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Research Article

Identification and Characterization of Green Microalgae, *Scenedesmus* sp. MCC26 and *Acutodesmus obliquus* MCC33 Isolated from Industrial Polluted Site Using Morphological and Molecular Markers

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Abstract

Two unicellular green microalgae, were isolated from Amani Shah Nallah (Jaipur, India) that receives discharge of dyeing industries. Preliminary morphological characterization using light microscopy showed ovoid, fusiform shape of cells with single to two celled coenobia in a culture suspension. Transmission electron microscopy (TEM) analyses depict abundant chloroplast located at the periphery of the cell, a pyrenoid as well as nucleus is visible in the centre. A well developed cell wall with many layers is also evident in TEM. Phylogenetic position and genetic variabilty among the isolated microalgal strains were investigated by 18S rDNA sequence analysis. The results suggest that the isolated microalgae belongs to family chlorophyceae and corresponds to genus *Scenedesmus* and genus *Acutodesmus*. Growth profile of both microalgal strains showed a typical sigmoid curve with specific growth rate of µmax, 0.839 day^{-1} and 0.654 day^{-1} for *Scenedesmus* sp. and *Acutodesmus obliquus*, respectively. The strains were allotted with MCC numbers by IARI, New Delhi as MCC26 for *Scenedesmus* sp. and MCC33 for *Acutodesmus obliquus*.

Keywords: Microalgae; Microscopy; 18S rDNA; MCC; Phylogeny

Introduction

Microalgae constitute one of the major group of photosynthetic eukaryotes and exist worldwide in different habitats including fresh and marine water to terrestrial ecosystems (Vilar *et al*., 2005). Microalgae are ubiquitous in soil and exist in high amount ≈150 to 500kg/ha in phylogenetically diverse forms (Shtina, 1974). Microalgae have attracted the attention of many researchers worldwide due to their wide applications in different areas. Their ecological position at the base of most aquatic food webs and their essential roles in nutrient cycling and oxygen production are critical in many ecosystems (Demirbras,

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2009). Microalgae is reported to be a promising source of high valuable products as well as feedstock for biofuels (Stephens *et al*., 2010; Arbib *et al*., 2013). It can be used as a good source of vegetable oils due to their high rate of biomass and oil production compared to plants (Chisti 2007; Groom *et al*., 2008). Microalgae are also a better alternative to limited fossil fuels. Different types of algal biomass have been reported as promising biosorbent for removal of contaminants including heavy metals (Volesky and Holan 1995; Vannela and Verma 2006; Sarwa and Verma 2014), nitrogen and phosphorus (Abe *et al*., 2008; Khan and Yoshida, 2008), dyes (Omar 2008; Sarwa and Verma 2013; Sarwa *et al*., 2014) and other pollutants from industrial wastewater. Despite of huge algal diversity, with approximately one million microalgal species (Andersen, 1992), less than 3000 are currently available in algae culture collections (Wijffels and Barbosa, 2010). Thus, the isolation, identification and characterization of new algal strains from various natural sources would be a pertinent to increase gene bank and that can serve as resource to select suitable strain for any practical applications such as suitable biosorbent for wastewater treatment and biofuel generation.

To characterize microalgal community, standard microbiological approaches based on the isolation and characterization along with molecular approaches are required. Identification and characterization of several algae based on morphological and physiological features have been reported by many researchers (Liu *et al*., 2006; Wu *et al*., 2013). In recent years, the use of molecular markers for identification and characterization of microalgae has gained considerable significance. Molecular markers can effectively complement morphological identification and characterization of an organism. 18S rDNA and Internal Transcribed Spacer (ITS) regions sequences has most frequently been used for phylogenetic analysis (An *et al*, 1999; Vanormelingen *et al*, 2007; Hagewald *et al*, 2010). 18S rDNA and ITS (Internal Transcribed Spacer) sequences are located in the nuclear DNA (Schlötterer and Tautz, 1994; Liu *et al*., 2006). The non translated ITS of transcriptional unit, present between 18S and 5.8 S and between 5.8 and 28 S rDNA subunits, are divergent and distinctive (Turenne,1999).Therefore, ITS regions are generally used for distinguishing closely related species (Abou-Shanab *et al*., 2011). The phylogenetic analysis of rDNA has been successfully employed to know the evolutionary relationship between closely related species of algae (Marks and Cummings, 1996; Beck *et al*., 1998; An *et al*., 1999).

The goal of this research work was to isolate, identify and characterize microalgal strains that are existing in the water bodies contaminated with industrial effluents, using morphological and molecular techniques.

