatography (DEAE-cellulose). The same authors also demonstrated the baseline separation of α-, β- and γ-subunits of PE by a reverse-phase HPLC gradient semi-preparative method. Rossano *et al*[36] (2003) extracted and purified R-PE

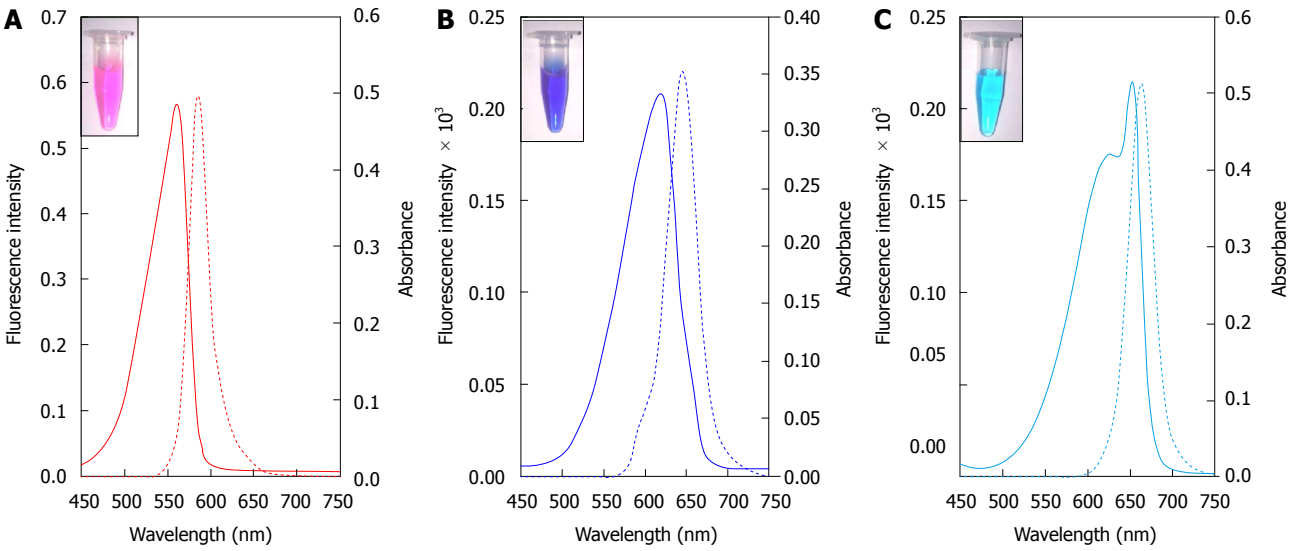

Figure 3 UV-visible absorbance (solid line) and fluorescence emission (dotted line) spectra, and appearance (inset) of A: Phycoerythrin, B: Phycocyanin and C: Allophycocyanin.

