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# Study of temperature and food-grade preservatives affecting the *in vitro* stability of phycocyanin and phycoerythrin extracted from two *Nostoc* strains

## Vpliv temperature in živilskih konzervansov na *in vitro* stabilnost fikocianina in fikoeritrina, ekstrahiranega iz dveh sevov vrste *Nostoc*

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**Abstract:** Cyanobacteria have many bioactive compounds. In the present study, we investigated the degree of purification and free radical scavenging ability of phycocyanin (PC) and phycoerythrin (PE), and compare their stability against selected preservatives at different temperatures with the aim of achieving the best and most stable preservative in increasing shelf life of PC and PE. After collecting and culturing *Nostoc* sp. strains FSN and ASN in BG-$11_0$ medium, the pigments phycocyanin and phycoerythrin were extracted and purified with 56% ammonium sulfate followed by dialysis. The antioxidant activity of pigments was evaluated by DPPH and ABTS assays. Their stability was compared with food-grade preservatives citric acid, sodium chloride, sucrose, and calcium chloride at two temperatures of 5 °C and 35 °C over time period from 3 to 30 days of cultivation. The results showed that the concentration and purity of the pigments increased after the dialysis, the pigments had antioxidant properties and were more stable at 5 °C. In addition, among different preservatives, citric acid caused more stability over time.

**Keywords:** bioactive compound, cyanobacteria, food-grade preservative, *Nostoc,* pigment

**Izvleček:** Cianobakterije vsebujejo mnoge bioaktivne spojine. V naši razskavi smo ekstrahirali barvili fikocianin (PC) in fikoeritrin (PE) in preučili njuno sposobnost odstranjevanja prostih radikalov ter primerjati njuno stabilnost v primerjavi z izbranim konzervansom pri različnih temperaturah z namenom doseganja najboljšega in najstabilnejšega konzervansa za podaljšanje roka uporabnosti PC in PE. Uporabili smo dva seva FSN in ASN vrste *Nostoc* sp., jih gojili v gojišču BG-110, ekstrahirali barvili in ju nadalje očistili s 56% amonijevim sulfatom in dializo. S testoma DPPH in ABTS smo ovrednotili antioksidativno aktivnost barvil. Njuno stabilnost smo primerjali z živilskimi konzervansi citronsko kislino, natrijevim kloridom, saharozo in kalcijevim kloridom pri 5 °C in 35 °C in v časovnem obdobju od 3 do 30 dni gojitve. Rezultati so pokazali, da sta se koncentracija in čistost barvil po dializi povečala, da imata barvili antioksidativne lastnosti in da sta bolj stabilni pri 5 °C. Izmed uporabljenih konzervansov je bila najbolj stabilna citronska kislina.

**Ključne besede:** bioaktivna spojina, modrozelene bakterije, *Nostoc*, barvilo, živilski konzervans

## Introduction

Cyanobacteria are a diverse group of photosynthetic prokaryotes. They have the ability to photosynthesize oxygen, which is similar to ability of plants. However, instead of chloroplasts, cyanobacterial photosynthesis takes place in thylakoid membranes (Vothknecht and Westhoff 2001). Worldwide attention is drawn towards cyanobacteria for their possible use as food, feed, biopolymer, biofuel, and bio-fertilizer, for production of various secondary metabolites including vitamins, toxins, enzymes, pharmaceuticals, pharmacological probes, and pollution abatement (Righini et al. 2022). Phycobilisomes are light sensitive complexes in cyanobacteria that are located on the thylakoids and are essentially made up of protein pigment complexes known as phycobilia proteins (MacColl 1998). Phycobiliproteins (PBP) are divided into three groups based on their properties: phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC). They are also considered as environmentally friendly, non-toxic and have anti-cancer activities (Eghtedari et al. 2021). Among these pigments, PC and PE have been shown to have broad health properties that are used in food, pharmaceutical and cosmetic industries (Nowruzi and Porzani 2021). Recently, more attention is given to the application of PC and PE utilizing their biochemical and biophysical structural properties (Annibal et al. 2016). PC and PE are also used in fluorescent labelling of antibodies that are applied in diagnostic kits in immunology, cell biology and biomedical research (Sekar and Chandramohan 2008). PC, an accessory pigment to chlorophyll, is a pigment-protein complex of the phycobiliprotein family that occurs in many cyanobacteria and some red algae. Also, PC is a nutraceutical compound with antioxidant properties. It prevents oxidative damage and is used for human consumption as a colorant in food and milk shakes, and as an ingredient in cosmetic and pharmaceutical formulations (Mishra et al. 2010). PE is also good for human health, having antioxidant, radical scavenging, anti-inflammatory and anticancer properties. PE can be used as nutrient ingredient and natural dye for food and cosmetics besides, being potential therapeutic agent in oxidative stress-induced diseases and as a fluorescent marker in biomedical research (Mishra et al. 2010).

PC is formed by α- and β-subunits and has linear tetrapyrrole phytochromobilin covalently attached to its polypeptides via a thioether linkage to a conserved cystein residue (Stadnichuk et al. 2015). PC, exists as a complex interacting mixture of trimer, hexamer, and decamer aggregates which has been reported to be a function of pH and ionic strength of the medium, protein concentration, and algal origin (Mishra et al. 2008, Giannuzzi 2019). The stability of PC depends on its origin, pH, temperature, light, and some exogenous substances (Wu et al. 2016). Minor differences in the amino acid composition of PC affect the stability and structural properties of these proteins (Teng et al. 2010). Stabilizing agents are usually added to PC solutions to ensure long-term stability (Wu et al. 2016).

Antioxidants can prevent the production of reactive oxygen species (ROS) and scavenge them (He and Häder 2002). Excess formation and/or insufficient removal of ROS causes oxidative stress, which is related to various diseases such as cancer, diabetes mellitus, inflammatory diseases, as well as neuro-degenerative diseases (Yang et al. 2011). Both the apoproteins and the prosthetic group, which are structural components of PBP, are involved in ROS stabilization. Apoproteins contributes to the removal of hydroxyl radical, as well as hypochlorous acid radical by reacting with its cysteine and methionine residues (Pleonsil et al. 2013). Other amino acids such as tryptophan, tyrosine and histidine can remove peroxyl radicals (Fernández-Rojas et al. 2014a). Fernandez-Rojas et al. (2014a) reported that phycocyanobilin removes most free radicals, the singlet oxygen is stabilized by the oxidation of double bonds of the tetrapyrrole. Phycocyanobilin can also scavenge peroxynitrite, hypochlorous, hydroxyl and peroxyl radicals (Fernández-Rojas et al. 2014a). Evidence is accumulating on the hydroxyl, and peroxyl free radical-scavenging properties of PC suggesting that its therapeutic effects are largely attributed to its antioxidant potentials (Farooq et al. 2014). It has been found that PC prevents apoptosis in mice with Cisplatin-induced nephrotoxicity. The protective effect of PC against CP-induced nephrotoxicity was associated with the ROS scavenging ability of phycocyanin (Fernández-Rojas et al. 2014c).

It is suggested that the human consumption of PC may be useful for the prevention and/or treatment of kidney diseases associated to oxidative stress (Fernández-Rojas et al. 2014b, Wu et al. 2016).

Although several methods have been developed for the separation and purification of PC and PE from cyanobacteria, the purity and recovery is relatively low because these pigments are highly sensitive to light, oxygen and moisture, hence, it is needed to process it along with efficient preservatives (Mishra et al. 2008). Sodium azide and dithiothreitol are commonly used as preservatives of PE for analytical purpose, but they are toxic (Mishra et al. 2010); so for developing process of food grade PE only edible preservatives with unique properties can be used (Mishra et al. 2010). It is therefore, desired to develop a simple, but more well-organized method for the separation, purification and stabilization of the food grade PE from cyanobacteria (Patil et al. 2006). Food additives have been used by mankind for centuries, e.g. salt; sugar and vinegar were among the first to be used as preservatives in food (Mishra et al. 2010). PE can be used as a food colorant which is not only safe but, has an extra advantage of being potential antioxidant. Preservative for the food grade PE is indispensable for making the process commercially viable due to its extreme sensitivity towards light, oxygen and temperature under aqueous condition (Mishra et al. 2010).

