Maximising phycocyanin extraction from a newly identified Egyptian cyanobacteria strain: *Anabaena oryzae* SOS13

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Abstract

A potentially promising Egyptian cyanobacterial isolate *(Anabaena oryzae)* was identified by morphological, biochemical approaches as well as sequencing a fragment of the 16S rDNA gene as new strain denoted as *Anabaena oryzae* SOS13. The new strain was used to produce phycocyanin through extraction using a physical (freeze-thaw) or an enzymatic (lysozyme) approach. Combined freezing-thawing treatments were associated with different levels of phycocyanin yield, where the combination -50ºC x 25ºC achieved complete extraction of the three pigments (100% each). The enzymatic extraction of the three pigments was significantly influenced by the temperature giving maximum yields at 40ºC but was much less effective than the physical freeze-thaw method that is more economic and less liable to potential chemical changes. The resulting extract could be easily purified by the two step ammonium sulfate fractionation giving a product with two SDS-PAGE bands (>15 kD and <25 kD).

Introduction

Cyanobacteria are prokaryotic photoautotrophs capable of simultaneously carrying out photosynthesis and nitrogen fixation. These microorganisms contain only chlorophyll a while almost 50% of the light required is captured by the phycobiliproteins (Samsonoff and MacColl, 2001) which represent about 20% of the dry weight (Romay and González, 2000). Anabaena is a filamentous cyanobacterium broadly distributed in the nature that has relatively simple nutritional requirements as it is able to fix atmospheric nitrogen (Bergman *et al.*, 1997).

Phycocyanins (Phycobiliproteins) are light harvesting antenna pigments, consisting of chromophores called bilins attached to cysteine residues of the apoprotein (Samsonoff and MacColl, 2001). They have an apparent molecular mass of 140-210 kD and two subunits, α and β. Phycocyanin is the most important natural blue pigment for the food industry, e.g. in bubble gum process, milky products and jelly. Recently, phycocyanin has been observed to possess certain therapeutic properties, such as antioxidant providing 20 times more antioxidant activity than ascorbic acid (Romay and González, 2000).

The quantity and quality of extracted phycocyanin (C-PC) are crucial to its effectiveness and potential applications. C-PC can be extracted from cyanobacteria by different procedures, combining the breakage of cell walls and aqueous extraction. Hot water method and ultrasonic extraction were among the methods of extracting phycocyanin (Patil and Raghavarao, 2007). However, hot water method was time-consuming and achieving only a low extraction rate, while ultrasonic extraction was difficult to control and liable to induce changes in the structure of phycocyanin, leading to degraded quality (Yang *et al.*, 2008). C-PC has also been extracted after mechanical cell disruption (Boussiba and Richmond 1979; Schmidt *et al.*, 2005), high pressure exposure (Patil and Raghavarao 2007) and lysozyme treatment (Boussiba and Richmond 1979). Recently, the extraction of phycocyanins was conducted using repeated freezing and thawing method (Su *et al.*, 2012). The dried biomass of *A. platensis* biomass, was used for the extraction of C-PC by re-suspending in 0.1 M phosphate buffer, pH 7 (Oliveira *et al.*, 2008) or 0.5 M $(NH_4)_2SO_4$ (Niu *et al.*, 2007). The freeze/thaw cycle method has been reported as the most efficient way to extract C-PC from wet cyanobacterial biomass (Abalde *et al.*, 1998). The wet biomass was used for C-PC extraction by subjection to cycles of freezing at -25 to -15°C or in liquid nitrogen, then thawing at 4 to 30°C (Abalde *et al.*, 1998; Soni *et al.*, 2006).

The purity of C-PC preparations is achieved through ammonium sulphate precipitation as combined with a variety of chromatographic procedures to produce food, reactive and analytical

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grades of C-PC (Boussiba and Richmond 1979; Niu et al., 2007). Two phase aqueous extraction was recently developed into efficient C-PC purification procedure (Rito-Palomares et al., 2001), resulting in both high purity and high yield (Patil and Raghavarao, 2007). Two-phase aqueous extraction followed by ion exchange chromatography was recently reported to result in extremely pure C-PC with A_{620}/A_{280}=6.69 (Soni et al., 2008).

