



Original article

Isolation and characterization of two novel plasmids pCYM01 and pCYM02 of *Cylindrospermum stagnale*Venkatesan Ganesan^{a,b,*}, Rathinam Raja^c, Shanmugam Hemaiswarya^d, Isabel S. Carvalho^c, Narayanaswamy Anand^b^a Acme ProGen Biotech (India) Private Limited, Balaji Nagar, Salem 636 004, India^b Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai 600 025, India^c Food Science Laboratory, Center for Mediterranean Bioresources and Food, FCT, University of Algarve, Gambelas, Faro 8005 139, Portugal^d AUKBC, MIT Campus, Anna University, Chrompet, Chennai 600 044, India

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ABSTRACT

Cyanobacteria play a vital role in supplying nitrogen into the soil and aquatic ecosystem. It has an extra chromosomal DNA, whose role is not yet defined well. Isolation and characterization of extra chromosomal DNA in cyanobacteria might help to understand its survival mechanism. *Cylindrospermum stagnale* isolated (and deposited in NRMCF 3001) from soil showed presence of four plasmids namely pCYLM01, pCYLM02, pCYLM03, and pCYLM04. The following plasmids pCYLM01 and pCYLM02 were subjected to restriction digestion using *Hind*III restriction enzyme and cloned into pBlueScriptSK(-) vector. The sequence of pCYLM01 contained 4 potential open reading frames (ORFs) that have amino acids in the range of 59–299. Among them, ORF1 shows high sequence homology to the bacterial replication initiator family protein as evident from BLASTP analysis. The analysis of 4359 bp plasmid pCYLM02 sequence revealed 7 ORFs which are longer than 50 amino acids in length. The ORF2 of pCYLM02 has 243 amino acids and is represented in the plasmid sequence from 3045 to 3776 bp. The ORF3 of pCYLM02 corresponds to the plasmid sequence from 2323 to 2976 and codes for a putative protein of 217 amino acids long. A number of small ORFs below 50 bp were also found in the sequence analysis.

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1. Introduction

Cyanobacteria belong to one of the oldest group of microorganisms which are photoautotrophic, prokaryotic, and exhibit greater diversity when compared to unicellular, colonial, filamentous and heterotrichous forms (Black et al., 1995; Hess and Kopfmann, 2013; Stucken et al., 2013). Cyanobacteria have two types of DNA, chromosomal DNA and self-replicating plasmid DNA. The existence of plasmid DNA in cyanobacteria was first observed in *Anacystis nidulans* (Asato and Ginoza, 1973). The number of plasmids per cyanobacterial cell may vary from one to eight with sizes

ranging from 1.3 to 130 kb (Marsac and Houmard, 1987; Houmard and Tandeau de Marsac, 1988; Goyal, 1990; Ma et al., 2012). For instance, *Nostoc* sp. PCC 7524 has three plasmids, pDU1, pDU2 and pDU3 with sizes of about 6.1, 11.8, and 37.3 kb, respectively. Restriction digestion pattern analysis revealed that the plasmid pDU2 was the dimeric form of pDU1 (Reaston et al., 1980).

Though many cyanobacterial plasmids have been identified so far, the exact functions of these plasmids are yet to be elucidated. However, several researchers have proposed their probable functions which include gas vacuolation (Walsby, 1977), toxin production (Dittmann et al., 2013; Cirés et al., 2017), resistance to high salt concentrations, heavy metals (Van den Hondel et al., 1979; Huertas et al., 2014), resistance to antibiotics (Vachhani et al., 1993; Hahn et al., 2012), synthesis of restriction modification enzymes (Nelson and McClelland, 1987; Lubys et al., 1996) and sophisticated poisoning system (Hess and Kopfmann, 2013). In the present study, we have identified four plasmids (pCYLM01, pCYLM02, pCYLM03 and pCYLM04) from *Cylindrospermum stagnale*. This is the first report on the presence of plasmids in the genus, *Cylindrospermum*. The current study has been aimed to

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understand the two plasmids, pCYM01 and pCYM02 of *C. stagnale* and their characterization.

2. Materials and methods

2.1. Isolation, growth and culture conditions of *Cylindrospermum stagnale*

Cylindrospermum stagnale (Kütz.) Born. et Flash was isolated from arid soil at P.M. Palayam, Namakkal District (Tamil Nadu, India). The morphological features of the isolates were identified by field microscope. The isolated culture was axenized by imipenem (Merck) and maintained in BG11 medium under a light intensity of $40 \mu\text{Em}^{-2} \text{s}^{-1}$ at $25 \pm 2 \text{ }^\circ\text{C}$ (Rippka et al., 1979; Ferris and Hirsch, 1991). The growth of *C. stagnale* was assessed by estimating its total Chlorophyll *a* (Mackinney, 1941) and protein content (Bradford, 1976).

2.2. Isolation of total DNA from *C. stagnale*

Exponentially growing cyanobacterial cells (10 mL) were harvested by centrifugation and washed twice with the washing buffer (Tris 100 mM, EDTA 50 mM, and NaCl 100 mM, pH 8.0). The washed cells were resuspended in 200 μL of P1 buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 10 μL of 10 mg/mL RNase A, and 2.5 mg/mL lysozyme) and incubated at $37 \text{ }^\circ\text{C}$ for 1 h. To this solution, SDS (Sodium Dodecyl Sulfate) was added to a final concentration of 2% and mixed vigorously by inversion. An aliquot (100 μL) of 5 M NaCl was added and the contents were mixed by inversion followed by refrigeration at $-20 \text{ }^\circ\text{C}$ for 10–20 min. The mixture was centrifuged at 12,000 rpm for 5 min and to the supernatant equal volume of phenol: chloroform (1:1, v/v) was added, mixed well and centrifuged again at 12,000 rpm for 5 min. Another extraction with chloroform alone was carried out and the upper aqueous phase was transferred to a fresh microfuge tube. To this aqueous phase, 2.5 vol of ice-cold 95% ethanol was added and incubated at $-20 \text{ }^\circ\text{C}$ overnight for precipitation. The DNA was pelleted by centrifugation at 12,000 rpm for 5 min and after washing with 70% ethanol, the pellet was air-dried and DNA was dissolved in 0.1X TE buffer. The DNA was analyzed by electrophoresis on a 0.8% agarose gel in 0.5X TBE buffer (Sambrook et al., 1989).