The main and the simplest way to improve the stability of PBP is by using additives. Most studies have focused on the use of additives to improve their thermal stability (Martelli et al. 2014; Gonzalez-Ramirez et al. 2014; Braga et al. 2016). This method of stabilization is easy to apply and does not require sophisticated or expensive equipment. However, low toxicity additives must be used because large amounts of additives may be necessary. Temperature and pH play an important role in the stability of PC (Freitas et al. 2022). Systematic investigations showed the maximum stability of PC was in the pH range of 5.5–6.0. Incubation at temperatures between 47 °C and 64 °C caused the concentration and half-life of PC in solution to decrease rapidly (Chaiklahan et al. 2012). In case of PE, the maximum stability was in the pH range of 4.0-10.0 (Gonzalez-Ramirez et al. 2014). In addition, all PBPs were thermostable up to 4-40 °C while their concentration decreased rapidly at 60-80 °C (Rastogi et al. 2015a).

Temperature is an important factor that strongly influences the oxygen-evolving activity of photosystem II and affects nutrients availability (Markou and Georgakakis 2011). Also, the fluidity of the cell membrane of microalgae is affected by temperature (Daliry et al. 2017). The optimum temperature for cyanobacterial cultivation depends on the species and strain (Mehnert et al. 2010). Increase in temperature above the optimum decreases the microalgae biomass primarily due to the denaturation of essential proteins and enzymes. In addition, if the temperature is below the optimum state, the growth of cyanobacterial cells slows down and enters the stationary phase (Hsieh-Lo et al. 2019). PC and PE are stable at low temperatures (4-5 °C) with addition of preservatives like citric acid (e.g., acidic or basic solutions) and therefore they could be utilized as a food colorant (chewing gum, jellies), beverages, and coloring agent in the sweet confectionery and cosmetics (Patel et al. 2004, Mishra et al. 2008, Eriksen et al., 2008).

The use of PC in food and other applications is limited due to its sensitivity to heat treatment, which results in precipitation and fading of the blue color. Sodium azide and dithiothreitol are commonly used as preservatives for PC for analytical purposes, but they are toxic and thus cannot be used for food-grade PC production (Mishra et al. 2008). Sugars and polyhydric alcohols have been used to stabilize proteins, and are being used widely at present as stabilizing agents in the food industry as well as in pharmaceutical formulations since they are safe for consumption (Petersen et al. 2004). Moreover, many studies have reported that the modification of the protein conformation itself can improve the stability of proteins (Deller et al. 2016). Fukui et al. (2004) reported that dithio- bis (succinimidyl propionate)) DSP) modified phycocyanin was resistant to bleaching by urea treatment because the amino groups of modified lysine residues were cross-linked, thus maintaining the protein's high-order structure (Fukui et al. 2004). Another method used to improve stability was reported by Li et al. (2009). The study of Chaiklahan et al. (2012) showed that the measured photodamage rate constant of PC entrapped in a silica matrix (immobilized biomolecules of protein with silica)

was 25-times lower than that of PC in a buffer solution (Chaiklahan et al. 2012, Li et al. 2009).

Although a number of reports are available for PE and PC purification and characterization from different cyanobacterial and red algal strains, exploitation and optimization of PE and PC production from the genus *Nostoc* extracted from paddy fields with different pigments are limited (Mishra et al. 2010; Tan et al. 2016). However, the main challenges for PE and PC commercialization and implementation in food and cosmetic applications are their low yield during production and limited chemical instability (Freitas et al. 2022). For this reason, the present study aimed isolation and purification of the PE and PC from *Nostoc* sp. strains FSN and ASN, and evaluation of their antioxidant activity for biotechnological applications. In addition, no research has been done on the optimum temperature. So, in this study, two temperatures of 5 °C and 35 °C degrees were studied during 35 days of growth. Also, shelf times of two pigments with or without additives were evaluated.

## Materials and methods

*Cyanobacterial isolation and growth conditions*

*Nostoc* sp. strain FSN and *Nostoc* sp. strain ASN were isolated from paddy fields of Golestan province, Iran (36° 54′ 41″ N, 54° 47′ 25″ W). In order to obtain a cyanobacterial monoculture, soil samples were spread into sterile Petri dishes containing liquid BG-$11_0$ (two 10-days-old cyanobacterial strains) medium (Allen 1968), without a nitrogen source, pH 7.1, and incubated in a growth chamber (Merck, Germany) for two weeks at 28 ± 1 ºC under constant cool white fluorescent light (100-150 $\mu E/m^2s$). After 14 days of growth, selected colonies were transferred to a fresh solid BG-$11_0$ medium. For bacteria-free cultures, colonies were tested for bacterial contamination in dextrose-peptone broth and caseinate-glucose agar media according to Rajabpour et al. (2019). The selected bacteria-free colony was maintained on different agar slants media. After 20 days, the isolate was washed with sterile deionized water, and transferred to 1L of freshly prepared liquid BG-$11_0$ medium.

*Morphological characterization of the studied strains*

Cyanobacterial strains were examined under a light microscope (Leica DM750). After cultivation, the morphological characteristics were investigated according to the classification system devised by Komárek (2013). The strain FSN was green, and the strain ASN was brown. The reason for the color change is due to the higher concentration of one pigment than the other and it is common among cyanobacterial strains. When there is more PE, the strain is brown and when the level of PC is higher, the strain is green.

*Molecular analysis*

Because it is not possible to use the morphological characteristics of cyanobacteria to accurately identify the genus, the most reliable method to identify the genus is to use the genetic sequences. To do this, genomic DNA was extracted from living cells using the EZNA SP Plant DNA mini kit (Omega Bio-tek) according to the manufacturer's instructions. PCR reactions were performed on the genomic DNA using the oligonucleotide primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and B23S (5'-CTTCGCCTCTGTGTGCCTAGGT-3') (Stoyanov et al. 2014) that target the 16S rRNA gene sequence. Reactions were cycled with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C, and final extension step at 72 °C for 5 min. Amplicons were verified by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. PCR products were purified using the Geneclean® Turbo kit (Qbiogene, Inc.) prior to sequencing.

Sequencing reactions were carried out using the refined PCRs products in a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Life Technologies). A total volume of 10 µL of the PCR master mix included 1 µL of forward or reverse primers (10 µM), 1 x sequencing buffer, 1 µL of Big dye and 100 ng (1µL) of DNA. The cycle sequencing reaction was carried out using 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 minutes, followed by storage overnight at 4 °C. After the completion of

the sequencing reactions, the sequenced products were precipitated by adding 40 µL of 0,125 M NaCl and 2.5 x volume of cold 100% ethanol, followed by vortexing and centrifugation at 13,000 rpm for 10 min at 4 °C. Once the supernatant was removed, a 5 x volume of 70% ethanol was added, and the sample centrifuged at 13,000 rpm for 5 minutes (4 °C). The supernatant was removed, and the pellet was dried at 37 °C. The purified reactant was resuspended in 12 µL of HiDi-formamide, the mixture was spun down, denatured for 2 min at 94 °C and subjected to sequencing. The runtime for each reaction was 45 min with a running voltage of 15 kV at a temperature of 50 °C and the polymer used was POP-6™ (Applied Biosystems, Life Technologies). The 16S rRNA gene sequences obtained in this study was used to construct a consensus sequence in BioEdit version 7.0. Positions with gaps, as well as undetermined and ambiguous sequences were removed. BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) of the partial 16S rRNA gene sequence were used to identify similar sequences available in the GenBank database of National Center for Biotechnology Information (NCBI).