In Egypt many strains of cyanobacteria are ubiquitous in different water media. Very limited studies were oriented to the isolation and identification of these potentially useful bacteria or their exploitation for the production of phycocyanins. Hence, the current work is initially dedicated to the isolation of cyanobacteria and the identification of a potentially promising Egyptian cyanobacterial strain (Anabaena oryzae) through morphological and biochemical characterization and by sequencing a fragment of the 16S rDNA gene. Secondly, this work is trying optimize the enzymatic (lysozyme) or physical (freeze-thaw) extraction conditions of phycocyanin from this strain. The repeated freezing and thawing method was conducted through a factorial design to reach the most appropriate extraction conditions.

Materials and Methods

Micro-organism and culture preparation

The cyanobacterial isolate used in the present study was previously obtained from rice fields in Sharkhia governorate, Egypt (Ali, 2012). The nitrogen free medium (BG011) used to cultivate the pure culture of this isolate consisted of (g/L) 0.040 $K_2HPO_4.3H_2O$, 0.075 $MgSO_4.7H_2O$, 0.036 $CaCl_2.2H_2O$, 0.06 of ferric ammonium citrate, 0.4 g of anhydrous sodium carbonate (Waterbury and Stanier, 1981). Ten mL citric acid stock solution (0.06 g/100 mL), 1 mL EDTA (1 mg/mL) and 1 mL trace metal mix adjusted to pH 7.4 were added to the previous mixture and the total volume was made up to 1 liter. The trace metal mix was composed of (g/100 mL) 0.286 H_3BO_3, 0.181 $MnCl_2.2H_2O$, 0.022 $ZnSO_4.7H_2O$, 0.039 $Na_2MoO_4.2H_2O$, 0.005 $Co(NO_3)_2.6H_2O$ and 0.008 $CuSO_4.5H_2O$. The fresh cyanobacterial culture was prepared by inoculating 250 mL of BG011 medium with 10 mL of 10 days old culture in 500 mL Erlenmeyer flasks. Inoculated flasks were incubated at 26 ± 2ºC for 28 days under continuous illumination (600-800 lux) using 36W white fluorescent lamp.

Identification of the cyanobacterial Isolate
Morphological and biochemical characteristics

The morphological examination of the selected cyanobacterial isolate was carried out at different stages of growth in N-free BG011 medium. The isolates were investigated under light microscope to specify the general shape and the color of isolates culture and the vegetative cells. The shape, width and length of the vegetative cells, heterocysts and akinets were also specified. The site of heterocyst and the presence or absence of sheath were also defined using the keys provided by Desikachary (1959), Rippka et al. (1979) and Bergey's Manual of Systematic Bacteriology (2001).

The nitrogenase enzyme activity was estimated using the acetylene reduction assay technique (ARA) according to the standard procedures by Hardy et al. (1973). Total N content in cyanobacterial culture was determined by microkjeldahl method (Bremner and Mulvaney, 1982) and the total protein content was calculated according to Bremner and Mulvaney (1982). Chlorophyll a (Chl a) in cyanobacterial culture was estimated by the method of Meeks and Castenholz (1971). Amount of cyanobacterial phycocyanin (C-PC) was estimated according to Singh et al. (2009).

16S rDNA gene sequencing

Preparation of DNA sample for electrophoresis
The bacterial DNA was extracted according to standard procedures (Sambrook et al., 1989). Exponentially growing (50 mL) cells were pelleted by centrifugation, re-suspended in 0.5 mL of lysis solution (25% sucrose, 50 mM Tris – HCl, 100 mM EDTA) and treated with 5 mg of lysozyme for 30 min at 37°C. Sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 1% and 100 µg mL, respectively and the samples were incubated at 56°C overnight. The bacterial DNA was extracted three times with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and twice with Chloroform: Isoamyl alcohol (24:1) then was precipitated and washed with 70% ethanol. The precipitate was resuspended in 100 µL of Tris – EDTA buffer, and stored at -20°C until Polymerase chain reactions (PCRs) were performed using PCR thermocycler machine from Biometra (T-Gradient Thermoblock).

Amplification of the 16S rDNA gene fragment
PCR amplification was performed on purified DNA extracted from the cyanobacterial isolate (Iteman et al., 2000). Amplification of the 16S rDNA gene was carried out by PCR using forward primer A2 (AGAGTTTGATCCTGGCTCAG) and reverse primer GGTTACCTTGTTACGACTT.