Materials and Methods

Sampling site: Sanganer (Jaipur)

Soil samples were collected from Amani Shah Nallah (drainage) of Sanganer town situated at 26° 49´-26° 59´N and 75° 46´-75° 50´E near Jaipur district of Rajasthan, India. The Amani Shah Nallah is surrounded by many small and large scale textile and dyeing industries, discharging their toxic colored effluent directly in to it. The tie and dye industry effluent of this region is reported to be contain high concentration of heavy metals (Ni, Zn, Pb, Cr, Cd, Fe) and different types of dyes (Mathur *et al*., 2005; Sarwa and Verma, 2014). Therefore, it is expected that the microalgae community growing in this area will be having natural resistance against these toxicants and can be employed as a biosorbent for pollutants.

Isolation of Axenic Cultures

Wet soil (mud) samples were collected aseptically during the month of July, from sites that appeared to contain algal growth and was homogenized using a sterile spatula and 1.0g of dry sample was dispensed into 500 ml conical flasks containing 200 ml of BG-11 liquid medium (pH 6.8 ± 0.5) (Wu *et al*., 2013). Flasks were incubated on a rotary shaker at 150 rpm and 25±2ºC under continuous illumination using white fluorescent light at intensities of 40µmol m^{-2} s^{-1} for two weeks. Every two days, the flasks were examined for algal growth using an optical microscope. Axenic cultures of microalgae were maintained in BG-11 medium under controlled lab conditions for further analysis. The algae were stored in slants containing BG-11 medium solidified with 2% agar. The slants were kept at light intensity of 20µmol photons $m^{-2}s^{-1}$and a temperature of 15°C, and were transferred aseptically twice a month.

Strain Identification and Characterization

The isolated axenic microalgal strains were identified and characterized morphologically by studying the morphological features under light microscope and by using transmission electron microscope (TEM). After morphological characterization, the phylogenetic analysis was carried out by using 18S rRNA sequence analysis.

Morphological Characterization

Light microscopic analysis

Micro algal strains in rehydrated bio films and in liquid culture were examined under light microscope using bright field and differential interference contrast. Microalgal strains were identified according to the morphological features of species using standard botanical approaches. Classic morphological taxonomy includes the use of traits observable with light microscopy such as arrangement of cells, shape and size of cells, pattern of cells, mode of division (Lurling M, 2003). Morphological features of isolated microalgae were recorded photographically at 10–40X magnification using Olympus BL51 microscopy connected with a digital camera system.

Electron microscopic analysis

The ultra structural features of the cells were studied using Transmission Electron Microscope (TEM) (PHILIPS Morgagni 268). For TEM analysis, the samples were prepared by harvesting microalgal strains by centrifugation (4000 rpm, 10 min) and washing with 0.1M Phosphate buffer (pH 7.4). Initially samples were fixed in 2.5% glutaraldehyde in phosphate buffer for 6 hrs at 4°C followed by post fixation with 1.0% OsO_4 in 0.1% M phosphate buffer for 2 hrs at 4°C. The samples were washed several times using the same buffer and then dehydrated in ethanol series (50-100% v/v) and acetone to critical point drying. Samples were embedded on araldite and thin sections of 60-70 nm were cut by an ultra microtome. Observations were carried out with a transmission electron microscope (PHILIPS Morgagni 268) at 80kV (Ramachandran *et al*., 2009).

Molecular Characterization

The molecular characterization of isolated microalgal strains was done by 18S rRNA sequence analysis using standard methods (Liu *et al*., 2006). For 18S rRNA sequence analysis, following procedure was applied to get the phylogeny of isolated strains.

Isolation of Genomic DNA

Total genomic DNA from microalgal cells was extracted according to the modified hexadecyltrimethyl ammoniumbromide (CTAB) method as described by Doyle and Doyle, 1990.

PCR (Polymerase Chain Reaction) Amplification

PCR amplification of the isolated DNA was done according to method described by Ferrer *et al*., 2001. Universal eukaryotic primers, ITS1 and ITS4 (Table 1) as proposed by White *et al*. (1990) were used in the present study to amplify the internal transcribed space (ITS) region of ribosomal DNA, which comprises of 5.8S gene and the ITS1 and ITS2 regions. PCR amplification was performed using a Veriti 96 well Thermal cycler (Applied Biosystems, Warrington, UK). The reaction mixture contained 100ng of chromosomal DNA, 50 pmol of each oligonucleotide primer, 200µM of total dNTP mix and 1U of *Taq* DNA polymerase in a volume of 50 µl. PCR products were observed by agarose gel electrophoresis using 1% agarose and 5µl of PCR product (DNA) including 1kb DNA ladder BIORON (Bioron International). The thermal cycling protocol consisted of an initial denaturation step of 2 min at 94°C, followed by 30 cycles. Each cycle began with a denaturing step of 1 min at 94°C followed by 1.5 min at the annealing temperature of 57°C for the specific primer pairs and ended with extension step of 2 min at 72°C. 1 cycle for final extension was set at 72°C for 4 min (Jain *et al*., 2013). From gel, elution of the DNA fragment (bp) was done using Quagen Gel extraction kit according to the manufactures instructions. To check the purity of eluted DNA, 1µl of sample was used in 1% agarose gel.