*Chemicals*

All chemicals and protein molecular weight marker used in this study were of analytical grade, purchased from the Hi-Media, Merck and Sigma manufacturers. All buffers and reagents used were prepared in double distilled water.

*Extraction and purification of PC and PE*

As two *Nostoc* strains have different colors, we assumed that they have PC and PE. PE and PC were extracted from 500 mL of homogenized log phase (14 days old) culture after centrifuging at 4,000 rpm to obtain a pellet. The pellet was suspended in 100 mL of 20 mM acetate buffer (pH 5.1). Extraction was carried out by repeated freezing (-20°C) and thawing (room temperature) method for 4 days until cell biomass became dark purple according to Afreen and Fatma (2018). Cell debris was removed by centrifugation at 5,000 rpm for 10 min, and a crude extract was obtained.

Purification was carried out according to Afreen and Fatma (2018) (Afreen and Fatma 2018). Solid ammonium sulphate was added to the crude extract slowly for achieving 65% saturation by continuous stirring. The resulting solution was allowed to stand for 12 h under cold room, and centrifuged at 4,500 rpm for 10 min. The pellets were resuspended in a small volume of 50 mM acetic acid–sodium acetate buffer (pH 7.1), and dialyzed overnight. The extract was recovered from the dialysis membrane and filtered through 0.45 µm filter (Chakdar and Pabbi 2012, Tiwari et al. 2015).

The absorption spectrum was determined by scanning the sample in a range of 300-750 nm wavelengths by Specord 200 spectrophotometer (Analytik Jena, Germany) (Mishra et al. 2008, 2010).

The amounts of PE, PC and APC in different extracts and biliprotein (PBPs) containing solutions were calculated from measurements of the absorbance at 565 nm, 620 nm and 650 nm using the following equations below (Nowruzi et al., 2020). Purity of PE and PC were calculated at each step as purity ratio (A555/A280) and (A620/A280), respectively.

$$\text{PE } (\mu g\ mL^{-1}) = \frac{(OD\ 565nm - 2.8[R-PC] - 1.34[APC])}{12.7}$$

$$\text{PC } (\mu g\ mL^{-1}) = \frac{(OD\ 620nm - 0.7\ OD\ 650nm)}{7.38}$$

$$\text{APC } (\mu g\ mL^{-1}) = \frac{(OD\ 650nm - 0.19\ OD620nm)}{5.65}$$

*Stability studies of purified pigments*

Stability of purified PE and PC were obtained by adding additives including citric acid, sucrose, sodium chloride, and calcium chloride as preservatives (0.193 and 0.653 mg/mL, respectively) at different temperatures (5 and 35 °C) by recording its absorption spectrum for 30 days (Setyoningrum and Nur 2015, Gonzalez-Ramirez et al. 2014, Rastogi et al. 2015b).

*Antioxidant activity of purified PE and PC*

DPPH assay

This test was conducted following the method described by Shanab et al. (2012) with

modifications. An amount of 710 µg/mL purified PE was mixed with 1 mL of DPPH reagent. After incubating for 30 min in the dark at room temperature, the absorbance was measured at 517 nm. Ascorbic acid (100 µg/mL) was used as a positive control.

$$\text{Activity (\%)} = \text{Ac} - \text{At/Ac} \times 100$$

where At was the absorbance of sample, and Ac the absorbance of DPPH.

ABTS assay

$ABTS^+$ radicals (7 mM) were produced by adding 2.45 mM potassium persulphate in the dark for 12–16 h. The resulting solution was diluted with ethanol up to an absorbance of 0.5 at 734 nm. An aliquot of 3 mL of $ABTS^+$ solution was added to 50 µL of the PE sample (710 µg/mL) and standards, and the absorbance was recorded at 734 nm against ethanol as blank (Re et al. 1999). $ABTS^+$ solution was taken as positive control and BHT as standard.

$$\text{Activity (\%)} = \text{Ac} - \text{At/Ac} \times 100$$

where At was the absorbance of sample, and Ac the absorbance of ABTS.

## Results

*Morphological and molecular characterization*

*Nostoc* sp. strains FSN and ASN were grown rapidly on liquid media culture, and changed the colour to dark brown and dark green after 15 days (Fig. 1). The unicyanobacterial culture and dry material were deposited into ALBORZ herbarium and Cyanobacteria Culture Collection (CCC) of Science and Research Branch, Islamic Azad University, Tehran, respectively. The phylogenetic position of the two *Nostoc* strains in relation to *Nostoc-carneum*-BF2 is shown in full circle (Fig. 2). Numbers near nodes indicate bootstrap values over 50% for NJ analyses.

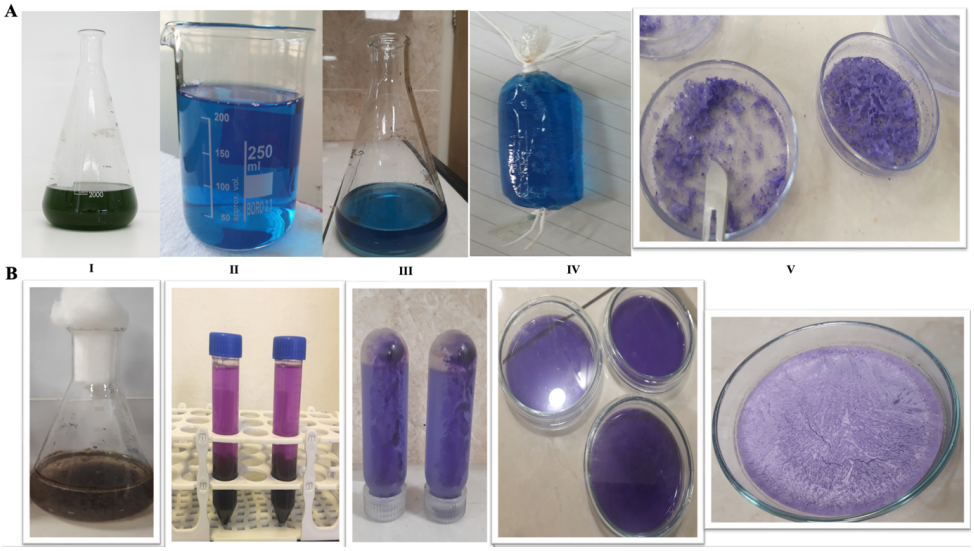



**Figure 1:** Pigment production process of (**A**) phycocyanin and (**B**) phycoerythrin. I, Primary cultivation; II, Preparation of the crude extract; III, Ammonium sulphate precipitation; IV, Dialysis; V, Freeze-drying.
**Slika 1:** Proces pridobivanja barvila (A) fikocianina in (B) fikoeritrina. I, Primarna kultura; II, Priprava grobega ekstrakta; III, Precipitacija z amonijevim sulfatom; IV, Dializa; V, Zamrznjeno sušenje.