2.3. Isolation of plasmid DNA from *C. stagnale*

The plasmid extraction was performed according to the method with few modifications (Birnboim and Doly, 1979). Exponentially grown (16 days old) *C. stagnale* culture (100 mL) was harvested by centrifugation and washed twice with wash buffer. The washed cells were resuspended in 0.5 mL of P1 Buffer (50 mM Tris, 10 mM EDTA and RNase A, 100 $\mu\text{g}/\text{mL}$ (pH 8.0) containing 2.5 mg/mL of lysozyme and incubated at $37 \text{ }^\circ\text{C}$ for 1 h. A 0.5 mL of P2 Buffer (200 mM NaOH, 1% SDS (w/v) was added and mixed thoroughly by inverting the tube 10–15 times, and incubated at room temperature ($25\text{--}30 \text{ }^\circ\text{C}$) for 10 min. A 0.5 mL of pre-chilled P3 buffer (3.0 M potassium acetate (pH 5.5) was added, mixed immediately and vigorously by inverting 10–15 times, and incubated at $-20 \text{ }^\circ\text{C}$ for 1 h. The tubes were centrifuged at 12,000 rpm for 5 min at 4 and the supernatant was transferred to fresh tubes. To this supernatant solution, equal volume of phenol: chloroform (1:1, v/v) was added, mixed well and centrifuged at 12,000 rpm for 5 min. The remaining procedures were same as mentioned for the isolation of total DNA.

2.4. Heat treatment of the isolated plasmid DNA from *C. stagnale*

To determine whether the DNA bands on the agarose gel contain covalently closed circular (CCC) double stranded DNA, a 25 μL of plasmid prep in TE buffer containing 0.1% Sarkosyl was heated at 100°C for 2 min. The plasmids were allowed to cool in ice and immediately subjected to electrophoresis before renaturation (Van den Hondel et al., 1979). The following plasmids pBR322 and pUC18, and λDNA were obtained from ThermoFisher Scientific, treated in the same way and used as controls.

2.5. Gel elution of plasmid DNA from *C. stagnale*

The isolated plasmids of *C. stagnale* from 1 L culture were pooled together and loaded onto 0.8% agarose preparative gel and ran at 70 V in 1X TBE buffer (Sambrook et al., 1989). The plasmid DNA bands were excised from the agarose gel and the cut pieces dialysed against 1X TBE buffer. The solution was precipitated with 1/10th volume of 3 M sodium acetate along with 2.5 volumes of 95% ethanol and incubated at $-20 \text{ }^\circ\text{C}$. The plasmids were pelleted by centrifugation at 12,000 rpm for 10 min and washed with 500 μL of 70% ethanol and air dried. The pellet was dissolved in 20 μL of 0.1X TE buffer. The isolated plasmids were named as pCYLM01, pCYLM02, pCYLM03 and pCYLM04. The plasmids, pCYLM01 and pCYLM02 were further cloned into pBlueScriptSK(-) vector.

2.6. Cloning of *C. stagnale* plasmids

The vector pBlueScriptSK(-) was digested with *Hind*III restriction enzyme, dephosphorylated with Calf Intestinal Alkaline Phosphatase (NEB) and purified by phenol: chloroform (1:1; v/v) extraction method. The plasmids pCYLM01 and pCYLM02 were digested with *Hind*III restriction enzyme, purified and ligated into the pBlueScriptSK(-) vector. The ligation mixture was transformed into *E. coli* DH5 α cells and plated onto agar plates incorporated with ampicillin and X-gal. After incubation, the clones were selected by blue/white colony selection (Black et al., 1995). The white colonies were initially screened for the plasmids (pCYLM01 and pCYLM02) by colony-PCR method (Sandhu et al., 1989; Zon et al., 1989) using M13 forward and reverse primers.

2.7. Sequence analysis of plasmids

The plasmids pCYLM01 and pCYLM02 were sequenced and analysed using online bioinformatic tools such as BLAST (Altschul et al., 1990) for similarity search, ORF finder for detecting ORFs (Stothard, 2000), EMBOSS (Rice et al., 2000) for finding repeats and DNA motifs, Tandem Repeats Finder (Benson, 1999), Snappgene (<http://www.snappgene.com/>) to draw circular map of the plasmids and NEB cutter V2.0 (Vincze et al., 2003) for restriction site analysis.

2.8. Newly reported sequence accession numbers

pCYLM01 - Accession No. EF221636 and pCYLM02 - Accession No. EF452232.