Oligonucleotides were purchased from Biron, cl857 Sam. The PCR mixture contained 25 µL of Maxima® Hot Start PCR Master Mix (2X), 1 µL (20uM) of 16S rDNA forward primer, 1 µL (20 uM) of 16S rDNA reverse primer (of each 8 primer), 5 µL of template DNA and 18 µL of water, nuclease-free. Total reaction volume is 50 µL after an initial cycle consisting of 3 min at 95°C, 1 min at 65°C and 30s at 72°C, 35 cycles of amplification were started (1.5 min at 95°C, 2.5 min at 65°C and 3 min at 72°C). The termination cycle was 10 min at 72°C. The PCR products were migrated either on 1.0% (w/v) agarose gel containing 0.5 X TBE and visualized by staining with 0.5 µg/mL ethidium bromide.

A clean up was applied to the PCR product using GeneJET™ PCR Purification Kit (Thermo) as follows; An aliquot (45 µL) of the binding buffer was added to completed PCR mixture and mixed thoroughly. The resulting mixture was transferred to the GeneJET™ purification column which was centrifuged for 30-60 s at >12000 xg and the flow-through was discarded. An aliquot (100 µL) of the wash buffer was added to the GeneJET™ purification column and centrifuged for 30-60s at the same speed. The flow-through is discarded and the purification column was placed back into the collection tube. The empty GeneJET™ purification column was centrifuged for an additional 1 min to completely remove any residual wash buffer. The GeneJET™ purification column was transferred to a clean 1.5 mL micro centrifuge tube and 25 µL of the elution buffer was added to the centre of the GeneJET™ purification column membrane before centrifugation for 1 min at the same speed. The GeneJET™ purification column was discarded and the purified DNA was stored at -20°C.

Sequencing of the 16S rDNA gene and phylogenetic analyses

Sequencing of the PCR amplified product was conducted by GATC Company using ABI 3730xl DNA sequencer and forward/reverse primers. The traditional Sanger technology is combined with the new 454 technology (Thompson *et al.*, 1994). Phylogenetic tree was constructed including the available cyanobacterial gene sequences along with the sequences determined in this study using the neighbor-joining method. In this program, bootstrap analysis was used to evaluate the tree topologies by performing 1000 resemblances.

Phycocyanin extraction

Biomass preparation

Phycocyanin was extracted from the fresh cyanobacterial strain biomass using the modified methods of Sarada *et al.* (1999). Thirty-four day grown cyanobacterial cells were harvested by centrifugation at 3000 xg for 5 min (Jouan, MR 1822, France) at 20°C. Cell pellets were washed with 1M Tris–Cl buffer (pH 8.1). One volume of washed cell mass was re-suspended in five volumes of the same buffer and treated for the extraction of phycocyanins either physically (freeze-thaw) or enzymatically (2 µg/mL lysozyme). The levels of the phycocyanins (C-PC, C-APC and C-PE) concentrations were calculated using the spectrophotometric method of Boussiba and Richmond (1979) according to the following equations.

$$C\text{-}PC \ (mg \ mL^{-1}) = (OD_{620} - 0.7 \ OD_{650})/ \ 7.38 \qquad (1)$$

$$C\text{-}APC \ (mg \ mL^{-1}) = (OD_{650} - 0.19 \ OD_{620})/ \ 5.65 \qquad (2)$$

$$C\text{-}PE \ (mg \ mL^{-1}) = (OD_{540} - 2.8 \ [C\text{-}PC] - 1.34 \ [C\text{-}APC])/ \ 12.7 \qquad (3)$$

Physical extraction

The experiment was carried out to examine the effect of physical (freeze-thaw) and enzymatic extraction methods of phycocyanin produced by the cyanobacterial strain. In the physical approach, the wet biomass was frozen at -4, -20 and -50°C (for at least 2 hours) then thawed at different temperatures each (4, 25 and 40ºC) in four cycles, arranged in 9 different combinations. The experimental design was a factorial design 3 x 3 in 9 treatments and 3 replicates. In the enzymatic approach, the wet biomass was incubated with lysozyme (2 µg/mL) at three different temperatures (4, 25 and 40ºC) for 24 hours.