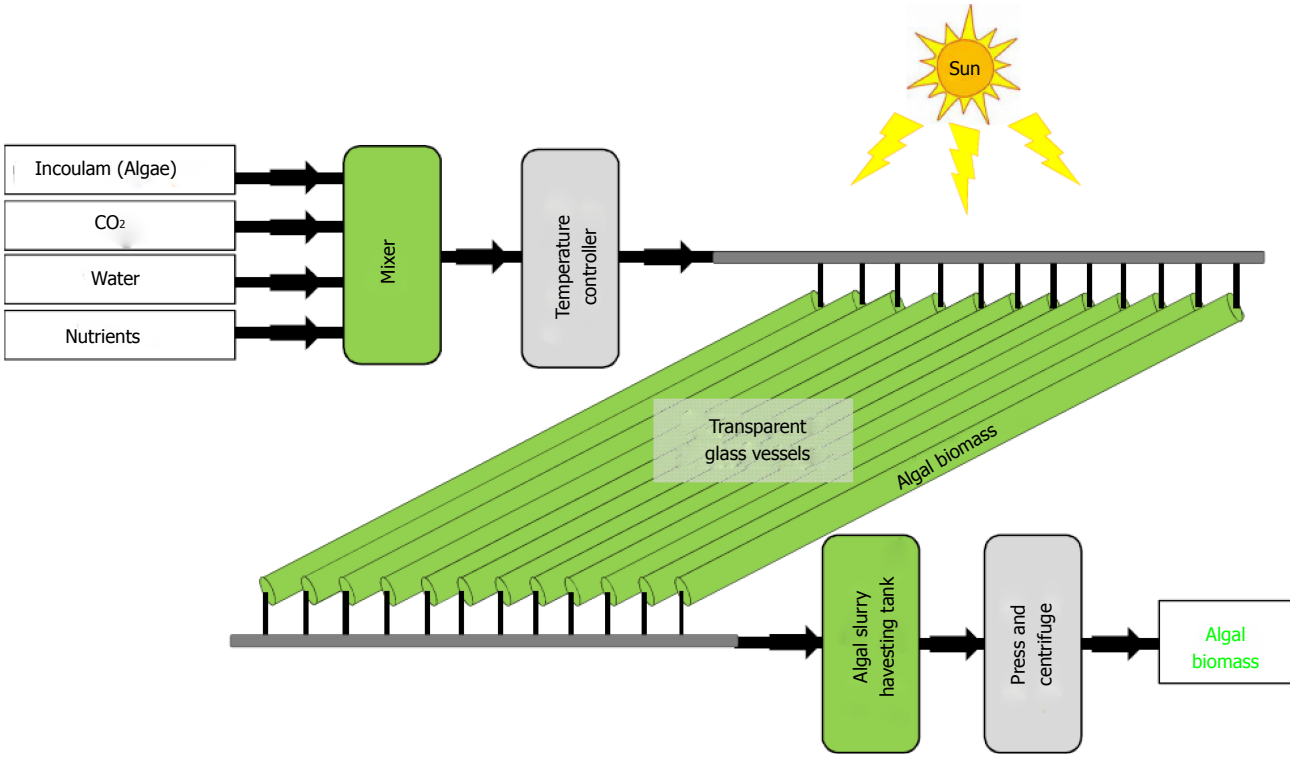

Figure 4 Photo-bioreactor: The graphical representation of the structure and operation of widely used sun light based enclosed photo-bioreactor for the large-scale production of algal biomass.

from Mediterranean red algae *Corallina elongata*, using hydroxyapatite column. Isailovic *et al*[37] (2004) isolated and characterized R-PE subunits and its enzymatic digests. Their results showed efficient absorption and fluorescence of the R-PE subunits and digested peptides, originating from the incorporation of PEB and PUB chromophore groups in them. Tripathi *et al*[38] (2007) extracted and purified an unusual PE from a terrestrial and desiccation-tolerant cyanobacterium *Lyngbya arboricola*. PE was purified using acetone precipitation, gel filtration and ion-exchange chromatography resulting in PE with high purity ratio (> 5). Soni *et al*[39,40] (2006, 2008) purified and characterized PC by using ammonium sulphate precipitation coupled with ion-exchange and hydrophobic interaction chromatography, respectively. Zhang *et al*[41] (1999) purified PC and APC using ion-exchange chromatography, however they used pH gradient to elute proteins rather than slat gradient. Patil and Raghavarao[42] (2007) separated PC and APC using aqueous two phase separation techniques. Recently,

Table 1 List of various procedures employed for phycobiliptoteins purification from cyanobacteria and red algae

