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PROTEIN FRACTION OBTAINED FROM EGYPTIAN ISOLATES OF CYNOBACTERIA HAS RESTRICTION ACTIVITIES

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Abstract

Six cyanobacterial strains isolated from different locations in Egypt were screened for the presence of restriction enzyme(s) where the extraction of restriction enzymes was performed using two-buffer system. Species of *Phormidium autumnale*, *Cyanothece aeruginosa* and *Spirulina platensis* were found to possess restriction activity that linearized plasmid pUC18 DNA. To identify the protein fragments of restriction activity, a DEAE cellulose column fractionation was performed and elution was carried out by gradient concentrations of NaCl. Fractions isolated with concentration of 0.1 and 0.4 M NaCl were found to possess restriction activity. Biotechnological potential of those fractions was tested using genomic DNAs from two different plants. Both the genomic DNAs from *Lactuca sativa* and *Solanum tuberosum* were successfully cut thereby confirming their biotechnological potential.

Introduction

Cyanobacteria are oxygenic photosynthetic prokaryotes that lack plastids but possess thylakoids. All cyanobacteria contain chlorophyll *a* as the main photosynthetic pigment in addition to other accessory pigments such as carotenoids and phycobiliproteins (Douglas 1994). Due to lack of enough diagnostic phenotypic characters of cyanobacterial strains, protein profiling using whole cell protein pattern analysis (SDS-PAGE) was used as a way of protein fingerprinting for comparing closely-related isolates (Vandamme *et al*. 1996). Cyanobacteria inhabiting extreme niches of hot or saline habitats in Egypt, have coped to those conditions through metabolic adaptation by producing a wide spectrum of bioactive compounds/entities (El Semary and Abd El Nabi 2010). Some of those compounds showed antimicrobial action (El Semary 2011, El Semary and Mabrouk 2013, El Semary *et al*. 2015) while others have anticancer potential (El Semary and Fouda 2015).

The most important biological entities are enzymes, particularly restriction enzymes. Restriction enzymes are one of the most important tools in the molecular genetics (Madigan *et al*. 2000). Nevertheless no studies were found on the isolation of those enzymes from cyanobacteria present in Egypt. Cyanobacteria are particularly considered as a rich source of type II restriction endonucleases (Reaston *et al.* 1982, Whitehead and Brown 1985). In many cyanobacteria, there are multiple restriction enzymes in a single strain (e.g., strain of *Nostoc* sp. PCC7524 contained five enzymes (Reaston *et al.* 1982). Piechula *et al.* (2001) discovered two different strains of mesophilic *Phormidium* sp. that produced thermophillic restriction endonucleases *Pta I* and *Ppa AII* with their optimum activity at 65 - 80ºC. A new type II restriction endonuclease, *Ofo*I from *Oscillatoria foreaui* was reported for the first time by Saravanan *et al.* (2003). A putative nicking endonuclease was isolated from unicellular cyanobacterium *Chroococcus minutes* that cleaves only one strand of the duplex DNA forming a nick or a gap (Sundararajan *et al*. 2010). Unfortunately, reports on those enzymes from cyanobacteria from Middle East are missing due to theunder-exploitation of microorganisms from this region. Therefore, this study was conducted to investigate the restriction activity of three Egyptian cyanobacterial strains.

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Materials and Methods

Synechocystis salina, Phormidium tenue, Oscillatoria sp., *Phormidium* sp., *Cyanothese* sp. and *Spirulina* sp. which were previously collected from different sites of Egypt and kept in Helwan culture collection were selected and grown on modified BG11 medium (Watanabe *et al*. 2000). The cultures were incubated in growth room at 27 ± 2ºC in (12 : 12) dark/light cycle.