In all treatments, the cell debris were removed by centrifugation at 10,000 xg and the supernatant was pooled and labelled as crude extract. The most optimal extraction method was determined according to the maximum phycocyanin recovery.

Enzymatic extraction

Lysozyme enzymatic preparation was incorporated into the cyanobacterial biomass at a ratio of 2 µg mL^{-1}. The mixtures were incubated at different temperatures (4, 20 and 40ºC) for 24 hours before removing the cell debris by centrifugation and calculating the amount of the extracted pigments as previously described.

Purification of C-PC by ammonium sulfate precipitation

Purification procedure of C-PC was followed as described by Soni *et al.* (2006). Finely powdered ammonium sulfate was gradually added into the crude extract of cyanobacteria to obtain 20% saturation and kept under continuous stirring for 1 hour. The

resulting solution was kept overnight and centrifuged at 17,000 xg for 20 min. The supernatant was pooled and subjected to 70% ammonium sulfate saturation as previously described. After overnight incubation, the solution was centrifuged at 17,000 xg for 20 min and the resulting pellets were resuspended in a small quantity of 20 mM Tris–Cl buffer (pH 8.1) and subjected to dialysis for 48 hours against 100 times volume of the same buffer, with a change of buffer four times.

SDS-PAGE

SDS–PAGE was performed on a discontinuous buffered system according to Laemmli (1970). Stacking and separation gels (3% and 12%) were prepared from 30% acrylamide and 0.8% N, N-bis methylene acrylamide solution. The electrode buffer (pH 8.3) contained 0.025 mol Tris, 0.192 mol glycine, and 0.1% SDS. Five milligrams of phycocyanin were dispersed in 1 mL of 0.03 M Tris buffer (pH 8.0) for 15 min with vortexing and the extract was then centrifuged for 10 min at 11000 xg. An aliquot of the extract (20 µL) was mixed with 20 µL of SDS-sample buffer, heated at 96 °C for 3 min and an aliquot (10 µL) from the final mixture was electrophoresed. After running at 10 and 20 mA at the stacking gel and running gel respectively, staining was performed with Coomassie Brilliant Blue R-250 dye.

Statistical analyses

The data recorded in three replicates for the parameters in various treatments were subjected to analysis of variance (ANOVA) in accordance with the experimental design (factorial deign 3x3) according to Snedecor and Cochran (1980) using SPSS statistical package to evaluate the sources and magnitudes of variation. Duncan's multiple range test (DMRT) was applied to compare the mean performances of different treatments for the specific parameters under study and the rankings were denoted by superscripts in the relevant Tables.

Results and Discussion

Identification of the cyanobacterial isolate

Morpholgical and biochemical characterization

Identification of the cyanobacterial isolate was primarily investigated through morphological features; e.g. color of cultures, shape, width and length of vegetative cells, heterocysts and akinetes as well as the presence or absence of sheath (Table 1 and Figure 1). Biochemical properties such as total protein, nitrogenase activity, chlorophyll a,

phycocyanin contents and percentage of chlorophyll a/ phycocyanin (Table 1) were also considered. The investigated isolate is characterized by filamentous, blue green color culture. In addition, the shape of the vegetative cells was cylindrical, and their diameters ranged from 1.5-3.0 µm in width and from 3.0-5.0 µm in length. Moreover, the heterocysts are characterized by an extended spherical shape with two different diameters; 2.5-3.5 µm width and 2.5-4.0 µm length. Meanwhile, akinetes were present in their cylindrical shapes and their diameters were in the range; 5.0-6.0 µm width and 7.0-8.0 µm length. The biochemical traits of this cyanobacterial strain are characterized by 13% protein content, 16.05 µmole C_2H_4/h/mL of nitrogenase activity and 27.81%. of chlorophyll a / phycocyanin. Traditionally, *Anabaena* species are identified according to their morphological characters such as morphology of filaments, vegetative cells, heterocysts, and akinetes (Rajaniemi *et al*., 2005). Significant differences were previously observed among *Anabaena* strains with regard to the shape and size of trichomes and individual cells, as well as the qualitative and quantitative aspects of phycobiliprotein accumulation and activities of enzymes involved in nitrogen metabolism (Prasanna *et al*., 2006). The aforementioned morphological characteristics and the biochemical attributes of this cyanobacterial isolate globally support its classification as *Anabaena oryzae* according to Desikachary (1959), Rippka *et al*., (1979) and Bergey's Manual of Systematic Bacteriology (2001).