PBPs	Ref.	Species	Purification procedure	Purity	Yield (%)
PC	Boussiba *et al*[70]	*S. platensis*	Ammonum sulphate precipitation; Hydroxyapatite chromatography; Ion exchange chromatography	4.15	-
APC	Reuter *et al*[71]	*Rhodella violace*	Gradient centrifugation; Hydroxylapatite chromatography; Preparative "native" PAGE	-	-
PC	Abalde *et al*[72]	*Synechochoccus* sp.	Hydrophobic interaction chromatography; Ion exchange chromatography	4.85	-
PC	Zhang *et al*[41]	*S. platensis*	Ammonum sulphate fractionation; Ion exchange chromatography; Gel filtration chromatography	5.06	-
PE	Galland-Irmouli *et al*[73]	*Palmaria palmate*	Preparative "native" PAGE	3.2	-
PC	Rito-Palomares *et al*[74]	*S. maxima*	Two-phase aqueous extraction; Ultrafiltration; Ammonum sulphate precipitation	3.8	29.5
PC	Minkova *et al*[75]	*S. fusiformis*	Rivanol treatment; Ammonum sulphate precipitation; Gel filtration chromatography; Ammonum sulphate precipitation	4.3	45.7
PC	Soni *et al*[39]	*O. quadripunctulata*	Ammonum sulphate precipitation; Gel filtration chromatography; Anion exchange chromatography	3.31	44.2
PC	Benedetti *et al*[76]	*A. flos-aquae*	Ammonum sulphate precipitation; Hydroxyapatite (electrostatic interaction) chromatography	4.78	-
PC	Patil *et al*[77]	*S. platensis*	Chitosan adsorption; Two-phase aqueous extraction	5.1	66.0
PC	Patil *et al*[77]	*S. platensis*	Chitosan adsorption; Two-phase aqueous extraction; Ion exchange chromatography	6.69	-
PC	Niu *et al*[78]	*S. platensis*	Expanded bed adsorption chromatography; Ion exchange chromatography	3.64	8.7
PC	Patil *et al*[42]	*S. platensis*	Repeated two-phase aqueous extraction; Ultrafiltration	4.05	85.0
PC	Soni *et al*[40]	*P. fragile*	Ammonum sulphate fractionation; Hydrophobic interaction chromatography	4.52	62.0
PC and APC	Yan *et al*[79]	*S. platensis*	Ammonum sulphate precipitation; Ion-exchange chromatography	5.59 and 5.19	67.04 and 80.0
PE	Sun *et al*[80]	*Heterosiphonia japonica*	Ammonum sulphate precipitation; Gel filtration chromatography; Ion exchange chromatography	4.89	-
APC	Parmar *et al*[81]	*Geitlerinema* sp. A28DM	"Ethodin" precipitation; Gel filtration chromatography	3.2	66.0
PE	Niu *et al*[82]	*Porphyra yezoensis*	Expanded bed absorption; Ion-exchange chromatography	> 4	-
APC	Su *et al*[83]	*S. platensis*	Ammonum sulphate precipitation; Hydroxyapatite chromatography; Ion-exchange chromatography	5	43.0
PE	Parmar *et al*[84]	*Phormidium* sp. A27DM, *Lyngbya* sp. A09DM, *Halomicronema* sp. A32DM	Ammonum sulphate precipitation; Gel filtration chromatography	> 3.5	> 60.0
PE	Mishra *et al*[85]	*Pseudanabaena* sp.	Ammonum sulphate precipitation; Gel filtration chromatography; Ion exchange chromatography	6.86	47.0
PC	Johnson *et al*[86]	*Nostoc* sp.	Ion exchange chromatography; Two-phase aqueous extraction	3.55	-
PE, PC and APC	Sonani *et al*[43]	*Lyngbya* sp. A09DM	Triton X-100 mediated ammonium sulphate precipitation; Ion exchange chromatography; Gel filtration chromatography	6.75, 5.53 and 5.43	76.16, 60.23 and 71.91
PE	Johnson *et al*[86]	*Nostoc* sp.	Ion exchange chromatography; Two-phase aqueous extraction	-	-

PBPs: Phycobiliproteins; PC: Phycoerythrin; APC: Allophycocyanin; PE: Phycoerythrin; PAGE: Polyacrylamide gel electrophoresis.

Sonani *et al*[43] (2014a) and Rastogi *et al*[44] (2015c, 2015d) have developed a protocol, which facilitates the purification of all three major PBPs from marine isolate *Lyngbya* sp.. A09DM by Triton X-100 mediated ammonium sulphate precipitation. A novel microwave assisted extraction and pre-purification of PBPs from thick exopolysaccharide cell wall containing *Porphyridium purpureum* was demonstrated[45]. Cuellar-Bermudez *et al*[46] (2015) nicely reviewed various strategies and their successes in purification of PBP from algae. Several protocols employed so far for the purification of PBPs have been depicted in Table 1.

APPLICATIONS OF PBPS

Due to inherent bright color, non-toxic protein nature, easy availability and potential free radical scavenging capacity, the pigment PBPs are being employed in food, cosmetics, pharmaceutical industries and in several other biomedical researches (Figure 5).