3. Results

3.1. Isolation of *Cylindrospermum* strain

Cylindrospermum stagnale (Kütz.) Born. et Flah was isolated from soil identified by morphological observation (Fig. 1) and confirmed by 16S rDNA sequencing (Accession no. DQ897365). The

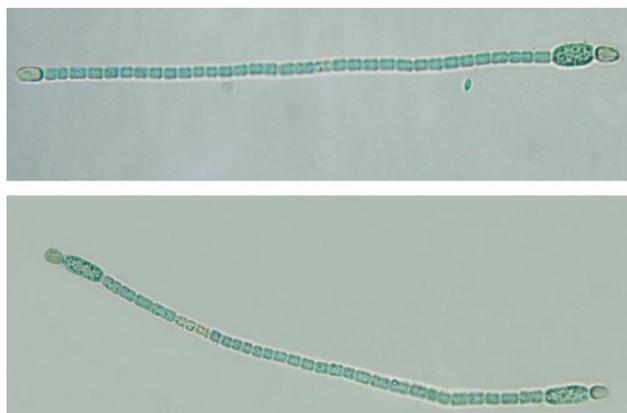


Fig. 1. Morphology of *Cylandrospermum stagnale* (Kütz.) Born. et Flah. (X 600).

isolated axenic culture of *C. stagnale* has been deposited (NRMCF-3001) at National Repository for Microalgae and Cyanobacteria-Freshwater (NRMCF-A separate wing of National Facility for Marine Cyanobacteria-NFMC), Bharathidasan University, Tiruchirappalli-620 024, India. The growth of *C. stagnale* was determined by the estimation of chlorophyll *a* and total protein contents at every fourth day for 60 days. The maximum Chl-*a* content (3.67 $\mu\text{g/mL}$) in *C. stagnale* was observed on 20th day. Thereafter the Chl-*a* content decreased marginally and reached a stationary phase. The protein content showed gradual increase up to 28th day and attained a steady state on 44th day followed by a stationary phase (Fig. 2).

3.2. Identification of plasmids from *C. stagnale*

Agarose gel electrophoresis of the total DNA of *C. stagnale* showed the presence of extra chromosomal DNA molecules of four plasmids (Fig. 3a). The CCC DNA molecules of these plasmids migrated at ~ 1.4 , ~ 4 , ~ 9 , and ~ 23 kb sizes and were named as pCYLM01, pCYLM02, pCYLM03 and pCYLM04 respectively (Fig. 3b). All the CCC plasmid DNA molecules were unaffected by the heat-treatment and appeared in the gel migrating along the same sizes as the untreated ones, whereas the heat-treated linear λ DNA was not visible. The mobility of the open circular form of plasmid DNA was also affected (Fig. 4).

3.3. Cloning of plasmids pCYLM01 and pCYLM02 into cloning vector

Among the four plasmids obtained, two smaller plasmids were chosen for further studies. Such smaller plasmids are easily

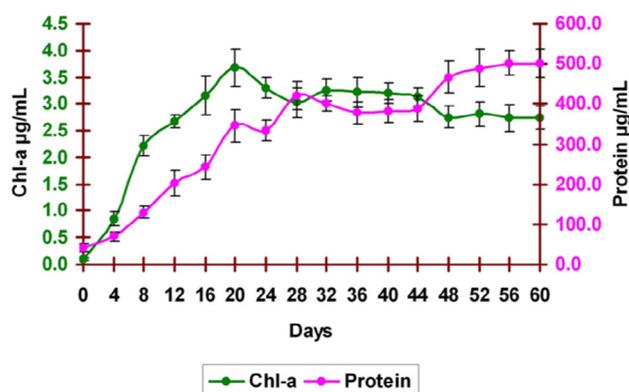


Fig. 2. Chlorophyll *a* and total protein in *Cylandrospermum stagnale*.

amenable for the construction of cloning, expression of vectors and, also for studies on transformation. The plasmids pCYLM01 and pCYLM02 were separately eluted from gel (Fig. 5a) and subjected to restriction digestion using common restriction enzymes viz. *EcoRI*, *EcoRV*, *BamHI*, *Sall* and *HindIII*. The plasmids were susceptible to *HindIII* digestion. The pCYLM01 plasmid was linearized with *HindIII* enzyme and gave a single band on 1% agarose gel. The other plasmid, pCYLM02 was digested into three fragments of different sizes about 2 kb, 1.9 kb and 700 bp on 1% agarose gel probably because it has three sites for *HindIII* enzyme (Fig. 5b). The *HindIII* digest of plasmid pCYLM01 was ligated into a pBSSK(-) cloning vector and the resulting hybrid plasmid was named as pBCY01 (Fig. 6a).

The following fragments 2 kb, 1.9 kb and 700 bp that arise from the restriction digestion of the plasmid pCYLM02 with *HindIII* were ligated into a pBS SK(-) cloning vector and the resultant plasmids were named as pBCY02A, pBCY02B and pBCY02C, respectively. The ligated plasmids were transformed into competent *E. coli* DH5 α cells and the colonies were screened by colony PCR method. The positive clones were initially confirmed by digestion with *HindIII* enzyme to release the insert and further confirmed by nucleotide sequencing (Fig. 6b). The obtained complete DNA sequences of two plasmids pCYLM01 and pCYLM02 were aligned using BioEdit software and the plasmid were reconstructed and analysed.

3.4. Reconstruction of pCYLM02 plasmid

The sequences of three different *HindIII* fragments of plasmid pCYLM02 were reconstructed by sequencing of plasmid pCYLM02 using specific primers CYLM02F and CYLM02R. The sequences were aligned using BioEdit software and the plasmid pCYLM02 was reconstructed. A 4359 bp complete sequence of plasmid pCYLM02 was obtained and submitted to GenBank of NCBI (Accession No. EF452232).