16S rDNA gene sequence analysis

The 16S rDNA gene encoding a component of the small subunit in the ribosome of all bacteria is used for the bacteria identification (Clarridge, 2004). Nearly full-length 950-bp nucleotide sequence of the 16S rDNA gene from the tested cyanbacterial isolate (Figure 1) was generated as presented hereafter:

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CGAAGCGTAGATCGGATCGGGGCTAGAGTTTCATCCTGGCTCAGTAAGTCGTAACAAGG
TATCCGTAAATCGTTTCCCGGGGGCCAGTTTCATCCTGGAAAGGACGCCAAAAAAAGGT
TCCCGTAAATGGTTTCCCTATTTCCCGACCAATCAACAACAATCACCCTAACAAGAAGTT
CCCGGAGTAATGTCCCTCCCATCCTAAAACGGGTACCCTGTGTGAGAACACGACCTGCG
ACGTCGGGACTGAGACACTGCCCGGACTTCTACAGAAGGCTGCAGTAACGAATCTTCCG
CAATGGGCACAAGCCTGACGGAGCAATGCCGCGTGGGGGATGAAGCGGCTACACCGTG
TAAACCACTGTCAGGGGCTAGAACCAATGATCAGCCCCAAAGGAAAAGCCGGCTAACC
CTGTGCCAGCAGCCGCGGAATACTGGGGGCTCGAGCGTTAATCGGAATCACTGGGCTTA
AAGGGTGCGTAGGTGGCTTGCCAAGCGTCCTGTGAAATCCCCCGGCTCAACCCCGGAAC
AGCAGGGCGAACTGGCAGGCTTGAGGCATGTAGGGGCGGACAGAACAGTCGGTGGAGC
GGTGAAATGCGTAGATATCGACTGGAATGCCGATGGGGAAACCGGTCCGCTGGGCATGT
CCTGACACTGAGGCACGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGTGCACTAGACTGGTGCGGTTTTTGACGCCTTATCGCCGAAGTAA
AAACGAATAAGTGCTCCGCCGGGGGAGTACGGTCGCAGGGCTAAATCTCAAAGGAATT
GACGGGGGCTCACGCAAGCGGGTGGAGCATGTTGCTTAATTTTAAGCACGGCGAAGAA
CCTTACGCGGGCGTTGACATGCTTGGAATTACTCTCATGATAGATAGGCCACCCCGTTCT
GGGTGTGAAC
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Table 1. Morphological and biochemical characteristics of the cyanobacterial isolate
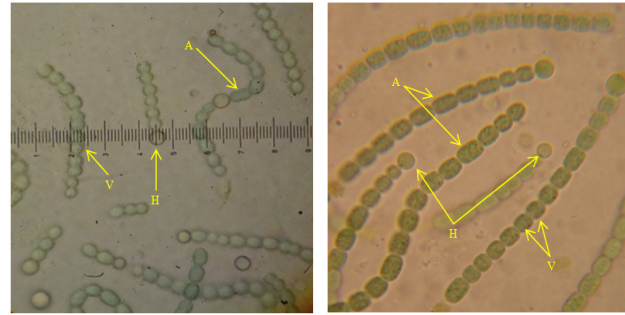
	Characteristic		Specification
Morphological	Vegetative cell	Color	Blue green
		Shape	Cylindrical
		Width (μm)	1.5-3.0
		Length (μm)	3.0-5.0
	Heterocyst	Shape	Spherical
		Width (μm)	2.5-3.5
		Length (μm)	2.5-4.0
	Akinetes	Shape	Cylindrical
		Width (μm)	5.0-6.0
		Length (μm)	7.0-8.0
	Sheath	Presence	No
Biochemical	Total protein	(%)[1]	13 ± 0.5 %
	Chlorophyll a	(mg/ mL)	0.64 ± 0.08
	C-PC	(mg /mL)	1.97 ± 0.2
	Pigments	(%)[2]	27.81 ± 1.5%