Protein profiling was made by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), (15% separating gel and 4% stacking gel (Acrylamid- Bisacrylamid , Gel buffer, 10% APS (freshly prepared), TEMED) according to Laemmli (1970) in a mini gel apparatus (Bio – Rad) according to the method of Hames (1990). The gel set was assembled to pour the gels. The separating gel layer was poured first and immediately after adding ammonium persulfate (APS) and tetramethyl ethylene diamine (TEMED) isopropanol was added on the surface of this layer. After polymerization, the isopropanol was removed. The prepared stacking layer was poured immediately after adding APS and TEMED and then the comb was added immediately. After polymerization, the comb was gently removed to keep the wells sound. After that the gel was put in the electrophoresis tank and filled with the run buffer "TGB (Tris - hydroxylamine, glycine, SDS and distilled water), next the samples were loaded. Finally the tank was connected to the power supply and the gel was run at 80V for 15 min. The volt was raised to 120 V to the end of run. The gel was then placed in large glass Petri dish and dehydrated with 70% methanol, rehydrated with water and then covered with staining solution overnight with homogenous shaking.

The biomass of six cyanobacterial strains grown in BG11 medium (about 5 g fresh weight**)** was collected after 35 days of growth and was centrifuged at 6000 rpm at room temperature for 20 mins. The cells were washed by distilled water with repeated centrifugation for three times. The cells were crushed in a mortar using extraction buffer. This extraction buffer actually consisted of two different buffer systems.

The first buffer was a general buffer for enzyme extraction which was modified from Venisse *et al*. (2001). Five ml of the first buffer (modified from Venisse *et al*. 2001) was added to cells and sonicated (Biologics, Inc. Ultrasonic Homogenizers model 150vt) at power 40, pulse 30 and kept at 4ºC using ice bath. The sample was centrifuged at 6000 rpm for 30 min and the supernatant was taken into a sterile 15 ml falcon tube and five ml of the second buffer was added. The second buffer (Sundararajan *et al*. 2010) contained 10 mM Tris pH 7.4, 50 mM NaCl, 0.1 mM EDTA and 5 mM β-mercaptoethanol). This was followed by enzymatic assay.

The restriction endonuclease assays were performed by incubating the enzyme extract with 1-2 µl the plasmid pUC18 DNA substrate in a 40 ml reaction buffer (10 mM tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl and 5 mM β-mercaptoethanol and 0.5 µl BSA) for 1.5 hrs at 37°C (Saravanan *et al*. 2003). The result was observed by running on a 0.8% agarose gel.

Partial purification of enzyme was performed on a DEAE column. DEAE cellulose (2.5 g) was added to 50 ml distilled water, stirred slowly and then was kept overnight in freeze for swelling. Swollen DEAE cellulose was filtered and incubated with 0.5N HCl (50 ml) for 1 hr. Then the DEAE cellulose was filtered after washing it with distilled water to remove excess HCl. Washing with water was continued until pH reached 3.0 of the HCl-treated DEAE cellulose. 0.5N NaOH (50 ml) was added and stirred on magnetic stirrer for 1 hr at 25ºC. Treated ion exchanger was washed again with distilled water till it attained neutral pH. Furthermore, it was suspended and stored in distilled water (50 ml) at 4ºC until use. The DEAE cellulose was poured onto column then poured on it about 20 ml tris buffer (10 mM tris pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol) more than two times. Five gram of fresh cyanobacterial biomass was collected and then ground in a mortar with 10 ml of 2x total protein buffer. The

sample was centrifuged at 6000 rpm for about 20 minutes. The supernatant was transferred into new falcon tube. The sample, cyanobacterial enzymatic extract using the two buffer systems was poured onto column and then 20 ml of tris buffer was added to it. The sample was eluted with a different gradient of NaCl concentration (0.1 - 0.5 M) using 10 ml fraction of each gradient. Each elute was then dialyzed against 0.5 M sucrose sugar to get rid of NaCl and different buffers in order to concentrate the enzyme. The dialyzed sample was then used for enzyme assay.

