A Simple Novel Agar Diffusion Method for Isolation of Indigenous Microalgae *Chlamydomonas* sp. CRP7 and *Chlorella* sp. CB4 from Operational Swampy Top Soil

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Abstract A simple agar diffusion method is developed where pure colony of *Chlamydomonas* sp. CRP7 was isolated from *Chlorella* sp. CB4 mixtures by passing through agar migration with a light exposure of 6,000 lux for 7 h. The main concept behind it is that *Chlamydomonas* has flagella and the rhodopsin pigment is attracted towards light. Thus the above two microalgae species can be separated from the mixtures as eye spot serves as a navigator and flagella serves as a propeller for *Chlamydomonas* spp. Further the genomic DNA was isolated and purified from the above mentioned two species after the separation from the mixtures. PCR amplification was carried out for ITS1, 5.8S and ITS2 regions. The amplified products were sequenced and the sequence analysis confirmed that they belong to *Chlamydomonas* sp. and *Chlorella* sp. This is an important augmentation for isolation and separation of microalgae.

Keywords Agar diffusion method · *Chlamydomonas* sp. CRP7 · Soil · Isolation · *Chlorella* sp. CB4

Isolation and purification of microalgae is based on various techniques including enrichment and single cell isolation. For non motile green microalgae scientists used Bold's basal medium and separated by routine dilution-plating

[1–3]. In addition with microscopic identification, recently researchers are using molecular tools in case of microorganism [4, 5]. Identification of microorganism, PCR amplification and sequencing of a universal amplified ribosomal region has been also studied [6, 7]. Interestingly, during this study two indigenous isolates *Chlamydomonas* sp. CRP7 and *Chlorella* sp. CB4 were isolated from the specific area of Global Positioning System coordinates viz. 23°24'51" N; 85°26'24"E. GPS coordinates of the sampling areas are listed in Table 1. One gram of upper layer of swampy soil was collected and mixed with 10 ml of sterilized water using vortex mixer (Remi India Pvt. Ltd.). It was left undisturbed for 1 h to settle coarse particles of soil. The upper portion of the muddy solution (6 ml) was inoculated in 50 ml of Tris Acetate Phosphate (TAP) medium in 250 ml conical flask [8]. The culture flask was incubated at 25 °C under light (8,000 lux) with photoperiod of 16:8 h. Seven days grown culture of *Chlamydomonas* CRP7 was observed under Olympus CH20 microscope (Olympus Corp., Japan) which was recorded with a cell density of 1×10^4 cells/ml. Then 50 µl of the sample was taken from the conical flask and was sub-cultured in 2 ml cryotube (Tarson, India) containing 0.7 ml of TAP medium. The cryotube containing the sample was incubated as described in the above conditions. After 72 h of incubation the *Chlamydomonas* cells showed phototaxis movements (Fig. 1a) towards the upper surface of the TAP medium and accumulated to form a greenish layers. The layers were vigilantly collected in sterile 2.0 ml Eppendorf tube without disturbing the lower part in the cryotube. The collected samples were incubated for 12 h in dark at 25 °C before performing the agar diffusion experiment in the transparent tubular pipe of 10 cm long (0.3 cm in diameter). This pipe was filled with 400 µl of TAP agar medium (0.45 % agar). The overnight (12 h) grown microalgae were injected

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Table 1 GPS coordinates of sampling site

Sl. no.	GPS coordinates	Algae observed
1	N 23°22'10" E 85°19'08"	<i>Scenedesmus</i> sp. CB1, <i>Chroococcus</i> sp. CB2
2	N 23°23'99" E 85°18'27"	<i>Pediastrum</i> sp. CB3, <i>Chlorella</i> sp. CB13
3	N 23°25'55" E 85°26'36"	<i>Euglena</i> sp. CB5
4	N 23°25'55" E 85°26'36"	<i>Anabaena</i> sp. CB6, <i>Nostoc</i> sp. CB7
5	N 23°24'51" E 85°26'24"	<i>Chlamydomonas</i> sp. CRP 7, <i>Chlorella</i> sp. CB4.

Fig. 1 Different step of isolation**Fig. 2** Phylogenetic analyses based on TS region of isolated algal strains

through sterilized syringe (Dispovan, India). During this experiment the utmost care was taken to wrap the whole setup with Freshwrap® aluminum foil (Hindalco Industries Ltd., India) leaving the upper part of the transparent pipe uncovered to allow continual entry of light (6,000 lux)

for the movement of *Chlamydomonas* sp. CRP7. The uppermost part (Fig. 1b) of the tubular pipe was cut with a sterile blade and the semisolid greenish agar piece (0.3 cm³) was sliced. The sliced piece was washed twice with 1.5 ml milli-Q water in 2.0 ml Eppendorf tube. The

sliced piece was pierced with autoclaved toothpick and used the mild vortex for proper suspension. The 10 µl of suspended culture was plated onto TAP agar medium (supplemented with 500 µg/ml of Cefotaxime) and was incubated at 25 °C with light intensity of 8,000 lux with 16:8 h light photoperiod for 4 days towards establishing axenic culture of *Chlamydomonas* sp. CRP7.

As we performed several steps based on phototaxis movements so the final culture contained only *Chlamydomonas* sp. CRP7 which was observed during microscopic studies (Fig. 1c) and plating. Accidentally only one colony of *Chlorella* sp. CB4 (arrow marked in Fig. 1d) was found in the plate. The rest of the other colonies belonged to *Chlamydomonas* sp. (Fig. 1d). The pure single colony of both the *Chlamydomonas* sp. CRP7 and *Chlorella* sp. CB4 were aseptically picked up and transferred to 10 ml TAP medium in 50 ml conical flask and thus forming a pure culture.

The algal DNA was isolated followed by Alvarez et al. [9]. PCR amplification of ITS1, 5.8S, and ITS2 regions was performed by PCR using the following primers: Forward: 5'-GAAGTCGTAACAAGGTTTCC-3' and Reverse: 5'-TCCTGGTTAGTTTCTTTTCC-3' [10]. The amplification conditions were 95 °C for 4 min followed by 35 cycles of 45 s denaturation at 94 °C, 1 min at 60 °C as annealing temperature and 1 min extension at 72 °C with a final extension of 72 °C for 10 min. The amplified products were separated by size on 1.0 % agarose gels which was found to be in the range of 700–800 bp and is purified according to pure link PCR purification kit (Invitrogen). The sequencing was done, analyzed and submitted to NCBI databank (Gene Bank ID: JQ408690-*Chlamydomonas* sp. CRP7 and JQ710683-*Chlorella* sp. CB4). The blast search was performed from the obtained sequences (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic tree was constructed and analysis was done by Neighbor Joining (NJ) method through MEGA 5 software with the bootstrapping of 1,000 replicates (Fig. 2). It was further confirmed the isolated algae showing clustering with *Chlamydomonas* sp. and *Chlorella* sp. clades [11, 12].

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