Purification of phycocyanin from isolated and identified hot spring cyanobacteria

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Received 15 March 2017; revised 13 March 2018

Phycocyanin is a phycobiliprotein used as a healthy food additive as well as an ingredient in cosmetic dye preparation. In this study, we attempted molecular and morphological identification of three therophilic filamentous cyanobacteria and also explored a method to separate and purify thermostable C-phycocyanin of analytical grade from them for biotechnological applications. Cyanobacteria strains were collected from hot springs in Karakoc-Seeferihisar, Gulbahce-Urla and Sifne-Cesme in Izmir, Turkey. The samples were identified both by morphological observations and genetic assay. Filamentous cyanobacteria DNA from the collected samples was isolated and extracted, and were analyzed using a 16S rRNA cyanobacteria-specific PCR and a denaturing-gradient gel electrophoresis (DGGE). Extraction and purification of phycocyanin was performed in two stages, ammonium sulfate saturation and dialysis, gel filtration and ion exchange chromatography. Conventional separation of Geitlerinema sp., Halospirolina sp. and Phormidium animale was successfully performed yielding C-phycocyanin at 493.760±3.610, 89.060±3.209, 32.978±0.350, 4.046±0.193, 8.303±0.511 and 4.196±0.090 mg/g purity from gel filtration and ion exchange chromatography processes, respectively. The SDS-PAGE demonstrated that the P. animale, Halospirolina sp. and Geitlerinema sp. were purified phycocyanin. Overall, in this study, three cyanobacteria were isolated and cultivated in laboratory conditions, and then the cyanobacterial cells was extracted and purified thermostable C-phycocyanin obtained, which may be used as raw material in food supplement and pharmaceutical industry.

Keywords: DNA isolation, Geitlerinema sp., Halospirolina sp., Phormidium animale, Phycobiliproteins

Cyanobacteria are prokaryotic microorganisms, which have multi-beneficial features, including nutrition and pharmacutic such as immuno-enhancing antioxidants, antiviral, and anti-inflammatory affects. Cyanobacterial diversity was initially discovered during the 19th century, and later was defined as a separate group of organisms. This classification is based on the morphology of the isolated strains and samples. Thus, most of the morphological traits are cell dimension, cell/filament morphology, the type of cell division, and the existence of sheath. Cyanobacterial systematics has been altered using molecular markers of significantly 16S rRNA since the 20th century although it should be emphasized that the significance of morphological features is renowned. Hence, the taxonomic system of the cyanobacteria is especially combined with the morphological and molecular methods determined characterization of the cyanobacterial taxa.

Phycobiliproteins (PBPs) located in the thylakoid membranes of the cyanobacterial cells are harvested by light used in the membrane-bound chlorophyll protein complexes. Based upon their spectral and specific properties, PBPs are classified in four major groups; phycoerythrin-pink colour (490-570 nm), phycoerythrocyanin-redish-purple (570-595 nm) phycocyanin-dark blue (610-625 nm) and allophycocyanin-brighter blue (650-660 nm). PBPs content in the cells is as high as 60% of the mass of water soluble proteins or 20% of the overall dry mass. Maximum absorption of the thermophilic cyanobacteria has a C-phycocyanin (C-pc) and allophycocyanin at a wavelength of 608 nm. Phycocyanin is commercially used potential applications as non-toxic and non-carcinogenic healthy blue protein in the food additive and cosmetic dye.

In this study, we explored three filamentous cyanobacteria cyanobacteria samples (Phormidium animale, Halospirolina sp., and Geitlerinema sp.) found in hot spring areas in Izmir-Turkey as a potential source of C-phycocyanin. Further, we attempted cultivation of these identified cyanobacteria for biomass and also purification of thermostable C-phycocyanin.