[1]g protein/100 g dry weight.   [2] % Chlorophyll. a phycocyanin

rDNA gene sequence of the tested isolate with genera representing heterocystous cyanobacteria *(Anabaena)* available in the Gene-Bank as well as other selected *Anabaena* species available in the databases, the studied isolate was concluded to be within the identity of *Anabaena* genus. The phylogenetic tree of the tested *Anabaena* strain was inferred by neighbour-joining distance method (Thompson *et al.*, 1994). Based on the 16S rDNA sequence and the comparison with other relevant sequences available in the public databases (Figure 2), BLAST search revealed maximum similarity scores with *Anabaena*. Meanwhile, it is generally noticed that the tested isolate is quite different from the previously identified and registered *Anabaena* strains. The nearest relatedness is only 83 and 84% with the cluster B and cluster A, respectively. With the rest of the known strains (C and D) the relatedness is further lower down to 78%. This wide separation from the previously identified strains indicates that the studied isolate is a new identified Egyptian strain genotypically different from other known identified ones and this is the first time to identify and report such strain. This new strain was given the code name SOS13. Additionally, the 16S rDNA gene sequencing analysis of the tested isolate confirms that heterocyst-forming cyanobacteria including the new identified strain belong to the monophyletic group. The obtained phylogenetic data correlate with the traditional classifications based on the morphological characteristics(filamentous heterocystous cyanobacteria without true branching) referring to the order Nostocales (Gugger and Hoffmann, 2004).

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Light micrographs

DNA sequencing images

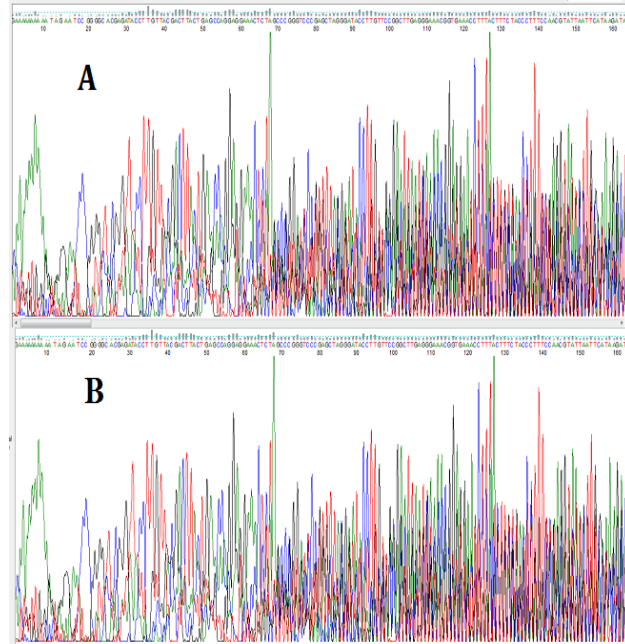
Figure 1. Light micrographs of *Anabaena* isolate (A: Akinete, H: Heterocyts, V: Vegetative cell) and its DNA sequencing image of a section of the 16S rDNA gene using forward (A) or reverse (B) primer.

ptimizing the extraction of phycocyanin

The data in Table 2 present the extraction yields of phycocyanins from the identified cyanobacterial strain (*Anabaena oryzae* SOS13) in response to different combinations of freezing-thawing treatments arranged in a factorial design (3 X 3). Based on the statistical analysis, the single freezing factor seems theoretically to proportionally augment the estimated extraction yield when lowering the freezing temperature from -4 down to -20 then -50ºC. Nevertheless, the single thawing factor seems only directly proportional to the extraction yield when raising the temperature from 4 to 25ºC. The extraction yield corresponded theoretically to ca. 95, 94 and 97% of the three pigments (C-PC, C-APC and C-PE, respectively) at 25ºC thawing temperature. Further increase in thawing temperature negatively affected the extraction yield of the three phycocynin pigments.