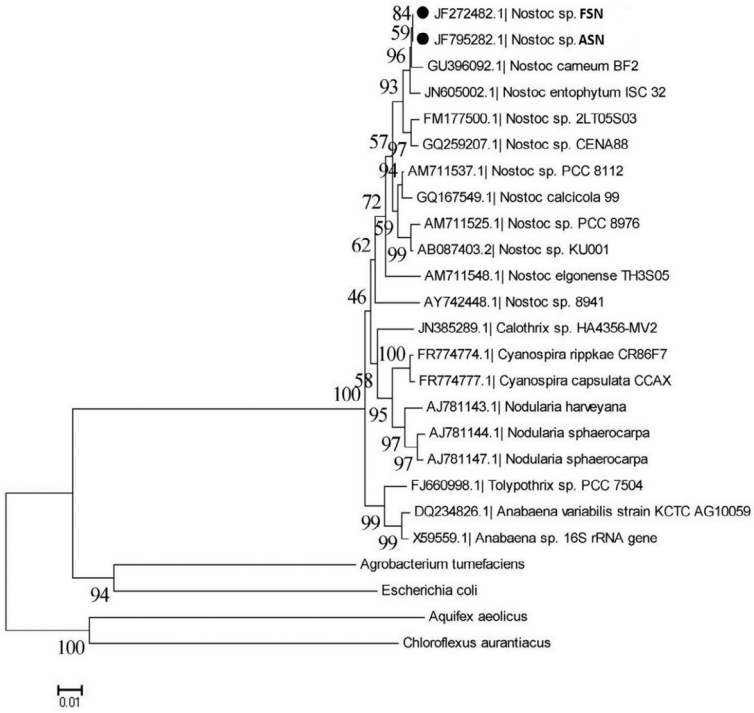



**Figure 2:** Consensus bootstrap tree on the basis of neighbour-joined distances of 1815 bp long full-length 16S rRNA genes sequences and sequences that were taken from the GenBank. Bar = 0.01 change per sequence position.

**Slika 2:** Filogenetsko drevo po metodi skupnega vezenja na osnovi 1815 bp dolgega genskega zaporedja za 16S rRNA in zaporedij iz baze GenBank. Merilce predstavlja spremembo 0,01 v legi zaporedja.

*Extraction, purification and characterization of PE and PC*

At each purification step, concentration and purity of PE and PC were checked as shown in Table 1. During successive steps of purification, the purity absorption rate increased from 0.797 nm up to 3.200 nm for PE and from 0.209 nm up to 0. 251 nm for PC. Purity ratio was found to be enhanced after each purification step. From crude extract to purified PE, purity was increased by almost 4-times for PE and almost 1.2-times for PC, which showed the efficiency of the method using successive steps of purification (solid ammonium sulphate and dialysis) to obtain high purity PE.

**Table 1:** Stepwise purification of phycoerythrin (PE) and phycocyanin (PC) from two *Nostoc* strains.
**Tabela 1:** Koraki čiščenja fikoeritrina (PE) in fikocianina (PC) iz dveh sevov vrste *Nostoc*.

| ***Nostoc* sp. strain** | **Step** | **Peak** | **PE (µg/mL)** | **Purity of PE (OD555/OD280)** |
|---|---|---|---|---|
| **FSN** | Crude extract | 566.2 - 616.9 | 0.108 | 0.797 |
| | Ammonium sulphate precipitation | 565.5 - 617.4 | 0.152 | 1.559 |
| | Dialysis | 567.6 - 617.7 | 0.193 | 3.20 |
| | **Step** | **Peak** | **PC (µg/mL)** | **Purity of PC (A620/A280)** |
| **ASN** | Crude extract | 619/8 | 0.058 | 0.209 |
| | Ammonium sulphate precipitation | 620.1 | 0.063 | 0.239 |
| | Dialysis | 621.9 | 0.653 | 0. 251 |

*Effects of different temperatures and preservatives on PE and PC*

The absorption spectrum was recorded with or without preservative at 5 °C and 35 °C. The visible absorption spectra of PE and PC showed that the loss of color was slightly lower at 5°C and very high at 35°C in 30 days (Tab. 2). The maximum rate of decrease in color was found with control (Fig. 3 and 4) at both temperatures, while the use of additives increased the absorbance of PE and PC. Among the additives, with citric acid at both temperatures, the absorbance increased (Fig. 5 and 6).

**Table 2:** Absorbance of purified phycoerythrin (PE) and phycocyanin (PC) at various days and temperatures of cultivation.
**Tabela 2:** Aborbanca očiščenega fikoeritrina (PE) in fikocianina (PC) ob različnih dnevih in temperaturah gojenja.

| | **Day 3 / 5 °C** | **Day 3 / 35 °C** | **Day 5 / 5 °C** | **Day 5 / 35 °C** | **Day 10 / 5 °C** | **Day 10 / 35 °C** | **Day 15 / 5 °C** | **Day 15 / 35 °C** | **Day 20 / 5 °C** | **Day 20 / 35 °C** | **Day 30 / 5 °C** | **Day 30 / 35 °C** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Nostoc* sp. FSN (PE)** | 0.20 | 0.13 | 0.19 | 0.12 | 0.14 | 0.06 | 0.10 | 0.042 | 0.10 | 0.04 | 0.08 | 0.03 |
| ***Nostoc* sp. ASN (PC)** | 0.63 | 0.33 | 0.37 | 0.33 | 0.27 | 0.22 | 0.26 | 0.20 | 0.24 | 0.19 | 0.21 | 0.16 |

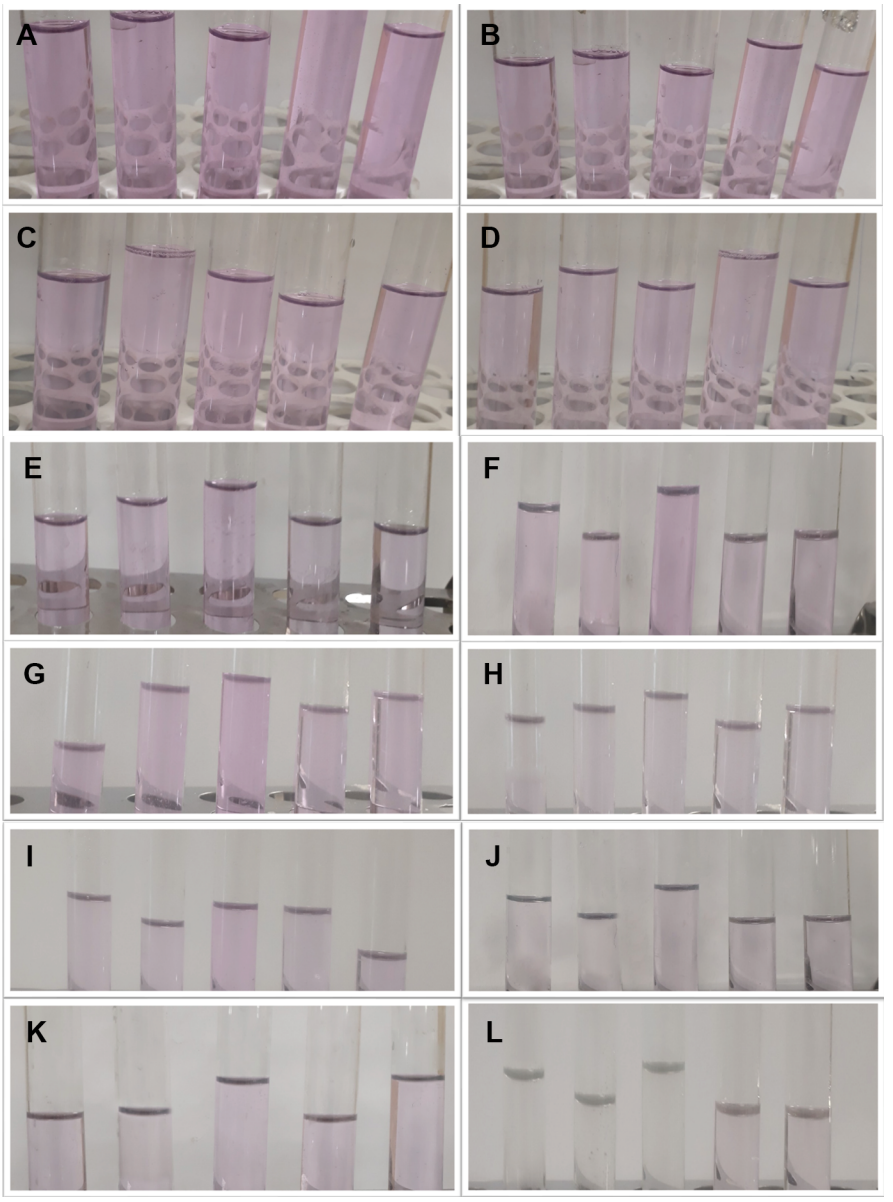



**Figure 3:** Coloured eluates of phycoerythrin at different days and temperatures. A, day 3 / 5 ◦C; B, day 3 / 35 ◦C; C, day 5 / 5 ◦C; D, day 5 / 35 ◦C; E, day 10 / 5 ◦C; F, day 10 / 35 ◦C; G, day 15 / 5 ◦C; H, day 15 / 35 ◦C; I, day 20 / 5 ◦C; J, day 20 / 35 ◦C; K, day 30 / 5 ◦C; L, day 30 / 35 ◦C.