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Material and Methods

Collection, isolation and cultivation of the sample

The organisms were collected from mats in Sıfne-Cesme, Gülbahce-Urla, and Karakoc-Seferihisar hot springs with water temperatures ranging from 40 to 75°C and intense mineral water such as bicarbonate, sodium chloride, etc. The cultures were cultivated in prepared of filtrated thermal waters in Blue-green algae medium (BG-11). The isolations of the strains were done using the serial dilution and the streaking plate method. All the isolates were grown under a light with photon flux densities 50 µE m⁻² s⁻¹ at 45±2°C. The cyanobacteria cells were transferred to a fresh medium several times, and then grown on BG-11 agar plates containing 1.5% agar for one to three weeks. The isolation of the filamentous cyanobacteria cells were picked on the BG-11 agar plates and transferred to a liquid medium. The isolates were incubated at 45±2ºC under the light intensity of 40 µE m⁻² s⁻¹ in a 1 L bottle, and aerated at a flow rate of 2 L min⁻¹ for 20 days.

Morphological identification

The cell morphologies were clarified with microscopes. The light, fluorescence, and scanning electron microscopy (SEM) images were used for the identification and morphological observation of the cultures. The living cells were photographed by a bright field microscopy using a Leica DMIL fluorescent microscope (Leica, Germany) with 40X. The freeze-dried species were coated with gold for 2 min, and then examined using a FEI Quanta 250 FEG scanning electron microscope (FEI Company, Czech Republic). The species classification was used as references by Desikachary, 1959 as well as Komárek & Anagnostidis 2005.

Molecular identification

A total of 10 mL cultures were harvested by centrifugation at 3500 rpm for 10 min. The concentrated cells were transferred into 1.5 mL micro tubes and stored at −20°C until the DNA extraction (Quick-DNA™ Fungal/Bacterial MiniPrep, ZymoResearch). A 16S rDNA amplification was performed in 25 mL reactions using primers 27F and 809R, PCβF and PCαR (phycoerythrin gene), CYA 359F and CYA 781R (a, b) in Table 1. For PCR amplification, we used PCR primers that were targeted to bind the nuclear 16S rDNA. The PCR reaction was performed with HelixAmp™ HyperSense DNA polymerase, Nannohelix at 95°C for 2 min, following 35 cycles of 95°C for 20 s, annealing temperature of 60-55-65°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 5 min (Table 1).

The resulting PCR products were electrophoresed in 1.0% agarose gel, stained with SYBR, and visualized by ultraviolet trans illumination. In the amplification step, the dye terminator sequencing was done using the primers, and the nucleotide sequences were determined by a genetic analyzer (Applied Biosystems 3130XL with16-capillary array). The nucleotide sequences were subjected to a BLAST analysis.

For DGGE, the 16S rRNA gene regions, approximately 400 bp in length, were amplified by PCR using the cyanobacteria-specific primers CYA359F and CYA781R [CYA 781R (a), CYA 781 R (b)] with a (GC)ₐ clamp added to the forward primer (Table 1). A second PCR reaction was subjected to DGGE analysis using DGGE (TV400-Scie-Plas). The polyacrylamide gel (8% w/v in 1× TAE buffer (Tris-acetate-EDTA) was 1 mm thick, and had a denaturant gradient of 30-70%, where 100% denaturant constitutes 7 M urea and 40% formamide. The samples (45 µL) were run at 60°C at 60 V for 16 h in 1× TAE). The gel was stained with Gel Green (Biotium, 1:10,000 dilutions in 1× TAE buffer) for 30 min and photographed.

Table 1 — List of primers used

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target population</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>Bacteria</td>
<td>5'-AGAGTTTGATCTTGCAGTCAG-3'</td>
<td>60</td>
</tr>
<tr>
<td>809R</td>
<td>Bacteria</td>
<td>5'-GCTTCGGCAGGCTCGGTCGATA-3'</td>
<td>60</td>
</tr>
<tr>
<td>PCβF</td>
<td>Cyanobacteria</td>
<td>5'-GGCTGCCTGTGTAGCGCGACA-3'</td>
<td>55</td>
</tr>
<tr>
<td>PCαR</td>
<td>Cyanobacteria</td>
<td>5'-CCACTAACAACAGCAACTAA-3'</td>
<td>55</td>
</tr>
<tr>
<td>CYA 359F*</td>
<td>Cyanobacteria</td>
<td>5'-GGGAAATYYTTCCGCAATGGG-3'</td>
<td>65</td>
</tr>
<tr>
<td>CYA 781R(a)</td>
<td>Cyanobacteria</td>
<td>5'-GACTACTGGGGTATCTAATCCATT-3'</td>
<td>65</td>
</tr>
<tr>
<td>CYA 781R(b)</td>
<td>Cyanobacteria</td>
<td>5'-GACTACAGGGGTATCTAATCCATT-3'</td>
<td>65</td>
</tr>
</tbody>
</table>