3.5. Sequence analysis of pCYLM01

The 2175 bp plasmid pCYLM01 sequence was submitted to the GenBank (Accession No. EF221636). The plasmid has five unique restriction sites viz. *HindIII*, *HincII*, *MboI*, *Sau3AI* and *EcoRV* and it has 43% GC and 57% AT content. The translations were done with a minimum threshold level of 50 amino acids. The sequence of pCYLM01 contained at least 4 potential open reading frames (ORFs) that were 50 amino acids or more in length. The circular representation of the plasmid was created using the online software SnapGene for the pCYLM01 sequence (Fig. 7). Individual sequences were blasted using the BLASTP program in NCBI website to predict the functions of these reading frames.

The ORF which represented the coding region from 946 to 1845 bp in the plasmid sequence consisting of 299 amino acids and it was the biggest among all the reading frames observed in pCYLM01. This hypothetical protein showed similar sequence as that of RepC1. The analysis of RepC1 protein using BLASTP showed a significant homology to the bacterial replication initiator family protein REP which is required for plasmid replication initiation. Domain analysis of the hypothetical protein RepC1 showed the presence of two partial domains in the ORF; one corresponding to the replication initiation factor (REP), pfam02486 protein from bacteria and the another one corresponding to putative phage replication protein RstA (COG2946). This replication initiation factor is thought to encode a putative topoisomerase protein, which creates a nick at the origin of replication of plasmids during the plasmid replication. The putative phage replication protein is involved in the replication repair and recombination of phage DNA. The plasmid region from 244 to 471 bp codes for PKc_like protein and consist of 72 amino acids (8.5 kDa). The ORF3 has 59

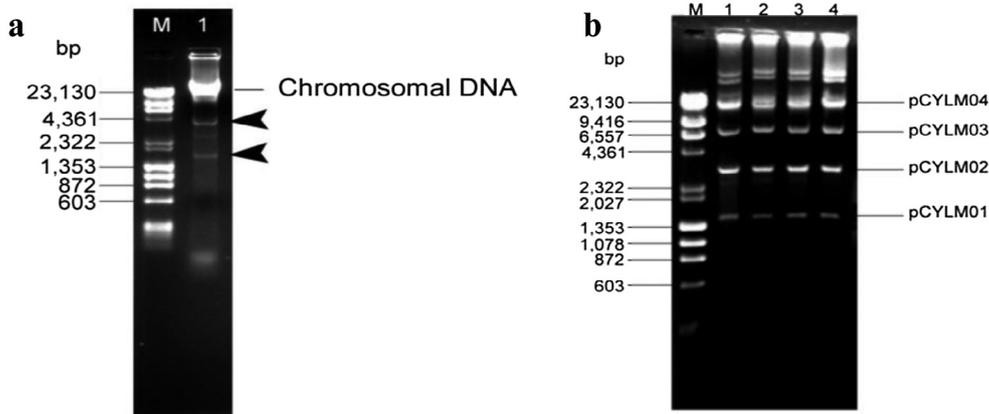


Fig. 3. (a) Agarose gel (0.8%) electrophoresis of total DNA extracted from *Cyindrospermum stagnale*. Arrows indicate the extra chromosomal DNA. M – λ DNA-*Hind*III digest and ϕ x174 DNA-*Hae*III digest Mix (Finnzymes); Lane 1 – Total DNA of *Cyindrospermum stagnale*. (b) Agarose gel (0.8%) electrophoresis of plasmid DNA isolated from *Cyindrospermum stagnale*. Lane M – λ DNA-*Hind*III digest and ϕ x174 DNA-*Hae*III digest Mix (Finnzymes); Lanes 1 to 4 – Plasmid profile of *Cyindrospermum stagnale* isolated from different batches of cultures.

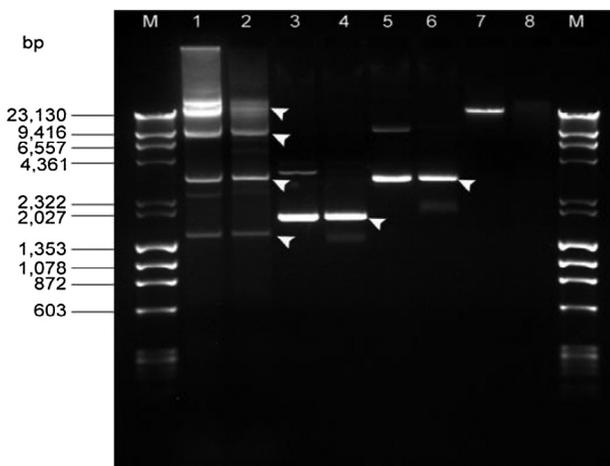


Fig. 4. Agarose gel (1%) electrophoresis of heat-treated and unheated plasmid DNAs from *Cyindrospermum stagnale* studied together with heat-treated and unheated bacterial plasmids pUC18 and pBR322 and linear λ DNA. Heat-treated samples of DNA were obtained by heating the samples in the presence of 0.1% (w/v) sarkosyl for 2 min at 100 °C followed by quick cooling. The arrows indicate Covalently Closed Circular (CCC) DNA. Lane M – λ DNA-*Hind*III and ϕ x174 DNA-*Hae*III digest Mix (Finnzymes); Lane 1 – Unheated *Cyindrospermum stagnale* plasmids; Lane 2 – Heat-treated *Cyindrospermum stagnale* plasmids; Lane 3 – Unheated pUC18 plasmid; Lane 4 – Heat-treated pUC18 plasmid; Lane 5 – pBR322 plasmid; Lane 6 – Heat treated pBR322 plasmid; Lane 7 – Unheated λ DNA; Lane 8 – Heat treated λ DNA.

amino acids (6.2 kDa) and codes from 618 to 797 bp. The ORF4 consists of 74 amino acids (8.8 kDa) that codes from 1639 to 1863 bp. There are no significant similarities found in BLAST for these two hypothetical proteins with existing databases. The plasmids also contain WELQut protease (574.6 Da) recognition and cleavage site from 1363 to 1374 bp.