Combined freezing-thawing treatments were

Table 2. Phycocyanin extractability (X ± SE) from *Anabaena oryzae* SOS13 using different combinations of freeze-thaw treatments

Treatments	C-PC		C-APC		C-PE	
	µg mL^{-1}	%	µg mL^{-1}	%	µg mL^{-1}	%
Freezing temperature						
-4°C	89.9±5.3[a]	79.8	59.2±3.4[a]	83.5	14.3±2.3[a]	84.6
-20°C	95.2±6.0[b]	84.5	62.2±6.7[b]	87.7	14.3±2.3[a]	84.6
-50°C	100.1±6.3[c]	88.8	64.5±6.8[b]	91.0	15.1±3.6[a]	89.3
Thawing temperature						
4°C	80.6±4.7[a]	71.5	55.7±4.5[a]	78.6	12.8±1.9[a]	75.7
25°C	107.3±6.6[c]	95.2	67.1±5.3[b]	94.6	16.4±2.4[b]	97.0
40°C	97.3±5.3[b]	86.3	63.1±4.8[b]	89.0	14.4±2.3[ab]	85.2
Interactions (Freezing xThawing)						
-4°C x 4°C	70.4±2.3[a]	62.5	51.9±1.3[a]	73.2	12.4±1.3[a]	73.4
-4°C x 25°C	103.9±3.7[c]	92.2	62.8±2.6[b]	88.6	15.8±2.1[b]	93.5
-4°C x 40°C	95.6±3.4[b]	84.8	62.8±2.3[b]	88.6	14.8±2.1[ab]	87.6
-20°C x 4°C	81.9±3.1[ab]	72.7	56.4±1.6[a]	79.5	12.5±1.3[a]	74.0
-20°C x 25°C	105.2±6.3[c]	93.3	67.4±1.8[bc]	95.1	16.4±1.7[b]	97.0
-20°C x 40°C	98.5±5.4[b]	87.4	62.6±1.6[b]	88.3	13.8±1.8[a]	81.7
-50°C x 4°C	89.5±2.5[b]	79.4	58.7±1.9[ab]	82.8	13.4±1.6[a]	79.3
-50°C x 25°C	112.7±6.6[d]	100.0	70.9±2.5[c]	100.0	16.9±2.3[b]	100.0
-50°C x 40°C	98.1±4.6[b]	87.0	63.7±2.3[b]	89.8	14.6±2.1[b]	86.4

Means in the same column with different superscripts are significantly (P≤0.05) different

associated with different levels of phycocyanin yield, where the combination -50ºC x 25ºC achieved the complete extraction of the three pigments (100% each). So, this combination is the most effective one and can be recommended for phycocyanin extraction. These results are in general agreement with Singh *et al*. (2010) who observed that the freezing/thawing method resulted in a maximum C-PC yield although they did not define the exact parameters of extraction. The enzymatic extraction of the three pigments was significantly influenced by the temperature giving maximum yields at 40ºC (data not shown). Under the experimental conditions followed in the current study, the extraction was far from completion. The highest obtained yields were only 63.2, 48.1 and 53.8% of the original contents of the three pigments; C-PC, C-APC and C-PE, respectively. The extraction of the three pigments by enzymatic approach was much less effective than the physical freeze-thaw method, achieving complete extraction of the three pigments. The pigment C-PC was the most extracted by the enzymatic method at the three applied extraction temperatures. The relative content of the aforementioned pigment was higher in the enzymatic than in the physical extraction due to the relatively lower extent of extraction of the other two pigments. Hence, the freeze-thaw extraction of the phycocyanin is more efficient, economic and probably less liable to potential chemical changes associated with other techniques.

The relative proportions of the pigment components after physical extraction as percentages of every component to the sum of pigments were calculated. The standard deviation inside each group

of interaction was also calculated (data not shown). For the first group of interaction (Freezing at -4ºC and thawing at three different degrees) the standard deviations (SD) of the contents of three components from the average was in the range 4-6, i.e. there were big differences between the different preparations according to the thawing temperature. Reducing the freezing degree to -20ºC while interacting with three different thawing temperatures resulted in values different from the average by a medium SD in the range 1.9-2.7, i.e. there were medium changes in the relative values of the three components according to the degree of thawing.

Further reducing the freezing temperature to the minimum (-50ºC) while still having the three different thawing degrees reduced the differences between the relative values of the components in the different preparations, recording the least SD values; 0.7-1.3, i.e. thawing temperature was not influencing when the freezing temperature was at the lowest value (-50ºC). So, even if the optimal condition for the highest extraction yield is -50ºC X +25ºC, the relative values of each component of the pigments will remain practically the same even if the thawing temperature deviated from +25ºC.