**Slika 3:** Obarvani eluati fikoeritrina ob različnih dnevih in temperaturah. A, dan 3 / 5 °C; B, dan 3 / 35 °C; C, dan 5 / 5 °C; D, dan 5 / 35 °C; E, dan 10 / 5 °C; F, dan 10 / 35 °C; G, dan 15 / 5 °C; H, dan 15 / 35 °C; I, dan 20 / 5 °C; J, dan 20 / 35 °C; K, dan 30 / 5 °C; L, dan 30 / 35 °C.

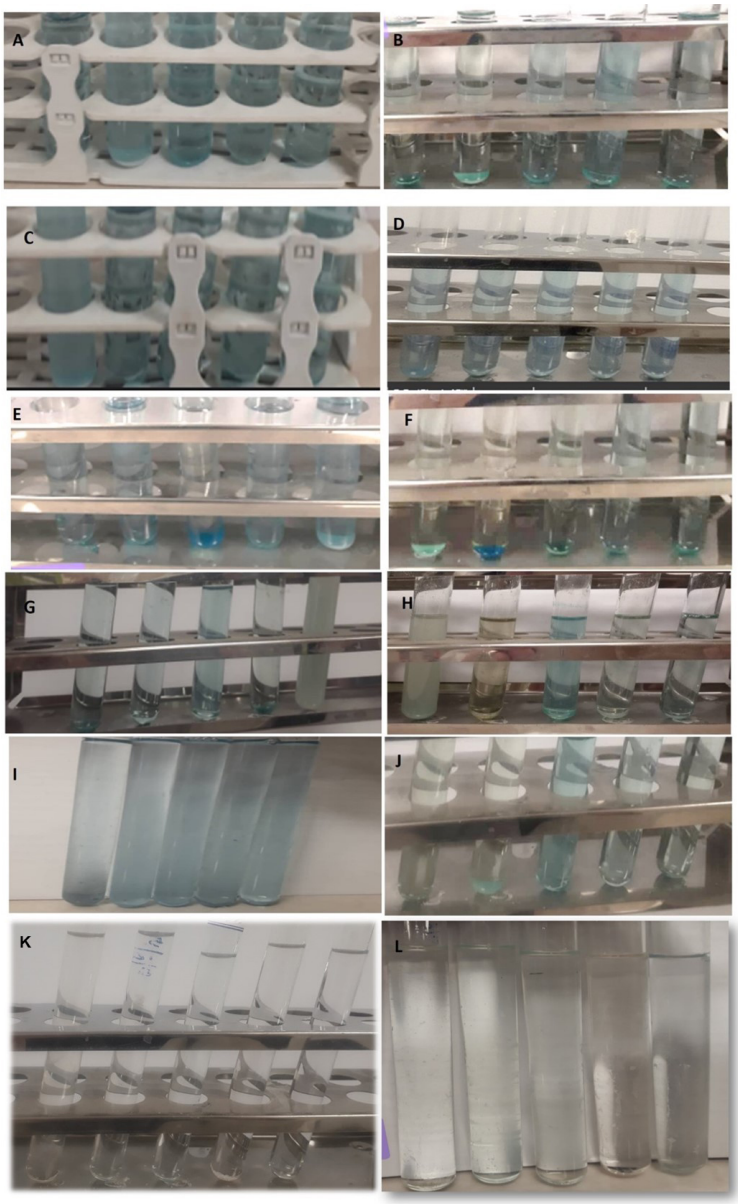
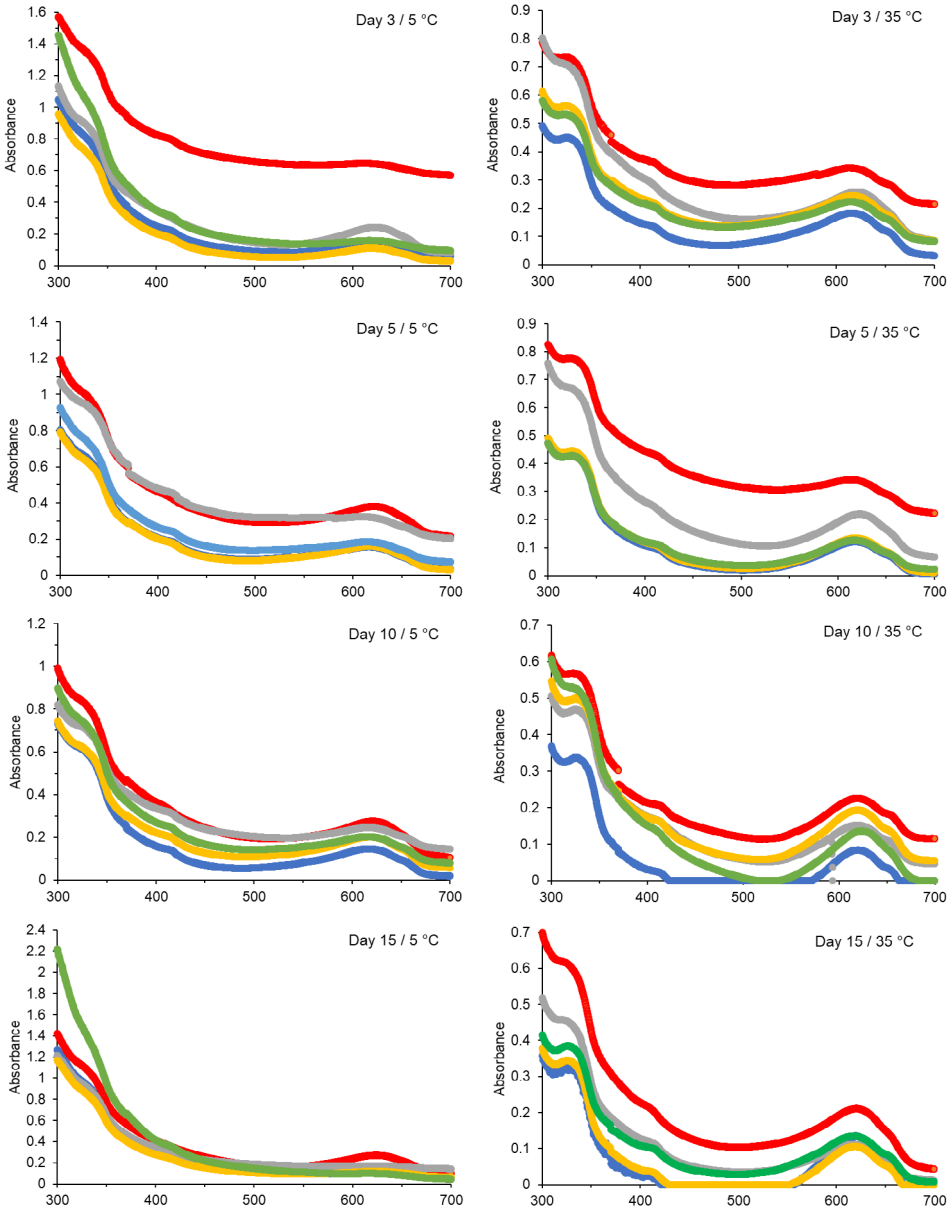



**Figure 4:** Coloured eluates of phycocyanin at different days and temperatures. A, day 3 / 5 °C; B, day 3 / 35 °C; C, day 5 / 5 °C; D, day 5 / 35 °C; E, day 10 / 5 °C; F, day 10 / 35 °C; G, day 15 / 5 °C; H, day 15 / 35 °C; I, day 20 / 5 °C; J, day 20 / 35 °C; K, day 30 / 5 °C; L, day 30 / 35 °C.