Table 1: Primer sequences of ITS1 and ITS 4

Name	Sequence (5′-3′)	Type
ITS1	TCCGTAGGTGAACCTGCGG	Forward Primer
ITS4	TCCTCCGCTTATTGATATGC	Reverse Primer

Sequencing

Amplified ITS regions of the ribosomal DNA were sequenced with the help of ABI Prism DNA sequencer (Applied Biosystems, Carlsbad,CA, USA) using either the ITS-1 and/or the ITS-4 primer for DNA labeling by the BigDye terminator method (Applied Biosystems, Foster City, CA, USA). The sequences obtained by the ITS-4 primer were inversed using Gene Doc software (Nicholas *et al*.,1997) and clubbed with the sequences obtained by the ITS-1 primer, to get the complete sequence of the ITS region (Jain *et al*., 2013).

Sequence Alignment and Phylogenetic Analysis

Sequence analysis of PCR products were performed using the computer programs NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997). The sequences were compared with the similar sequences in the GenBank database using Blast. Multiple alignment of the sequences was done with the CLUSTAL W program (Thompson *et al*., 1997) which allowed calculation of a distance matrix that was analyzed using the neighbour-joining (NJ) algorithm of CLUSTAL W. After complete sequence analysis, aligned sequences were used to generate a phylogenetic tree using neighbor-joining (N-J) method. The software MEGA 5 was used for construction of phylogenetic tree and bootstrap analysis of the same data (Swofford, 2000).

Growth Assessment

The growth of microalgal strains was monitored by estimating Chlorophyll *a* **(**Chl a**)** content of the cells according to the method described by McKinney (1941). 1.0 ml culture of the microalgae was taken and centrifuged at 2000 rpm (Eppendorf Centrifuge 5415D) for 3 minutes. After centrifugation, cell free supernatant was discarded and pellet was added with 1000 µl of 100% methanol (AR, Merk), vortexed and then incubated in dark at 4°C for 12 h. The contents were centrifuged at 8000 rpm for 5 minutes to remove cell debris. Chl-*a* in the supernatant was measured at 665 nm using UV-Visible Spectrophotometer (Jasco V640). The equation given by Jeffrey and Humphrey (1975) was used to calculate the amount of chlorophyll a content in microalgal culture. Specific growth rate of microalgal strains were calculated according to method described by Reynold, (1984) using equation (1),

$$\text{Growth rate } (\mu) = [\ln (X_2/X_1)/(t_2 - t_1)]\ (\text{day}^{-1}) \quad (1)$$

Where, X_1=Chl *a* content at time t_1 and X_2=Chl *a* content at time t_2.

Results and Discussion

Isolation and Identification of Microalgae

The survey of different locations of Amani Shah Nallah (drainage) showed scanty bloom formation in the month of July, due to extreme hot and dry weather conditions (45°C day temp). Two microalgal strains were isolated and purified after several plating and microscopic observations using standard microbial techniques. The preliminary morphological identification of isolated strains on the basis of their cell shape, size, indicate that they may belongs to Chlorophyta (*Scenedesmus* sp. and *Acutodesmus* sp.) as described by Hegewald and Hanagata (2000).

Morphological Characterization

The isolated *Scenedesmus* sp. and *Acutodesmus* sp. showed almost similar color in liquid medium ranging from pale green to dark green. It is reported that *Scenedesmus* species show diverse morphology under different environmental conditions (Trainor, 1998). Microscopic observations of *Scenedesmus* sp. and *Acutodesmus* sp. showed that both strains are unicellular and appeared to form colonies. Shape of the *Scenedesmus* sp. cells was observed to be ovoid or fusiform as shown in Fig 1(A). Whereas, cells of *Acutodesmus* sp. appeared to be round and oval in Fig 2(A). *Scenedesmus* sp. possess acute cell poles while no such acute cell poles were observed in *Acutodesmus* sp. as described by Hegewald and Wolf (2003). It was also observed that these algal stains normally divide by multiple fission forming more than two daughter cells connected in a coenobia. The colony analysis of *Scenedesmus* sp. revealed the presence of maximum number of unicells followed by two celled and a very less number of three to eight cells in their different developmental stages. Similar observations were also reported by Nilshammar and Walles (1974); Trainor (1998) and Ho *et al*. (2010) for characterization and colony formation in *Scenedesmus* sp. The number of cells in a colony has been correlated with the amount of protoplasm required for cell division. *Scenedesmus* cells reproduce by asexual method by the formation of auto-spores. Inside the parental cell wall, mother cell can divide into a number of daughter cells and these cells are released as unicells or as a colony (Morales and Trainor, 1999). In the present study, cells of *Scenedesmus* never exceed eight-celled coenobia which is in agreement with Van den Hoek *et al*. (1995), who reported colony size for *Scenedesmus obliquus.*