The sequence of pCYLM01 has two 45 bp tandem repeats, GAGCGTGGCGCTGTGGCATCAGGGTCTGGAGCGTGGTGCTGATGT were located beginning at position 2075. A 17 bp inverted repeat, AAGTCTAGAGACTTATT was located between positions 487 and 509. It also has sequence of CTTGATG at the position 453.

3.6. Sequence analysis of pCYLM02

The analysis of 4359 bp plasmid pCYLM02 sequence revealed 7 ORFs which were longer than 50 amino acids in length. Altogether

nine ORFs were found in the analysis using NCBI ORF finder with threshold level of 50 amino acids. The ORFs were designated as ORF-1 to ORF-7. Sequence analysis was carried out for the individual sequences. The plasmid has two unique restriction sites, *Xba*I and *Bgl*II and has 37% GC and 63% AT content (Fig. 8). The ORF1 of pCYLM02 contains 314 amino acids long (36.8 kDa) and is represented on the plasmid from positions 1270–2214 bp. This hypothetical protein is designated as, RepC2. On BLASTP analysis of RepC2 protein showed high similarity to replication associated proteins from a wide range of organisms ranging from cyanobacteria to other bacteria reported in the database.

The function of this putative protein is predicted to be in plasmid replication. The protein sequence consisted of two conserved domains one represented a pfam01446, Rep_1, replication protein (rep) involved in plasmid replication and the other was similar to a COG5655, plasmid rolling circle replication initiator protein. The ORF2 of pCYLM02 has 243 amino acids (26.9 kDa) and is represented in the plasmid sequence from 3045 to 3776 bp. It showed 32 to 59% similarity over partial length of hypothetical protein of different cyanobacteria. ORF3 of pCYLM02 corresponds to the plasmid sequence from 2323 to 2976 and codes for a putative protein of 217 amino acids long (24.6 kDa). It showed 48% identities and 63% positive over a total length of 217 amino acids with hypothetical protein of *Oscillatoria* sp. PCC 10,802 (WP_017718967). ORF4 of the plasmid pCYLM02 corresponds to the sequence from 540 to 1136 bp and codes for a putative protein of 198 amino acids in length (22.6 kDa). It showed 35 to 69% similarity over partial length of hypothetical protein of different cyanobacteria. The plasmid region from 2164 to 2451 bp codes for a putative polypeptide of 95 amino acids (11.3 kDa) in length and was designated as ORF5. The function of this hypothetical protein could not be predicted using the information available in the databases. The ORF6 of the plasmid from 4160 to 4336 bp codes for a small putative polypeptide of 62 amino acids in length (6.5 kDa). This putative protein showed 25/44(57%) and 24/58(41%) identity to hypothetical protein of *Chamaesiphon minutus* (WP_015329025) and *Limnothrix rosea* (WP_084176640), respectively. The ORF7 codes from 4160 to 4336 bp for a small hypothetical protein of 58 amino acids (6.6 kDa). It also showed 33 to 39% identities over a partial length of hypothetical protein of different cyanobacteria. A number of small ORFs below 50 bp were also found in the sequence analysis. However, these ORFs did not show any significant similarity to sequences reported in the databases.

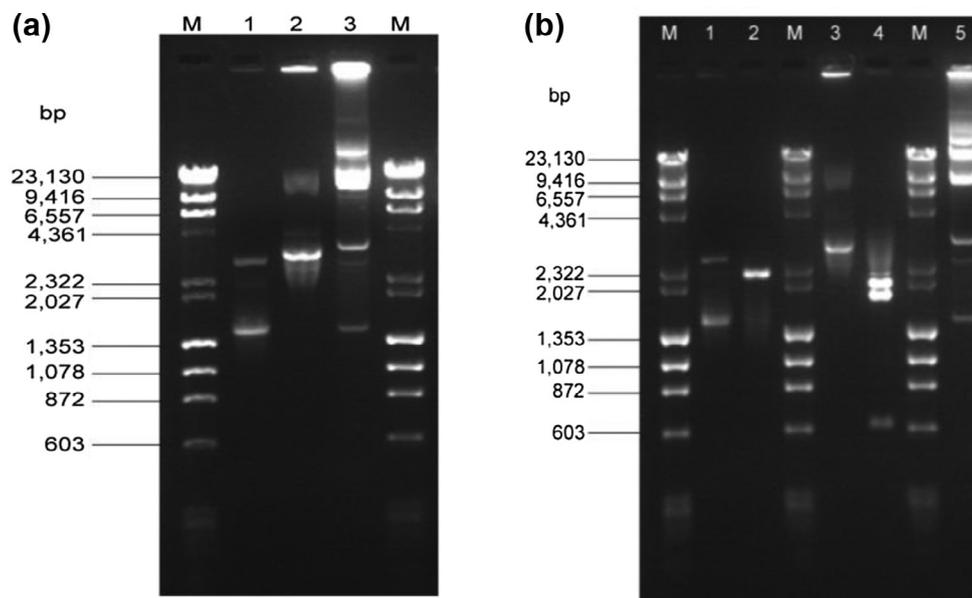


Fig. 5. (a) Agarose gel (1%) electrophoresis of plasmids pCYLM01 and pCYLM02 obtained by gel elution. Lane M - λ DNA-*Hind*III and ϕ x174 DNA-*Hae*III digest Mix (Finnzymes); Lane 1 - Plasmid pCYLM01; Lane 2 - Plasmid pCYLM02; Lane 3 - Plasmids of *Cylindrospermum stagnale*. (b) Agarose gel (1%) electrophoresis of plasmids pCYLM01 and pCYLM02 digested with *Hind*III restriction enzyme. Lane 1 - Undigested plasmid pCYLM01; Lane 2 - pCYLM01 plasmid digested with *Hind*III; Lane 3 - Undigested plasmid pCYLM02; Lane 4 - pCYLM02 plasmid digested with *Hind*III; Lane 5 - Total plasmid of *Cylindrospermum stagnale*.