**Slika 4:** Obarvani eluati fikocianina ob različnih dnevih in temperaturah. A, dan 3 / 5 °C; B, dan 3 / 35 °C; C, dan 5 / 5 °C; D, dan 5 / 35 °C; E, dan 10 / 5 °C; F, dan 10 / 35 °C; G, dan 15 / 5 °C; H, dan 15 / 35 °C; I, dan 20 / 5 °C; J, dan 20 / 35 °C; K, dan 30 / 5 °C; L, dan 30 / 35 °C.


Day 3 / 5 °C
Day 3 / 35 °C
Day 5 / 5 °C
Day 5 / 35 °C
Day 10 / 5 °C
Day 10 / 35 °C
Day 15 / 5 °C
Day 15 / 35 °C
Absorbance
300
400
500
600
700

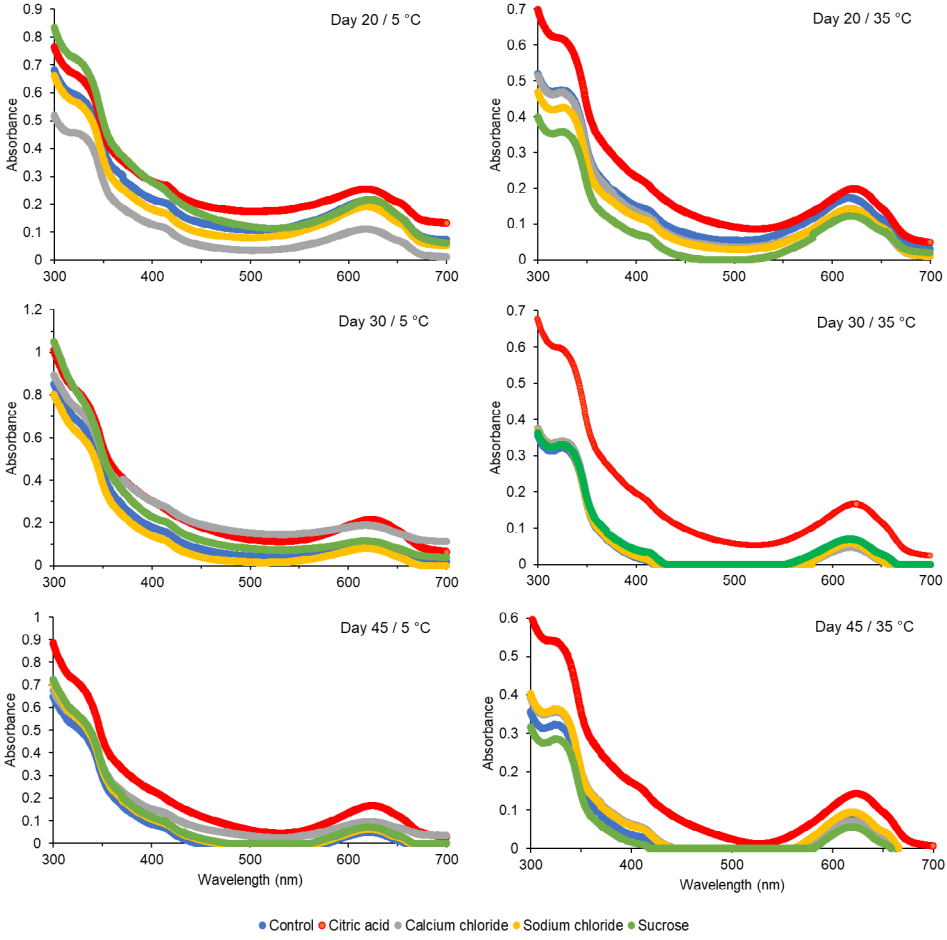
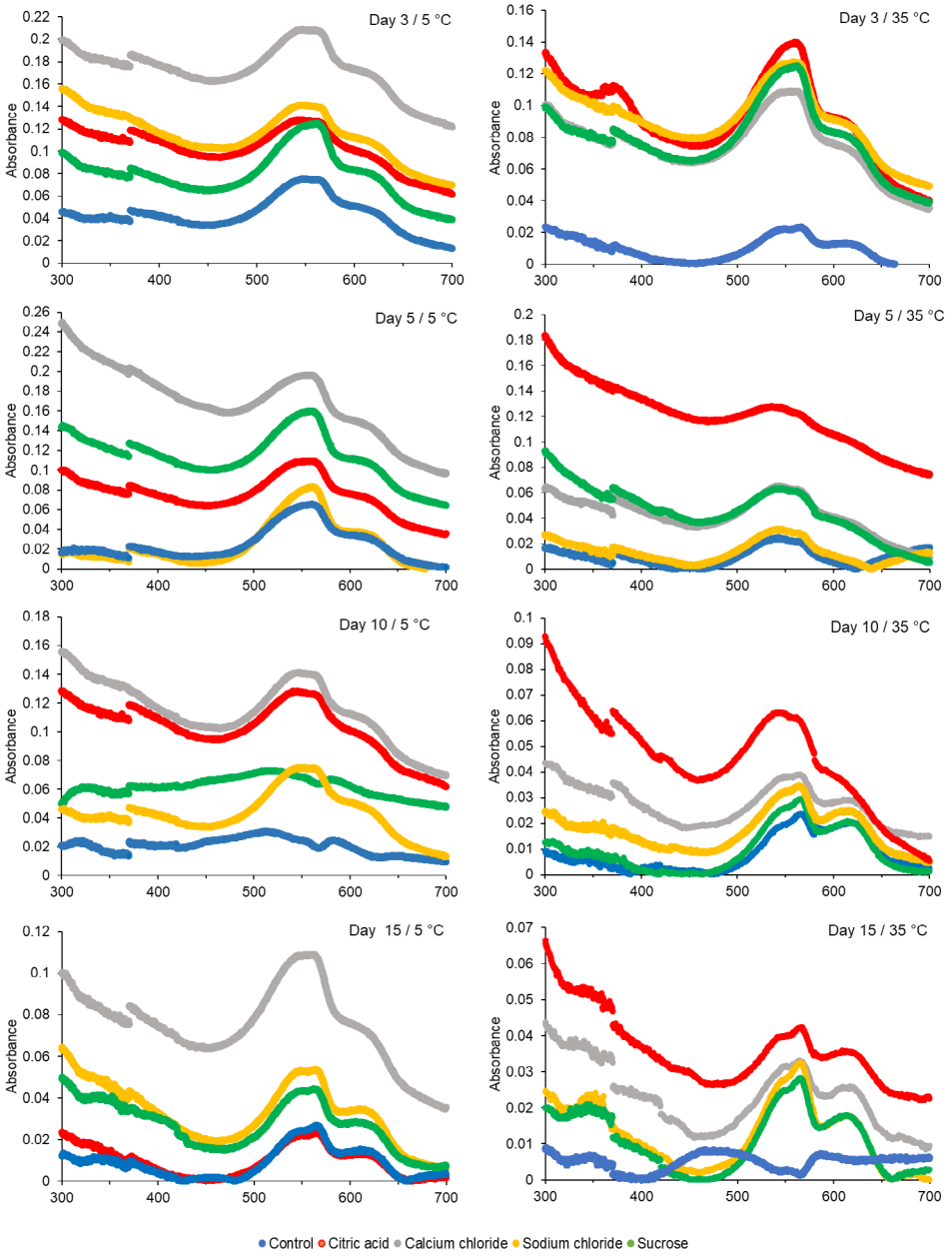



**Figure 5:** Absorbance spectra of purified phycocyanin at different days and temperatures.
**Slika 5:** Absorpcijski spektri fikocianina ob različnih dnevih in temperaturah.