Colorant in foods

PBPs are widely used as a natural protein dye in various food and cosmetic industry. PC isolated from *S. platensis* is widely used as a natural pigment in food such as chewing gum, dairy products and jellies[47]. Although, having lower comparative stability in the presence of heat and light, PC is considered more versatile due to its health-beneficial effects than that of gardenia and indigo in jelly gum and coated soft candies[48]. PBPs are also used in many other food products such as fermented milk products, ice creams, soft drinks, desserts, sweet cake decoration, milk shakes, maintaining the color for at least 1 mo at room temperature. Experiments of

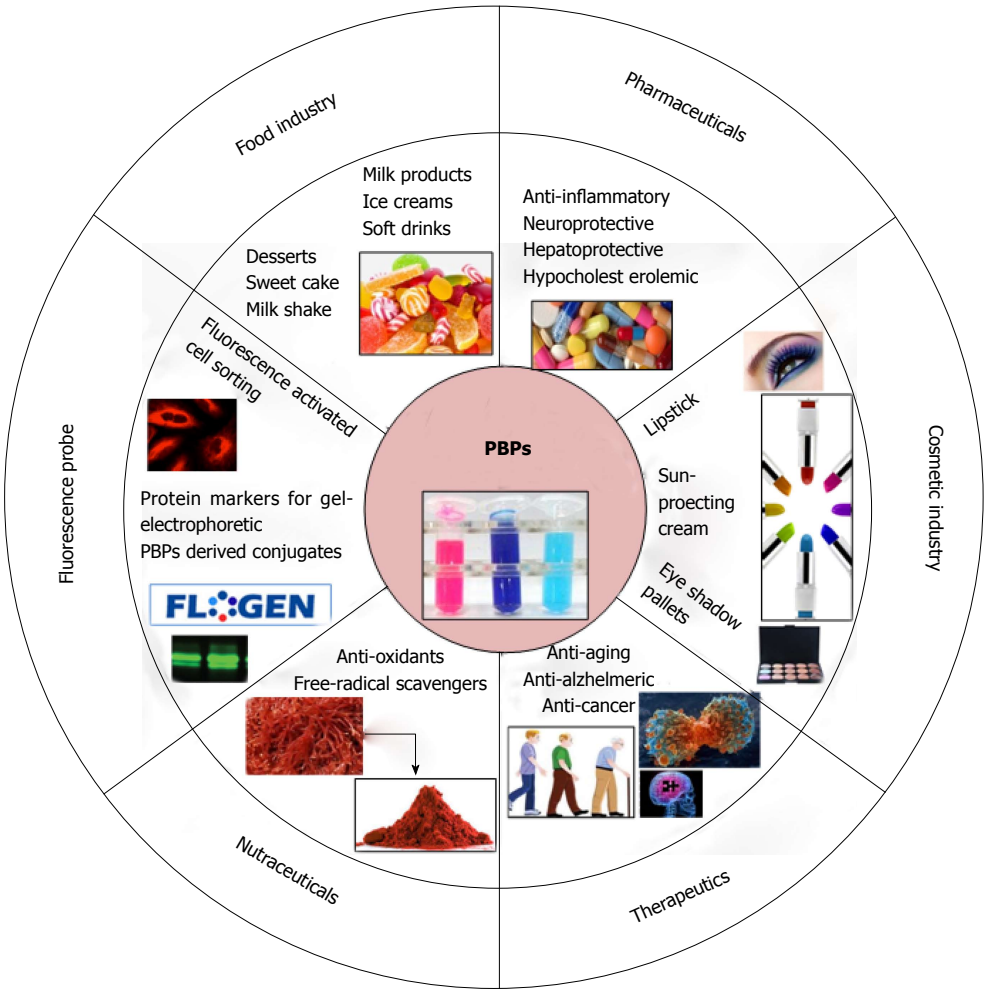

Figure 5 Application of phycobiliproteins in food, cosmetics, pharmaceutical and biomedical industries. PBPs: Phycobiliptoteins.

Jespersen *et al*[48] (2005) regarding the stability PC as colorants, stated that the shade of blue color produced from the red microalga *Phorphyridium aerugineum* does not change with pH (between 5 to 9 pH), temperature (up to 60 ℃) and in the presence of light. However, it is susceptible towards high temperature (> 60 ℃) and within the highly acidic and alkaline pHs. In addition to its colouring properties, PE possesses a yellow fluorescence. Transparent lollipops made from sugar solution, dry sugar-drop candies for cake decoration (that fluoresce under UV light), and soft drinks and fluorescence alcoholic beverages were prepared and tested as a results of enormous efforts to exploit this spectral characteristic[49].