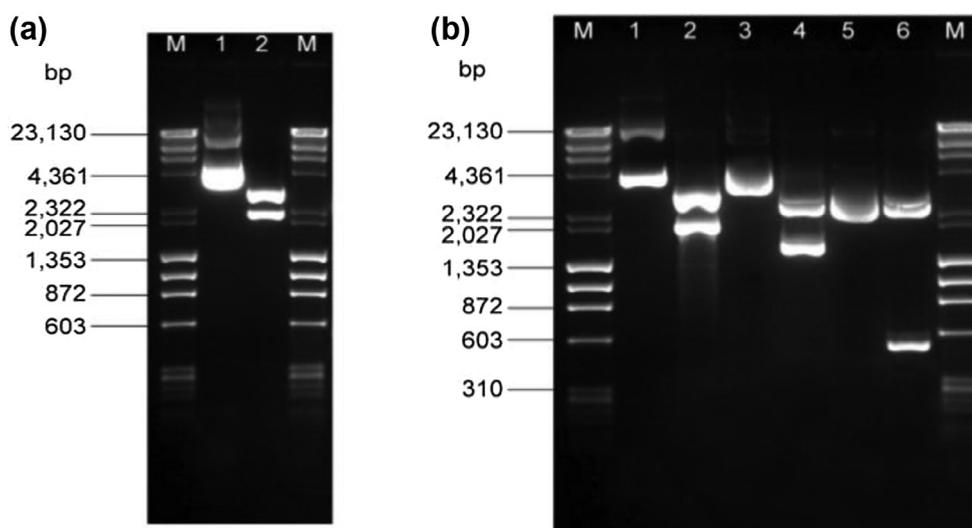


Fig. 6. (a) Agarose gel (1%) electrophoresis of the hybrid plasmid pBCY01 digested with the restriction enzyme *Hind*III showing the insert release. Lane M - λ DNA-*Hind*III digest and ϕ x174 DNA-*Hae*III digest Mix (Finnzymes); Lane 1 - Undigested plasmid pBCY01; Lane 2 - Plasmid pBCY01 digested with *Hind*III restriction enzyme. (b) Agarose gel (1%) electrophoresis of the hybrid plasmids pBCY02A, pBCY02B and pBCY02C digested with the restriction enzyme *Hind*III showing the insert release. Lane M - λ DNA-*Hind*III digest and ϕ x174 DNA-*Hae*III digest Mix (Finnzymes); Lane 1 - Undigested plasmid pBCY02A; Lane 2 - Plasmid pBCY02A digested with *Hind*III; Lane 3 - Undigested plasmid pBCY02B; Lane 4 - Plasmid pBCY02B digested with *Hind*III; Lane 5 - Undigested plasmid pBCY02C; Lane 6 - Plasmid pBCY02C digested with *Hind*III.

The plasmid pCYLM02 also has 10 bp inverted repeat, GGTTTTGGAG was located between the ORFs 6 and 5 at the position 32 and 96. An 11 bp inverted repeat, GGGTATCTGGG was located between the ORFs 2 and 6 at positions 3853 and 3965. Two 13 bp inverted repeats, AGGGATAACGGGC was located between the ORFs 4 and 1 at the positions 1136 and 1160 and AAAAATCCGTGAT at 1213 and 1252. 15 bp inverted repeat, CAACCTGCCCCCG was located between the ORFs 2 and 6 at the positions 3877 and 3925. It also has sequence of CTTAATA at the position 609.

4. Discussion

Cyanobacteria are versatile organisms that grow in a wide range of habitats since their physiological characteristics are very effective to

survive under diverse environments (Rampelotto, 2013; Singh et al., 2016; Raja et al., 2016). The organism has tolerance to desiccation and nutrient deficiency. Recently much attention is focused on the molecular aspects of various processes like photosynthesis, nitrogen fixation and protein synthesis. Molecular characterization to understand the genomics is being pursued in cyanobacteria as well. The filamentous cyanobacteria are known to possess plasmids (Ma et al., 2012; Shintani et al., 2015). However, the function of these plasmids has not been so far defined clearly. The present study was primarily aimed at investigating the *C. stagnale* for the presence of plasmids and if any, characterizes them based on their sequences using *in silico* tools. The *C. stagnale* possessed four plasmids which were covalently closed circular DNA molecules of ~1.4, ~4, ~9 and ~23 kb sizes and were named as pCYLM01, pCYLM02, pCYLM03 and pCYLM04.