*Contains a 40 base GC-clamp attached to the 5’ end, as follows:
CGCCCGCCGCGCCGCGCCGCGCCGCGCCGCGCCGCGCCG
Extraction of phycocyanin

The cold maceration method was used to optimize the isolation of the C-phycocyanin (C-PC). The C-PC was extracted from freeze-dried cyanobacteria powder 1:20 (w/v) in a 0.005M Na-phosphate buffer added in 1.5% CaCl₂ at 4°C for overnight

The C-PC, allophycocyanin (APC), and phycocerythrin (PE) concentration was measured by the optical densities at 562 nm (A₅₆₂), 652 nm (A₆₅₂), 615 nm (A₆₁₅) and calculated using the following equation. In each extraction stage, the concentration was calculated from the absorbance by using the method of Khazi et al.¹².

\[
C - PC (mg/mL) = \frac{A₆₁₅ - (0.474 \times A₆₅₂)}{5.34} \\
APC (mg/mL) = \frac{A₆₅₂ - (0.208 \times A₆₁₅)}{5.09} \\
PE (mg/mL) = \frac{A₆₅₂ - (2.41 \times PC) - (0.849 \times APC)}{9.62}
\]

The extraction yield was calculated as per Khazi et al.¹².

\[
Yield \left( \frac{mg}{g} \right) = \frac{(C - PC \times v)}{DB}
\]

where C-PC (mg/mL); v (mL), volume of the buffer; and DB (g), the dried cell mass. The assays were calculated in triplicate and the data were denoted as value ± standard deviation (SD).

Purification of C-phycocyanin

Ammonium sulfate (AS) and dialysis

AS precipitation was gradually added in 100 mL crude extracts to reach 35% with gently stirring. The resulting solution was kept for 4 h at 4°C and centrifuged at 6000 rpm for 30 min. The supernatant was pooled and subjected to 70% saturation of the AS similar to that in the first operation. The obtained blue precipitate was dissolved in a 0.005 M Na-phosphate buffer (pH 7.0)¹³. The obtained crude extract was dialyzed using dialysis tubing cellulose membrane (Sigma Aldrich) against the same buffer at 12 h. The dialyzed sample was centrifuged at 6000 rpm for 30 min and supernatant purified by passing through a gel filtration.

Gel filtration

The Sephadex G-50 column (Sigma Aldrich, Isolab 40 × 2 cm) was eluted with the same buffer. The fractions were collected at a 1 mL/min flow rate¹⁴.

Ion exchange chromatography (IEC)

The IEC was prepared using DEAE-cellulose in a column (Merk, Isolab 40 × 2 cm). The column was pre-equilibrated with a 0.005 M Na-phosphate buffer (pH 7.0). The column was first eluted with 0.15 M NaCl, and the C-PC was then eluted with 0.25 M NaCl in buffer. Finally, the column was eluted and cleaned with 1.5 M NaCl¹⁵.

Total protein determination and SDS-PAGE analysis

The protein contents of the extracts were determined by the Bradford methods¹⁶. Then, the purity of all the fractions was checked by equation and fractions showing maximum purity were selected for Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out according to Minkova et al.¹⁷ using a 12% polyacrylamide gel and coomassie brilliant blue R was used for visualization of the proteins. The molecular weights of subunits were determined by molecular weight markers (Fermentas SM1811).