To investigate the possible application of the restriction enzymes, restriction activities were tested. The restriction endonuclease assays were performed by incubating the enzyme extract with 4 µl of the genomic DNA substrate from two different plants namely: *Solanum tuberosum* and *Lactuca sativa* in a 40 ml reaction buffer (10 mM tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl and 5 mM β-mercaptoethanol) and 0.5 µl BSA for 1.5 h at 37°C (Saravanan *et al*. 2003). The result was observed by running on a 0.8% agarose gel.

Results and Discussion

Initially, different single buffer systems were used for enzymatic extraction but only the two buffer systems developed was successful. The single buffer systems tested were prepared according to methods previously published by Piechula *et al.* (2001), Saravanan *et al.* (2003) and Sundararajan *et al.* (2010). Unfortunately, those methods did not give positive results. A modified method for extraction that depends upon two different buffer systems were developed. The first buffer used for general extraction of enzymes was modified from Venisse *et al.* (2001) and the second was more specific for restriction enzymes (Sundararajan *et al.* 2010). The success of the two buffer system is most likely due to their balanced ionic concentrations together with the suitable pH of those buffers. Also the system contained polyvinylpyrrildone which acted as protein stabiliser (Hamada *et al*. 2010).

The comparison of protein profiling between the six cyanobacterial strains was made using SDS - PAGE. The results presented in Fig. 1A demonstrated that a characteristic band appeared in the protein profile of three strains; *Spirulina platensis* and *Cyanothece aeruginosa* (lanes 1 and 2) as well as band from *Phorrmidium autumnale* in lane 5. This band had a molecular weight of ~50kD. Those strains showed restriction activity later whereas the other strains that lacked this band showed no restriction activity. Therefore, it is most likely that this band is somewhat related to restriction enzymes.

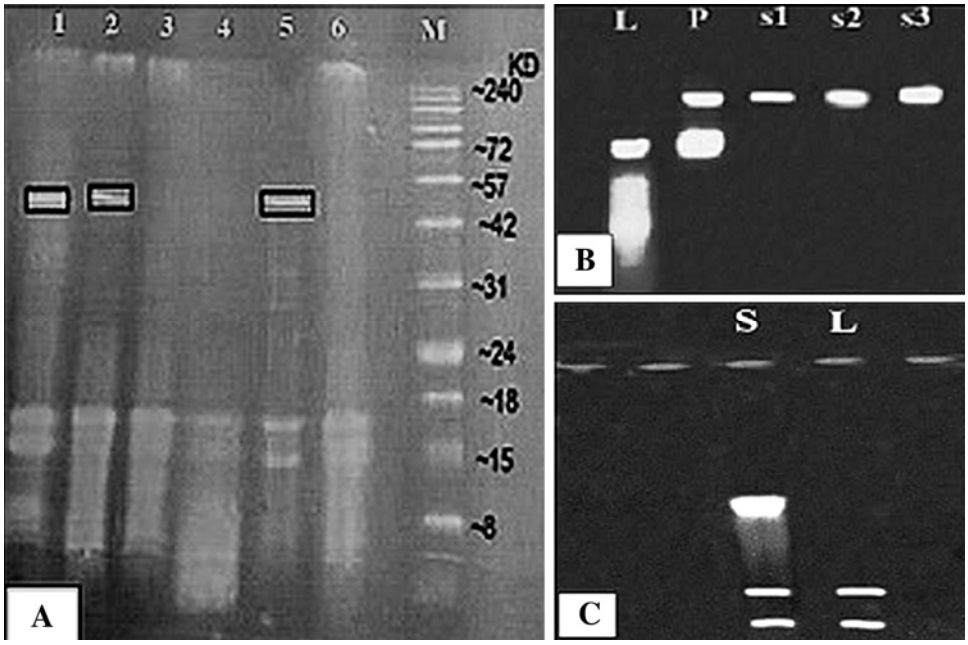
Phormidium autumnale, *Cyanothece aeruginosa* and *Spirulina platensis* cultures were screened for the production of restriction enzymes. The enzymatic bioassay showed the complete linearization of the plasmid in the three isolates and the complete disappearance of the supercoiled band characteristic of the plasmid in the uncut form (Fig. 1B). The complete linearization of the plasmid indicated the double-stranded break by the restriction enzyme (Fig. A-C).