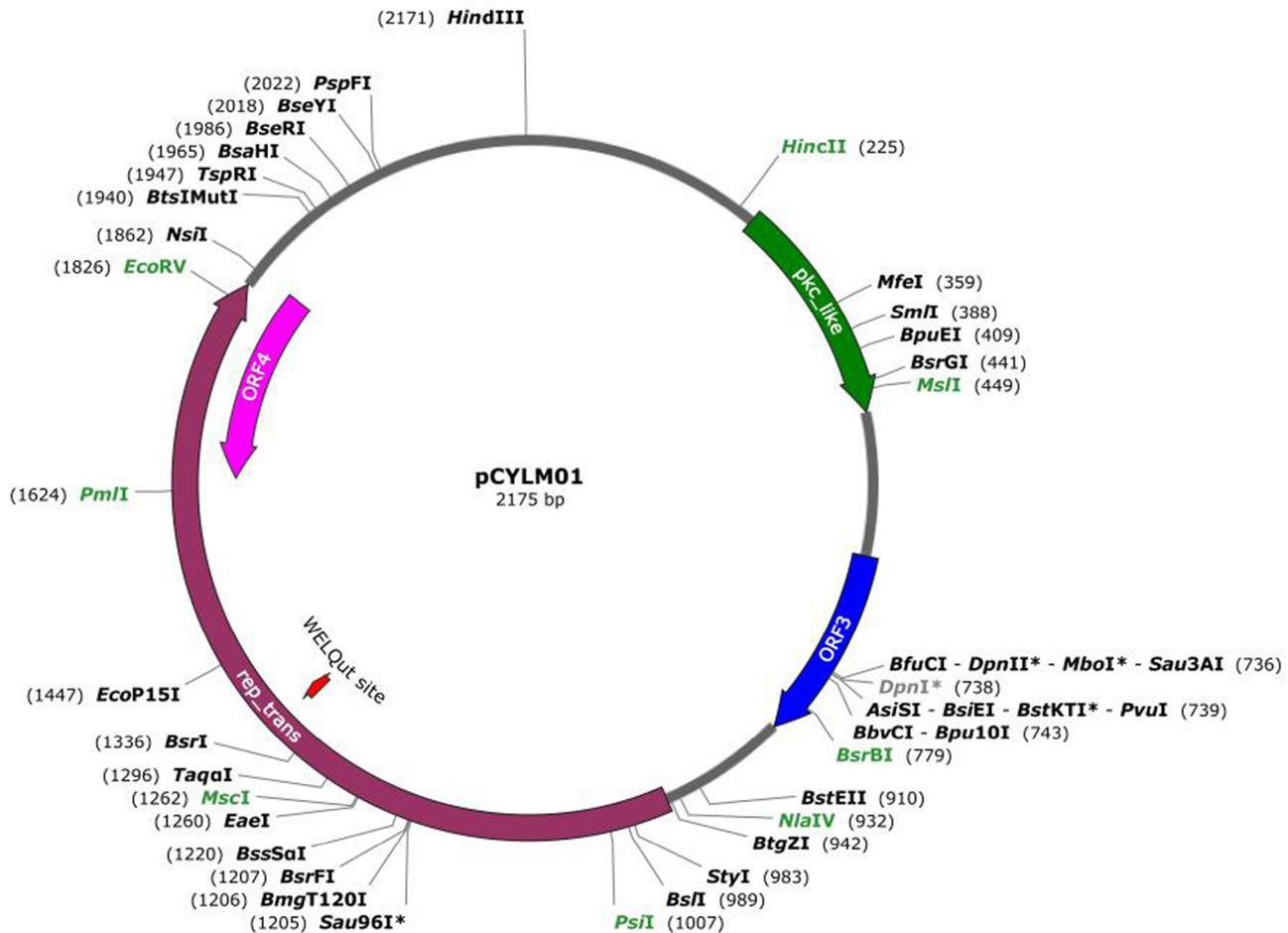


Fig. 7. Circular representation of the plasmid pCYLM01 created using SnapGene online software. The map showing restriction enzyme sites and Open Reading Frames (ORFs) of the plasmid. The ORFs a, b and d were present on the plus strand whereas the ORFs c and e were present on the minus strand of the plasmid.

The plasmid sequences were analysed for the presence of possible open reading frames using *in silico* tools (Black et al., 1995; Ma et al., 2012). The BLASTP search of hypothetical protein RepC1 showed significant similarity with hypothetical replication protein of *Synechocystis* sp. PCC 6803 plasmid pCB2.4, which replicated by rolling circle mechanism (Yang and Mc Fadden, 1994; Berlaa and Pakrasi, 2012; Jin et al., 2018). The protein was shown to possess two partial conserved domains. This family represents a probable topoisomerase, which has a strand and sequence-specific nicking-closing activity that acts at the origin of replication (Koepsel et al., 1985). The Rep protein binds to the plasmid DNA and nicks it at the Double Strand Origin (DSO) of replication. The 3'-hydroxyl end created is extended by the host DNA replicase, and the 5' end is displaced during synthesis. At the end of one round of replication, Rep introduces a second single stranded break at the DSO and ligates the ssDNA extremities generating one double-stranded plasmid and one circular ssDNA form. Complementary strand synthesis of the circular ssDNA is usually initiated at the single-stranded origin by the host RNA polymerase (del Solar et al., 1998; Khan, 2000).

Apart from this big open reading frame, the other open reading frames did not match with the proteins available in the various databases. The ORF-2 of the plasmid pCYLM02 encodes a putative protein of 243 amino acids. This putative protein did not show the presence of any conserved domains but a part of the sequence showed significant similarity to translation elongation factor (GTPases) reported in *Cyanotheca* sp. CCY0110 and an unknown

protein of *Nostoc* sp. ORF-3 encodes a putative protein of 217 amino acids and showed 47% identity to a reported hypothetical protein in *Cyanotheca* sp. CCY0110. The presence of a conserved domain similar to the phage replication initiator protein points to a possible single strand intermediate in the plasmid replication mechanism as in case of rolling circle mechanism (Xu et al., 1997; Ma et al., 2012). The plasmids remain cryptic has been reported in many other Cyanobacteria. Both the plasmids pCYLM01 and pCYLM02 have several direct as well as inverted repeats and the exact function of these repeats was not known. The wide variety of repetitive sequences has been shown to constitute around 10% or more of the total genome in prokaryotes. The repeats range from few bases to several kilobases and occur in many places like coding, non-coding, ORF's etc. Some of the repeats have been shown to be associated with pathogenicity in prokaryotes and pathological process in eukaryotes. Yet many of these repeats are not understood completely and needs to be characterized (Oliveira et al., 2010). The presence of repeats have also reported in cyanobacterial plasmids pRF1 of *Plectonema* sp. PCC 6402 (Perkins and Barnum, 1992) and the plasmid pAQ1 of marine cyanobacterium, *Synechococcus* sp. PCC7002 (Akiyama et al., 1998).