Results and Discussion

Collection and isolation of the sample

This study worked on the morphological, molecular identification and isolation of cyanobacteria collected from hot springs (Sifne-Cesme, Gulbahce-Urfa and Karakoc-Seferihisar) in Izmir, Turkey. The unicellular cyanobacteria tolerant to both high temperatures and intense mineral concentrations thrive in North America, Japan and the eastern Mediterranean however filamentous cyanobacteria teem in thermophilic areas worldwide¹⁸. The polyphasic taxonomic identification of thermophilic cyanobacteria studied in the world especially East Africa, Russia, Taiwan, Jordan, India, Chile, Algeria, Italy¹⁹. Eight different cyanobacteria species were identified using morphological characteristics in the hot spring at Pancharevo in Bulgaria²⁰. In addition, many hot springs are available in different regions across the country in Turkey; Aegean region of Turkey is located in the thermal waters that found remarkable geothermal fields²¹,²². The isolation of the cyanobacteria species was done by preparing from two different water media (distilled water in BG-11 and filtrated thermal water in BG-11). It was determined that the isolated colonies were best prepared from the filtrated water in BG-11 medium. Due to the thermal water has to contain a sodium chloride, bicarbonate, sulphate and calcium. Consequently, three different filamentous cyanobacteria strains (Phormidium animale, Halospirulina sp. and Geitlerinema sp.) were isolated and cultivated on the thermal water BG-11 agar medium.
Morphological and molecular identification

The phenotype of the strains was analysed by bright field, epifluorescence, scanning electron microscopy (SEM). *Phormidium* was a filamentous, non-heterocystous cyanobacterium, which represents morphologically in many various genera in all environmental conditions. The sample of *Phormidium animale* had straight trichomes with unconstricted cross-walls, unbranched, uniserial, thin sheaths, with cells 1.5-2.5 µm wide, and 4.5-5.5 µm long. Apical cells were conical or rounded (Fig. 1A). *Halospirulina* sp. was filamentous, closed helically coiled trichomes, no sheaths, gliding motility, helix widths between 1.2 and 1.5 mm, and trichome widths between 0.2-0.5 mm. These morphological features demonstrated that the isolate is related to *Arthrospira* (*Spirulina*) sp. (Fig. 1B). *Geitlerinema* was a non-heterocystous, filamentous cyanobacterium with thin (<4 µm) and motile filaments. *Geitlerinema* sp. had never branched trichomes with 1.5-1.8 µm wide, and 4.0-4.8 µm long. Trichomes varied in shape from a slope to instantly; they had not been constricted on the pass cell walls and had been seldom dwindled closer to their ends. Trichome cells were cylindrical. Trichomes had verified mobility through their sliding (Fig. 1C).

Considering the morphological characteristics of the apex of the filament, the *Phormidium* is divided into 8 groups. When there is similarly morphological variability in the genus, *Phormidium* cell is nonstriking that it has been located to be polyphyletic and consist of many dissimilar species utilizing

![Fig. 1](image-url) — Light (i), epifluorescence (ii) and scanning electron (iii) photomicrographs of (A) *Phormidium animale*; (B) *Halospirulina* sp.; and (C) *Geitlerinema* sp.
sequences of 16S rRNA and 16S-23S ITS\textsuperscript{21}. The members of the family of Phormidium to Lyngbya—Plectonema have similar morphological characteristics, so they have been confused many times\textsuperscript{24}.

Bittencourt-Oliveira \textit{et al.}\textsuperscript{25} studied 10 strains of Geitlerinema sp. examined by DNA sequencing and the transmission of electronic and optical microscopy. As a result, the identification of Geitlerinema was needed as many studies have shown it was far more polyphyletic\textsuperscript{26}. Loza \textit{et al.}\textsuperscript{24} explained that morphologically similar strains could also vary genotypically. Hence, molecular identification comprising of certain gene areas in combination with a morphological measure, would provide the higher taxonomic resolution of the filamentous cyanobacteria. The taxonomists were determined by using new taxonomic devices making re-evaluations and the taxonomic revisions of many cyanobacteria species and genera. The cyanobacterial mats were frequently dominated by way of morphologically defined cyanobacteria, such as Phormidium, Oscillatoria, Pseudanabaena, Calothrix and Fischerella at the lower end of the thermophile (40-50°C)\textsuperscript{27}.