Day 3 / 5 °C
Day 3 / 35 °C
Day 5 / 5 °C
Day 5 / 35 °C
Day 10 / 5 °C
Day 10 / 35 °C
Day 15 / 5 °C
Day 15 / 35 °C
Absorbance
Control
Citric acid
Calcium chloride
Sodium chloride
Sucrose

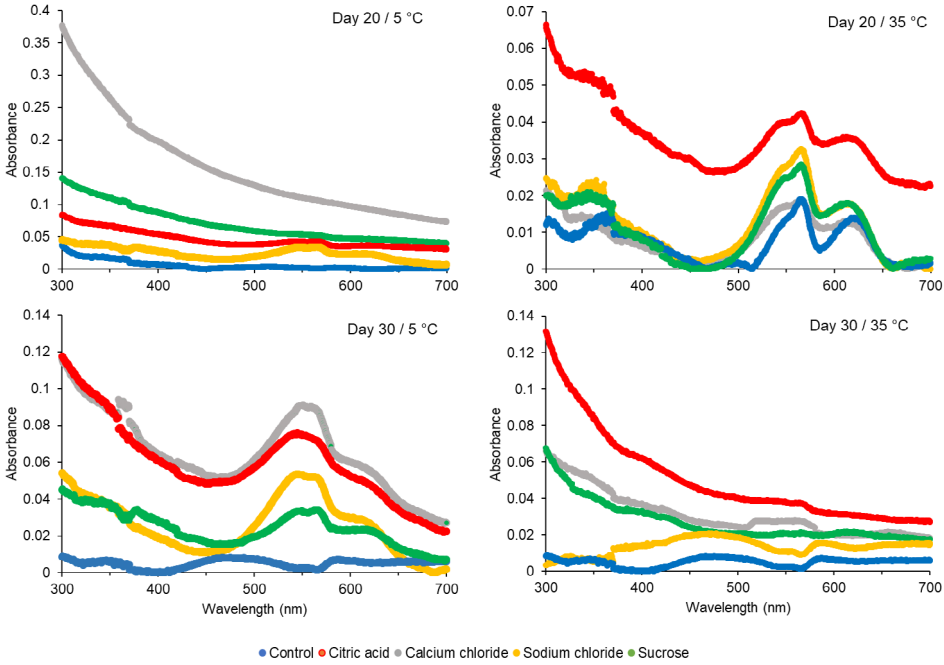



**Figure 6:** Absorbance spectra of purified phycoerythrin at different days and temperatures.
**Slika 6:** Absorpcijski spektri fikoeritrina ob različnih dnevih in temperaturah

*Antioxidant activity of purified PE and PC*

Free radical scavenging potential of two *Nostoc* strains in two methods was found to be concentration dependent. $IC_{50}$ value in DPPH and ABTS method were 0.03 and 0.04 mg/mL for FSN and ASN strain, and 0.02 mg/mL for ascorbic acid (vitamin C) and butylated hydroxyl toluene (BHT), that were used as standard (Fig. 7).

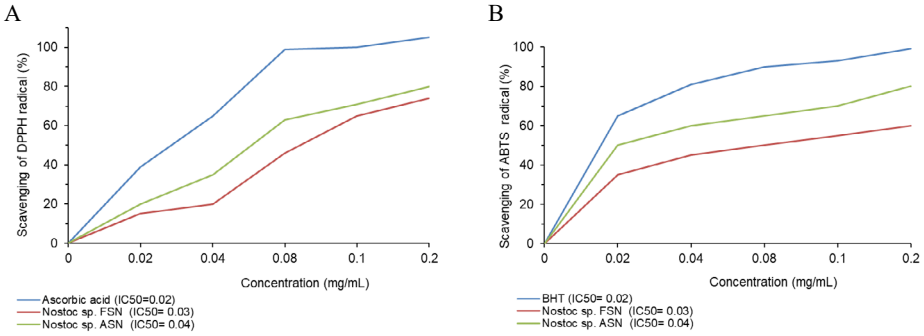



**Figure 7:** Free radical scavenging potential of two *Nostoc* strains FSN and ASN with DPPH method (**A**) and ABTS method (**B**).
**Slika 7:** Potencial odstranjevanja prostih radikalov sevov FSN in ASN vrste *Nostoc* po metodi DPPH (**A**) in ABTS (**B**).

## Discussion

PC and PE are photosynthetic pigments extracted from microalgae with great biotechnological potential due to their intense colors, fluorescent properties and potential health benefits (Hsieh-Lo et al. 2019). Their principal applications are as nutritional supplements, natural colorants in foods and cosmetics, and as a reagent for immunological assays (Hsieh-Lo et al. 2019). Different physicochemical factors such as pH, temperature, light, affect PC and PE stability (Johnson et al. 2014). Several authors suggest the main factors affecting PBP stability are pH and temperature (Chaiklahan et al. 2012, Wu et al. 2016). The optimum pH range for PC is slightly more acidic than for the PE. The values of pH is the main factor that affects the aggregation and dissociation of PC in monomers, trimers, hexamers and other oligomers in solution. The hexameric form predominates in pH near 7.0. This is the most stable structure and avoids the denaturation of the PBP (Chaiklahan et al. 2012). At higher or lower pH values, this structure dissociates easily, decreasing stability. According to Chaiklahan et al. (2012) at pH 6.0, 77% of PC was aggregated in its hexameric form, while at pH 7.0 only 18% was aggregated. Therefore, it is recommended to handle the pigments at their optimal pH to avoid degradation. On the other hand, it has been reported by Liu et al. (2009) and González-Ramírez et al. (2014) that PE is stable in a wide range of pH, from 4.0 to 10.0. They showed that the secondary structure of PE could adopt a stable conformation in that pH range. The stability is maintained by the formation of hexameric structures (Liu et al. 2009). Providing stability at a wide pH compared to other PE molecules can facilitate its application in the food industry (Gonzalez-Ramirez et al. 2014). In general, it is preferable to handle and preserve PBP at a low temperature. As these molecules are protein pigments, their primary cause of degradation is denaturation. Munier et al. (2014) mentioned that when the temperature increases, the amount of alpha helix decreases, resulting in the loss of stability. The optimum temperature for PC and PE is around 4 °C; however, it can be stable up to 40–45 °C but slow degradation still occurs. It is not recommended to preserve PBP at a higher than room temperature because of their susceptibility to degradation by microorganisms (Chaiklahan et al. 2012). Using preservative for PE and PC is indispensable due to high sensitivity to temperature, if the process should be commercially viable. Results from the present study showed that without adding additives, PE and PC absorbance decreased as the temperature changed. At 5 °C with the presence of acid citric, PE and PC stability was found to be better. Moreover, we have demonstrated that almost complete loss of PE and PC content occurred at 35 **°C. However,** Galland-Irmouli et al. (2000) have demonstrated the thermostability of PE from *Palmaria palmate* (Rhodophyta) up to 60 °C. Moreover, the purified PE from *Nostoc* sp. FSN exhibited more stability after 30 days in comparison to purified PC.