The plasmids pCYLM01 and pCYLM02 have the sequences CTTGATG and CTTAATA respectively which are closely similar to the sequence CTTGATA reported in the plasmid pCC5.2 of *Synechocystis* PCC 6803. The presence of several plasmids in cyanobacteria has been reported but the information on their functions are lacking. Using the modern sequencing and sequence analysis tools

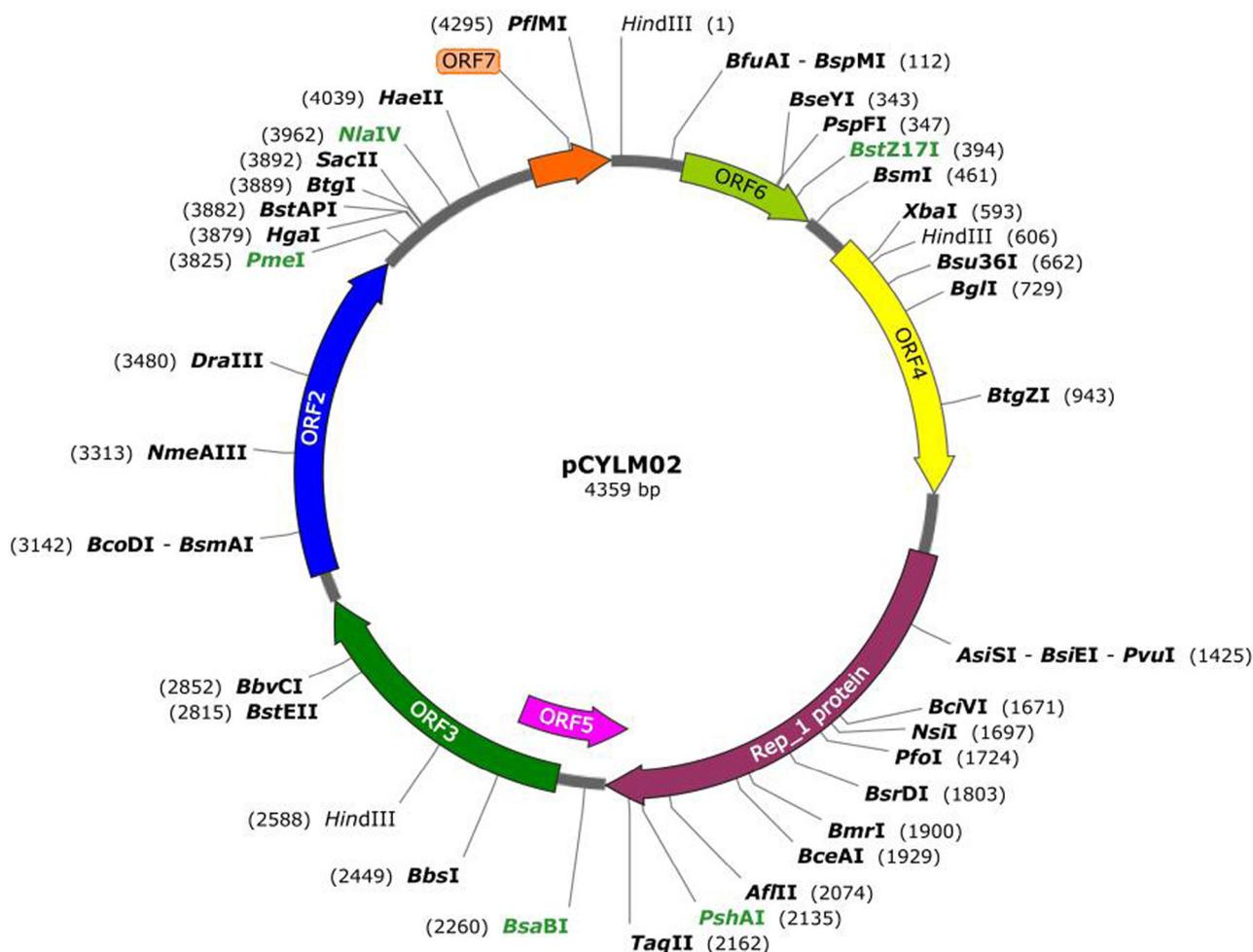


Fig. 8. Circular representation of the plasmid pCYLM02 created using SnapGene online software. The map showing restriction enzyme sites and Open Reading Frames (ORFs) of the plasmid.

along with the availability of a vast database of genes, predicted functions and other sequence information, the cyanobacterial plasmids and their role, if any, in the growth and development of these organisms have not been deciphered. From an evolutionary point of view also, these plasmids are mysterious. Bacteria are known to possess plasmids of varying sizes known to confer specific functions like antibiotic resistance to them and therefore, it is important for the bacteria to maintain these plasmids within them for their growth and survival especially under unfavourable environments. Cyanobacteria are also found in a wide range of environments and extreme conditions and they also maintain their plasmids. This similarity in the maintenance of the plasmids in bacteria and cyanobacteria points to the fact that these molecules are maintained because the organism requires them for their function in some context assigning of specific role to these plasmids will clear most of the cyanobacterial research uncertainties.

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Ethical statement

This is not applicable to this article since we have not used any animals or human model in this study.

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