At three genotypes of cyanobacteria were indicated the relationship of the 16S rRNA gene using universal primers stated that three isolates were related to the genus Halospirulina sp., Geitlerinema sp. and Phormidium animale. All of the 16S rRNA gene sequences (Genbank accessions \textit{Halospirulina} sp.-Gulbahce HQ916862, \textit{Geitlerinema} sp.- Sifne HQ197684, \textit{P. animale} Karakoc HQ916864) were blasted at NCBI GenBank database, and all of sequences identified to express a high level of resemblance were used to analyze relationships for the sequences obtained in this study. A specimen of this data set is declared in Fig. 2. The DGGE profiles of three different cyanobacteria isolated from thermal water and their matching field samples brought out bands that moved to identical positions (Fig. 3). All strains were put under protection from Ege University Microalgae Culture Collection-EGEMACC (http://www.egemacc.com/cultures.php), Izmir-Turkey (\textit{Geitlerinema} sp. EGEMACC 29, \textit{Halospirulina} sp. EGEMACC 31 and \textit{P. animale} EGEMACC 90).

\textbf{Extraction and purification of phycocyanin}

Thermophilic microorganisms are sources of thermostable enzymes and exopolysaccharides, which shown lots of features that might be suitable for using biotechnological application and pharmaceutical\textsuperscript{28}. A significant role for being the source of phycocyanin (the blue pigment) is utilized cyanobacteria, and so \textit{Spirulina} is rich supply of precious chemical substances commercialized for medical purpose as biomarkers, in the nutraceutical products as a natural blue pigment and in aquaculture feed as a protein-rich food residue\textsuperscript{29}. Phycocyanin is brilliantly coloured,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{(A) DNA isolation; and (B) PCR products of \textit{Halospirulina} sp., \textit{Geitlerinema} sp., \textit{Phormidium animale} electrophoresed in the agarose gel. [M: molecular marker 1 kb (New England Biolabs N3232S)-A, 100 bp (New England Biolabs N3231S)-B]}
\end{figure}
and is a stable fluorescent pigment protein soluble in water, which can be categorized into three essential groups: phycocyanin, phycoerythrin and allophycocyanin are attached to the inherent color and absorbance properties\textsuperscript{12}. While, this study has found that three different cyanobacteria species did not detect phycoerythrin. Accordingly, thermophilic cyanobacterium of *Synechococcus lividus* contained C-phycocyanin, allophycocyanin and absent in from phycoerythrin\textsuperscript{30}.

Extraction of phycocyanin was used in 0.005M Na-phosphate buffer added in salt concentration (CaCl\textsubscript{2}), 15 g 1\textsuperscript{1}, pH 6.8 at 4\textdegree C for the overnight period. Purification of phycocyanin was performed by two styles: ammonium sulfate precipitation and dialysis, gel filtration and ion exchange chromatography. The two step ammonium sulfate fractionation (35% and then 70%) procedure was used for the purification of the phycocyanin. Extraction of phycocyanin should be optimized by CaCl\textsubscript{2} treatment for cyanobacteria. Cell membranes absorb Ca\textsuperscript{2+} easily and the porosity of the membrane relevant to interactions of calcium ions. The binding depend on Ca\textsuperscript{2+} concentration to the lipid membrane bring about alters in the permeability especially rigidifies\textsuperscript{31}. Ilter et al.\textsuperscript{11} reported that the optimization of various phycocyanin extraction methods from *Arthrospira platensis* biomass and the highest phycocyanin content was extracted by CaCl\textsubscript{2} (1.5%). The ammonium sulfate procedure is especially useful in salting out unwanted proteins and at the same time in concentrating the principal phycocyanin. Many of procedures have been notified for the purification of phycocyanin from cyanobacteria not only chromatographic but also non chromatographic methods extraction\textsuperscript{12}. Hence, the purification stage of C-PC involves lots of process stages and as is known that at each step there may be a loss of product yield. Purity ratio is a determinant of purification in which above 1 is considered as food grade, above 4.0 as analytical grade\textsuperscript{32}. *Halospirulina* sp., *Geitlerinema* sp. and *P. animale* of the phycocyanin (C-pc), allophycocyanin (APC), phycocyanin yield and purity ratio ($A_{620}/A_{280}$) values obtained by extraction and two purification process are presented in Table 2. It was observed that among the extracted and purified phycocyanin from different species presented, *Geitlerinema* sp. found to be the highest C-pc (mg/mL) content and yield (mg/g). However, *Halospirulina* sp. of purity ratio reached the highest value (8.303±0.511). The C-pc yield decreased substantially with an increase in the number of purification process. Ammonium sulfate