Selection of a suitable extraction method is very important for the maximum recovery of phycocyanin from cyanobacterial cells. Many studies showed that the best methods for extracted of C-PC are freezing-thawing and lysozyme treatment (Soni *et al*., 2006; Singh *et al*., 2010). Nevertheless, neither the optimum temperature for freezing–thawing nor the optimal conditions for lysozyme extraction have been well specified. The observed highest
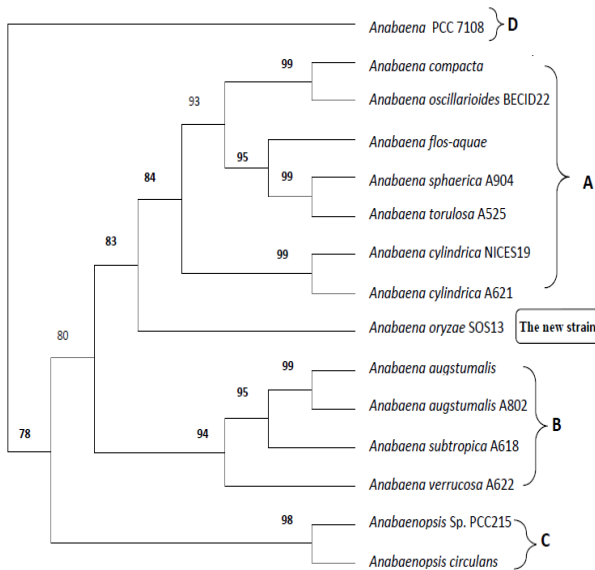
Figure 2. Neighbour joining phylogenetic tree for the tested cyanobacterial isolate 16S rDNA gene sequences as compared with other sequences available in public databases. Bootstrap values are indicated in the point at nodes.

Figure 3. SDS-PAGE of phycocyanin isolated from a newly identified *Anabaena oryzae* SOS13. St (protein standard), Lane 1 (crude extract of phycocyanin); Lane 2 (purified phycocyanin rich fraction).

phycocyanin extractability by the combination freeze-thaw treatment -50ºC X 25ºC is apparently due cell swelling and cell breakdown caused by the sharp crystals formed during freezing. The subsequent thawing step will cause cellular contraction, further cellular breakdown and leakage leading to the release of the cellular pigments. For complete pigment extraction, four cycles of this freezing-thawing step were required. No colored extract could be recovered upon further successive freezing-thawing cycles, indicating the absence of phycobiliproteins in cellular remnants and complete extraction. The obtained supernatant showed its highest absorption pick at 620 nm indicating the maximum concentrations of the three phcocynin components in the following order; C-PC > C-PE > C-APC. Although the extractability of C-PC obtained in this study by the combination freeze-thaw treatment -50ºC X 25ºC was not the greatest among the values reported in the literatures, it is still the highest among the genera of the order Nostocales.

Purification of phycocyanin with ammonium sulfate fractionation

The two step ammonium sulfate (20 % then 70%) fractionation procedure was used for the purification of the extracted pigments. This procedure is particularly useful at salting out unwanted proteins and simultaneously at concentrating the principal pigment (C-PC). Ammonium sulfate was used as a precipitating and purifying agent since it readily precipitates phycobiliproteins and maintains their
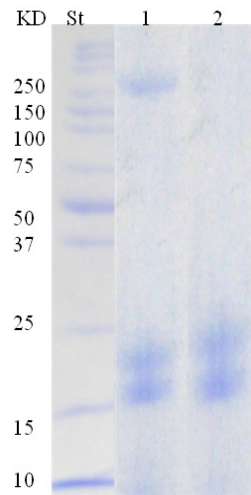
integrity. It is also highly water soluble at low temperatures and had bacteriostatic effect (Englard and Seifter, 1990). SDS-PAGE indicated the presence of three main bands in the crude extract. Two bands correspond to low molecular weights between 15 and 25 kD and one band correspond to high molecular weight ($\geq$ 250 kD). After purification the high molecular weight band, potentially representing protein impurities totally disappeared while the two low molecular weight bands potentially remained unchanged (Figure 3). These two low molecular weight (>15 kD and <25 kD) bands probably represent the pure phycocyanins in accordance with the results of Santiago-Santos et al. (2004) who defined the molecular weight of the two comparable SDS-PAGE bands as 17 and 21 kD each while the estimated molecular mass of native purified phycocyanin was 114 kD.

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