Pharmaceutical and neutraceutical

PBPs isolated from various cyanobacterial species have been reported to show a variety of pharmacological and health beneficial effects including antioxidant, anticancerous, neuroprotective, anti-inflammatory, hepatoprotective and hypocholesterolemic. A number of physiological abnormalities have been averted in various experimental animals by PBPs administration[50-54]. When PC was evaluated as an antioxidant *in vitro*, it was found to scavenge alkoxyl, hydroxyl and peroxyl radicals, and inhibits microsomal lipid peroxidation induced by Fe^{+2} – ascorbic acid or the free radicals initiators 2, 2′-Azobis (2-amidinopropane) dihydrochloride (AAPH)[55-58]. PC reduces the levels of tumor necrosis factor in the blood serum of mice-treated with endotoxin and it showed neuroprotective effects in the rat cerebellar granule cell cultures[59]. PC has been reported to inhibit the cell proliferation of human leukemia K562 cells in a dose- and time-dependent manner[57]. APC, at nontoxic concentration to the host cells (0.045 ± 0.012 μmol/L), was found to inhibit enterovirus 71- induced cytopathic effects, viral plaque formation, and apoptosis[60]. Bei *et al*[61] (2002) found the role of PE in improving the selectivity of photodynamic therapy treatment of mouse tumor cells S180 and human liver carcinoma cells SMC 7721. Sonani *et al*[43,62] (2014a, 2014b) has recently reported the anti-oxidant based anti-aging activity and anti-Alzheimeric potential of PE (isolated from *Lyngbya* sp. A09DM) in wild type and transgenic *Caenorhabditis elegans* (*C. elegans*) (Figure 6). Singh *et al*[63] (2014) have recently given the putative therapy of Alzheimer's disease (AD) by *in silico* molecular docking between the structures of PC and β-secretase (molecule assumed to