Wu et al. (2016) and Munier et al. (2014) reported that PC and PE are sensitive to light (Wu et al. 2016, Munier et al. 2014). Wu et al. (2016) observed that PC showed a higher level of degradation after being exposed to a light intensity of 100 $\mu mol\ m^{-2}\ s^{-1}$ compared to the treatment exposed to 50 $\mu mol\ m^{-2}\ s^{-1}$ (Wu et al. 2016). When PBP are exposed to light for long periods, they tend to lose their chromophores, thus losing their color and stability (Munier et al. 2014). Nevertheless, although some physicochemical factors can significantly affect the stability of PBP, there are alternatives for improving their stability such as the use of additives, encapsulation and other methods (Hsieh-Lo et al. 2019).

According to Chaiklahan et al. (2012), the addition of sorbic acid or sodium azide did not significantly increase the stability of PC compared to glucose, sucrose and sodium chloride (Chaiklahan et al. 2012). High concentrations of sugar can improve stability. Martelli et al. (2014) also reported that the high concentration of sugar strongly increases the thermal stability of the PBP and its stabilization depends on the final concentration of the added sugar rather than type. Sugars and salts such as glucose, sucrose and sodium chloride could act as protein stabilizing agents. They can cover the surface of PC, and maintain and protect its chemical structure. By adding sugars, the water surface tension increases, and consequently the thermal stability of the proteins increases (Chaiklahan et al. 2012). Likewise, Braga

et al. (2016) recommend the use of glucose to improve the thermal stability of the pigment and other additives such as 6% polyethylene oxide and 50% sorbitol. Furthermore, they reported that the addition of nanofibers could improve the thermal stabilization of the pigment by lowering the enthalpy of the system. Nanofibers can reduce the protein denaturation by forming multiple unions to the polymer, preventing conformational changes of the proteins in adverse environments. Another interesting additive to consider in order to improve PBP stability is benzoic acid. This additive can act as an antimicrobial agent inhibiting bacterial growth and has shown antioxidant activity, being able to preserve and increase pigments stability (Kannaujiya and Sinha 2016).

PE and PC can be largely useful as food colorant (Galland-Irmouli et al. 2000). Although its security has been questioned, it is a strong antioxidant, which highlights its advantage. Sonani et al. (2015) have reported the anti-oxidant based anti-ageing activity and anti-Alzheimer potential of PE isolated from *Lyngbya* sp. A09DM in wild type and transgenic *Caenorhabditis elegans* (Sonani et al. 2015). However, little investigation about the effect of PE and PC antioxidant activity is available in the literature.

Our results of antioxidant surveys suggest that PE and PC have efficient scavenging effects (IC50 = 0.03 and 0.04 for DPPH and ABTS respectively) compared to ascorbic acid and BHT (IC50=0.02) and is a potent free radical scavenger acting as antioxidant molecule. Similarly, Afreen and Fatma (2018) reported IC50 = 0.043 mg/mL using the extract of *Michrochaete* for DPPH test. Furthermore, it is demonstrated that extracts of *Nostoc linckia* (70% inhibition of ABTS radical) at 5 mg/L and ABTS radical scavenging capacity increased with the increasing phycobiliproteins concentration. Extracts of *Nostoc spheroids* showed considerable superoxide radical inhibiting activity according to Kuriakose (2014).

## Conclusions

PE and PC produced by *Nostoc* strains FSN and ASN were successfully extracted, purified, characterized and evaluated for *in vitro* stability under different temperatures and additives. Significant antioxidant activities of PE and PC shown its feasibility for future applications as colorant in food industries and for pharmaceutical purposes. However, toxicological studies must be carried out for commercial production, since *Nostoc* is known as a toxin-producing genus. Furthermore, studies on its conformational behaviour under high stress conditions will be necessary to explore and enhance its molecular stability for industry applications. In addition, *Nostoc*'s PE and PC also shown significant stability 35% over 30 days. This highlights the need to explore microorganisms from unfavourable environmental conditions due to their potential for a wide variety of biological activities.

## Summary

Cyanobacteria have a wide variety and richness in terms of bioactive metabolites, including cytotoxic, antifungal and antiviral compounds. Over the past few years, the isolation and detection of several new and diverse cyanobacterial metabolites with pharmacological activities have appeared, and the importance of the use of cyanobacteria became more apparent in the pharmaceutical industry. The aim of this study was to investigate the degree of purification and free radical scavenging ability of phycocyanin and phycoerythrin, and compare the stability of these pigments against selected preservatives at different temperatures with the aim of achieving the best and most stable preservative in increasing shelf life of PC and PE. In this study, after collecting and culturing *Nostoc* sp. strains FSN and ASN in BG-$11_0$ medium, the natural pigments phycocyanin and phycoerythrin were extracted by sequential melting and freezing the cyanobacterial cells. Then, purification of photosynthetic pigments was performed in two stages: extraction with 56% ammonium sulfate followed by dialysis. After that, antioxidant activity of photosynthetic pigments was evaluated by DPPH and ABTS assays. The purified pigments were then fermented and by adding sodium acetate buffer to the obtained powder, the resulting solution was used to measure the stability with the preservatives citric acid, sodium chloride, sucrose, and calcium chloride on the pigment at two temperatures of 5 °C and 35 °C in an aqueous solution. The results obtained after extraction and purification of natural pigments showed that the

concentration and purity of the pigment increased after the dialysis step. Examination of spear antioxidant activity of extracted pigments showed that they had antioxidant properties. The stability of phycocyanin and phycoerythrin pigments was higher at 5 °C than at 35 °C. In addition, among different preservatives, citric acid caused more stability over time.

## Povzetek

Cianobakterije so bogate z bioaktivnimi spojinami, vključno s citotoksičnim, protiglivnim in protivirusnim delovanjem. V zadnjih nekaj letih je bilo izoliranih in identificiranih več novih in raznolikih spojin iz cianobakterij s farmakološkim delovanjem, pomen uporabe cianobakterij pa je postal očitnejši v farmacevtski industriji. Namen te študije je bil raziskati stopnjo čiščenja fikocianina in fikoeritrina, njuno sposobnost odstranjevanja prostih radikalov ter primerjati njuno stabilnost v primerjavi z izbranim konzervansom pri različnih temperaturah z namenom doseganja najboljšega in najstabilnejšega konzervansa za podaljšanje roka uporabnosti PC in PE. V tej študiji smo po zbiranju in gojenju sevov FSN in ASN vrste *Nostoc* sp. v gojišču BG-110 ekstrahirali naravni barvili fikocianin in fikoeritrin s zaporednim taljenjem in zamrzovanjem cianobakterijskih celic. Nato je bilo izvedeno čiščenje fotosintetskih barvil v dveh stopnjah: ekstrakcija s 56% amonijevim sulfatom, ki ji je sledila dializa. Nato smo s testoma DPPH in ABTS ovrednotili antioksidativno aktivnost barvil. Očiščeni barvili smo nato fermentirali in z dodajanjem natrijevega acetatnega pufra dobljenemu prahu pripravili raztopino, ki smo jo uporabili za merjenje stabilnosti v primerjavi s konzervansi citronsko kislino, natrijevim kloridom, saharozo in kalcijevim kloridom v vodni raztopini pri dveh temperaturah, 5 °C in 35 °C. Rezultati, dobljeni po ekstrakciji in čiščenju naravnih barvil so pokazali, da sta se koncentracija in čistost barvila po koraku dialize povečala. Preiskava antioksidativne aktivnosti ekstrahiranih barvil je pokazala, da imata antioksidativne lastnosti. Stabilnost fikocianina in fikoeritrina je bila višja pri 5 °C kot pri 35 °C. Poleg tega je izmed različnih uporabljenih konzervansov citronska kislina povzročila večjo stabilnost skozi čas.

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