The retrieved protein fractions were initially purified using heparine sepharose column and 1X phosphate buffered saline (PBS) buffer. However, this gave negative result as fractions did not show any restriction bioactivity. Eventually purification using DEAE cellulose column was conducted where gradients of NaCl were applied on DEAE column. Ten ml of each fraction was collected and tested for their restriction bioactivity. The results presented in Table 1 demonstrated that fractions 0.1 and 0.4 showed restriction activity that confirmed that fraction eluted with 0.1 and 0.4 M NaCl gradient is the suitable gradient for purification of the enzyme on DEAE cellulose column. Partial purification of these enzymes from cyanobacteria was performed using DEAE cellulose column which is a weak anion exchanger resin. This type of separation using column chromatography is based upon ion exchange mechanism. The gradient concentration of NaCl acted as elution buffer as it is neutral salt that does not change pH, therefore, it is non-destructive

to proteins. This salt gradient can release first unbound proteins then bound proteins if the concentration is increased. The NaCl can then be removed by dialysis thereby not affecting eluted fragments. The fractions obtained at concentrations of 0.1 and 0.4 M showed restriction activity by cutting the super-coiled form of plasmid and linearizing it.

Table 1. Eluted enzymatic fractions using different NaCl concentrations and their restriction activity (plus sign indicates positive activity).

Fractions	NaCl gradient (M)	Restriction enzyme activity
F1	0.1	+
F2	0.2	-
F3	0.3	-
F4	0.4	+
F5	0.5	-



Figs. 1A-C: A. Total protein profiling for six strains of cyanobacteria. M is a low molecular weight protein marker (8-240 KD). The 50 KD band in the three isolates *Spirulina platensis*., *Cyanothece aeruginosa*, (Lanes 1 and 2) and *Phormidium autumnale* (Lane 5) is indicated by a rectangular box. B. L is DNA marker. P is PUC18 plasmid. Lanes S1, S2 and S3 denote extracts from strains *Phormidium autumnale*, *Cyanothece aerunginosa* and *Spirulina platensis*, respectively. C. application of restriction enzyme from these cyanobacteria on genomic DNA of *Solanum tuberosum* (S) and *Lactuca sativa* (L).

The investigation of the effect of the isolated protein fragments of previously shown restriction activities on genomic DNA from two different unrelated plants namely: *Solanum tuberosum* and *Lactuca sativa* gave positive results. Fig. 1C shows the genomic DNA after treatment with the eluted protein fragment with restriction activity. The genomic DNA of

S. tuberosum was cut into 3 pieces, which gave three bands of DNA (1000, 3000 and ~2600 bp) whereas DNA of *L. sativa* was cut by enzymatic extract of *Cyanothece aeruginosa* and resulted in two fragments which gave two bands (3000 ~ 2700 bp).

The cutting of genomic DNA of two different plants with retrieved protein fractions with restriction activities verifies that this type of restriction enzymes makes double stranded breaks and not nicking enzyme and also confirms the biotechnological potential of this enzyme.

The detection of restriction activities in cyanobacterial isolates is not surprising. The extreme environmental conditions of Egypt would act as selective pressure that allows only the microorganisms of exceptional metabolism and unique enzymes to survive and strive under those conditions. Unfortunately, the potentials of those microorganisms still await thorough investigations. The present research represents an attempt to investigate the restriction activities of cyanobacteria isolated from different localities of Egypt. Results indicate that some isolates do possess this activity. However, more isolates should be screened and exploited in future.

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