![Image](image_url)

**Fig. 3** — The polyacrylamide gel of DGGE (The gel: M molecular marker 100 bp (HelixPuler\textsuperscript{TM} HR100BL), *Halospirulina* sp. 1a, 359F-781R(a); 1b, 359F-781(b); 1c, 359F*-781R(a); 1d, 359F*-781(b); *Geitlerinema* sp. 2a, 359F-781R(a); 2b, 359F-781(b); 2c, 359F*-781R(a); 2d, 359F*-781(b); *Phormidium animale* 3a, 359F-781R(a); 3b, 359F-781(b); 3c, 359F*-781R(a); 3d, 359F*-781(b))

### Table 2 — Extraction and purification measure of C-phycocyanin, allophycocyanin, yield and purity ratio

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Dialysis</th>
<th>Gel filtration and Ion exchange chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Geitlerinema</strong> sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-pc (mg/mL)</td>
<td>APC (mg/mL)</td>
<td>Yield (mg/g)</td>
</tr>
<tr>
<td>24.450 ±0.153</td>
<td>3.517 ±0.028</td>
<td>1434.331 ±8.998</td>
</tr>
<tr>
<td><strong>Phormidium animale</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.925 ±0.198</td>
<td>0.107 ±0.002</td>
<td>54.159 ±11.619</td>
</tr>
<tr>
<td><strong>Halospirolina</strong> sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.987 ±0.164</td>
<td>0.239 ±0.004</td>
<td>233.431 ±9.615</td>
</tr>
</tbody>
</table>

*value mean±standard deviation (n=3); - not detected*
when followed by dialysis proved to be an effective precipitating and purification of phycobiliproteins. Accordingly, Sephadex G-50 column and DEAE-cellulose improved the purification ratio (620 nm to 280 nm absorbance) to increase from 0.77±0.001 up to 8.30±0.51 (Table 2). However, Bhaskar et al.\(^{13}\) used Sephadex G-25 and Sephadex G-100 column and gave a purity of 3.73 and 4.98. Kamble et al.\(^{14}\) have reported purity values the absorbance \(A_{620}/A_{280}\) ratio of ranging from 1.26-3.1 at 50% ammonium sulfate precipitation in Calothrix sp., Phormidium sp., Oscillatoria sp., Lyngbya sp., Anabaena marina, Nostoc sp., Synechococcus sp., Oscillatoria quadric-punctulata, P. ceylanicum, Aphanozomenon flosaquae and Arthronema africana strains. After running the fractions at the DEAE-cellulose, results of purified fraction showed the presence of C-pc on SDS-PAGE (Fig. 4). The SDS-PAGE results revealed that the proteins of the phycocyanin were purified and contained the two subunits (alpha and beta) and molecular weights of C-pc. The purified C-pc samples were further confirmed to specify the molecular weight by SDS-PAGE\(^{34}\).

Thermophilic cyanobacteria can thrive at both high temperature and high mineral concentration and also differ in stability when compared to mesophilic microalgae\(^{21,28}\). In addition, the identified thermophilic cyanobacteria species herein, have been demonstrated to be thermostable and also a high quality source of C-pc as a natural colorant for functional food industry.

**Conclusion**

The molecular and morphological investigations have confirmed the isolated samples from the hot spring water to be *Halospiroolina* sp., *Geitlerinena* sp. and *Phormidium animale*. The purification results suggest an easy and efficient method for purification of C-phycocyanin (C-pc) from these thermophilic cyanobacterial strains. Thermostable C-pc is a source of supplement material for various applications in food, cosmetics, medicine and biotechnology.

**Acknowledgement**

This study was financially supported by The Scientific and Technological Research Council of Turkey-TUBITAK (109T037) project and Ege University Science and Technology Centre-EBİTEM (09 BIL 027) project.

**Conflict of Interest**

None

**References**