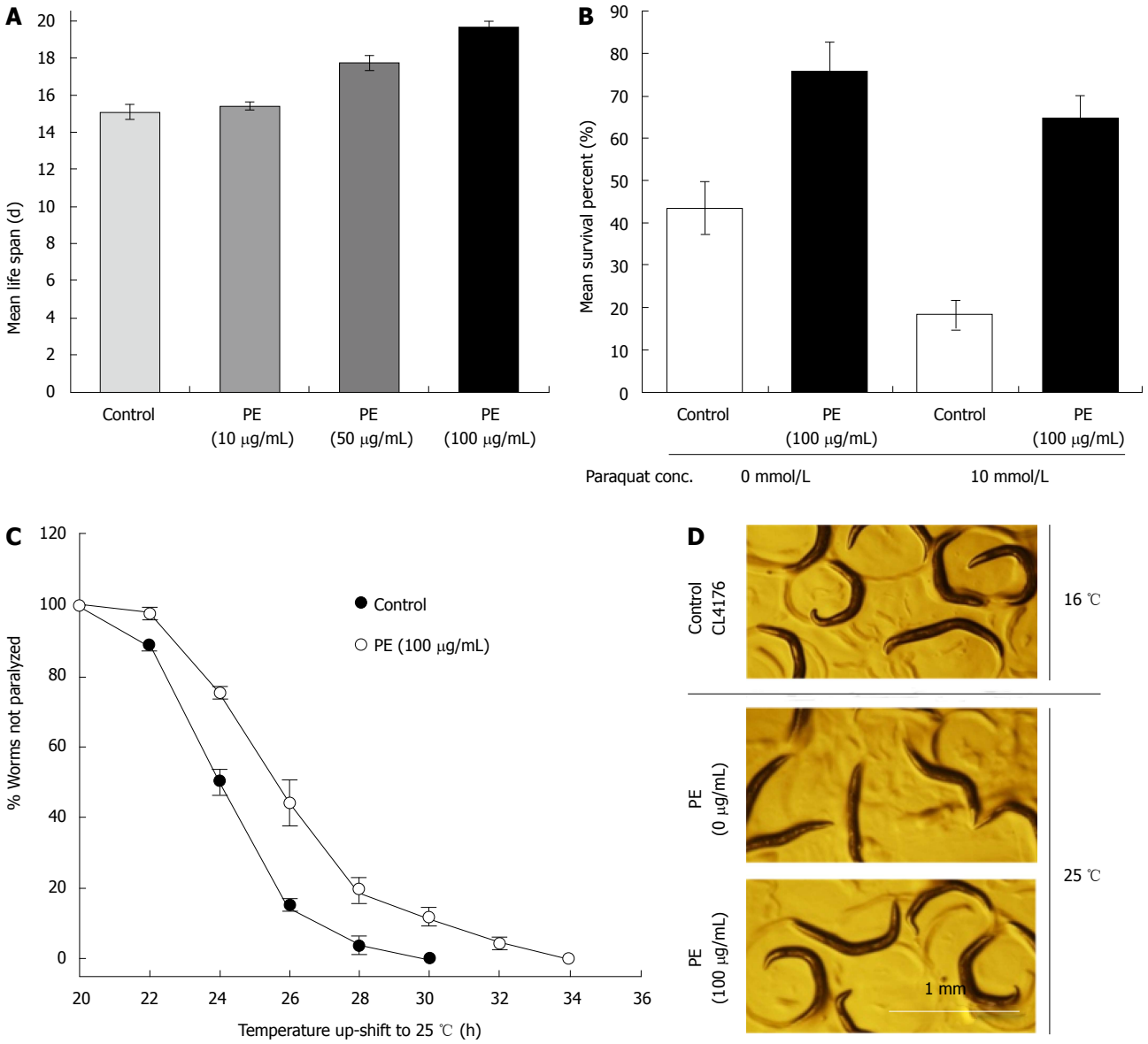

Figure 6 Phycoerythrin-feeding increases mean survival percentage of *Caenorhabditis elegans*. A: Phycoerythrin-feeding increases the mean life span of wild type N2 *C. elegans* in dose-dependent manner[43]; B: Phycoerythrin-feeding increases mean survival percentage of *C. elegans* in under normal and oxidative (paraquat) stressed conditions[43]; C: PE-treatment moderates the heat-induced Alzheimer associated paralysis in the muscles of *CL4176 C. elegans*[63]; D: Representative images of *CL4176* animal grown at 16 ℃, at 25 ℃ and at 25 ℃ with phycoerythrin. Phycoerythrin effectively moderates the Alzheimer associated phenotype in *CL4176 C. elegans*[63]. PE: Phycoerythrin; *C. elegans*: *Caenorhabditis elegans*.

be possible drug target for AD) (Figure 6C). However, the exact mode of actions of PBPs in various diseases is still unclear and further research is needed to elucidate the action point of PBPs in various metabolism pathways.

Fluorescence probe

High molar extinction coefficient of PBPs is attributable to trimeric and hexameric packing as well as higher number of chromophore attached to them in comparison with monomers[64,65]. Due to their unique fluorescent properties, PBPs get applications in flow cytometry, fluorescent immunoassays and fluorescence microscopy for diagnostics and biomedical research[66]. Moreover, they can be used as protein markers for electrophoretic techniques[67]. Synthesis of conjugates of PBPs with molecules having biological specificity, like immunoglobulins, protein A, biotin and avidin, were reported and showed that PBP conjugates are excellent reagents for two color fluorescence analysis of single cells using fluorescence activated cell sorter[66,68]. A series of low-molecular weight PBPs (derived from *Cryptomonad*) are evaluated for their utility for flow-cytometry labelling[69].

CONCLUSION

During last two decade, the field of PBPs research has made the significant progress. New purification methodology allows obtaining the ultra-pure PBPs at

large scale. Some new applications of PBPs in pharmaceuticals and nutraceuticals create more demand of PBPs in the commodity-markets. To meet such increasing demand of PBPs, it is very necessary to develop the new strategies for mixotrophic and heterotrophic production. A protein engineering or recombinant strategy also seems to be promising options to enhance the spectral properties and stability PBPs. Furthermore, more extensive research in the field of PBPs-application is still needed to elucidate the complete mode of action of PBPs during its anti-oxidants, anti-proliferative, anti-aging and neuro-protective effects using various model organisms.

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