Fig. 1 (B) shows the transmission electron micrograph of *Scenedesmus* sp. It is observed that in *Scenedesmus* sp. the chloroplast is located in the cellular periphery. In the central part of the chloroplast a pyrenoid was seen. Some starch granules were also observed in the periphery of cell. A nucleus is visible opposite to pyrenoid. The cell is surrounded by several layers of cell wall as shown in Fig 1(B). The present observations are in accordance with the findings of Staehelin and Pickett-Heaps, (1975) who reported three layered cell wall in *Scenedesmus* sp. in which the inner thick layer is the cellulose layer. The middle, three-laminar sheet resembles to the plasma lemma. The outer layer adhering to middle layer has an elaborate epistructure. Similarly, the TEM analysis of *Acutodesmus* sp. shows the presence of pyrenoid in the centre and chloroplast and the starch granules at the periphery as depicted in Fig. 2 (B). The present morphological observations of *Scenedesmus* sp. and *Acutodesmus* sp. are in agreement with An *et al*, (1999) who described the characteristics features of cocoid shaped microalgae.

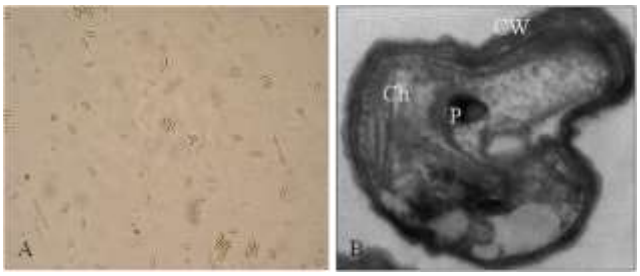

Fig. 1: (A) Micrograph of *Scenedesmus* sp. under 40 X magnification (B) Electron micrograph of *Scenedesmus* sp. (Abbreviations: Ch chloroplast; S starch; CW cell wall; P Pyrenoid)

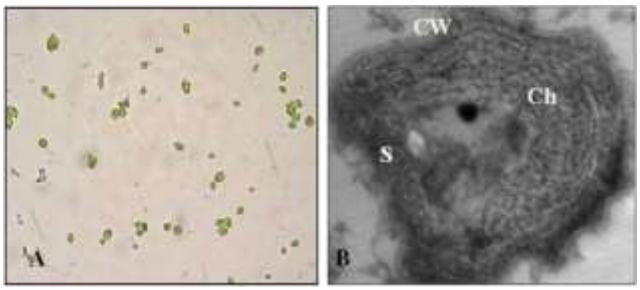

Fig. 2: (A) Micrograph of *Acutodesmus* sp. under 40 X magnification (B) Electron transmission micrograph of *Acutodesmus* sp. (Abbreviations: Ch chloroplast; CW Cell wall; S starch granules)

In the present investigation we observed that morphological features of both isolated microalgae, *Scenedesmus* sp. and *Acutodesmus* sp. showed similarity in some of the features. Therefore, for clear distinction, genetic analysis of both the microalgal strains was conducted by using 18S rDNA analysis.

Molecular Characterization

PCR Amplification and Sequence Analysis

The multi copy rRNA genes are considered as good candidates for identification of species as they are conserved during the course of evolution. The non translated Intergenic transcribed spacer regions (ITS) of transcriptional unit present between 18S and 5.8 S and between 5.8 and 28 S rDNA subunits, are divergent and distinctive (Turenne, 1999). Therefore, ITS regions are generally used for distinguishing closely related species (Abou-Shanab *et al*., 2011).

After morphological identification, the microalgal strains were identified at the molecular level by comparative sequence analysis of the ITS regions of ribosomal DNA. The primers ITS1 and ITS4, successfully amplified ITS region of microalgal strains (Fig 3). The sequencing of PCR products resulted in 719 bp long nucleotide sequences in microalgal isolate *Scenedesmus* sp. while 705 bp sequences were observed in microalgal isolate *Acutodesmus* sp. Percentage similarity values obtained after pair wise alignment of the sequences of the isolates *Scenedesmus* sp. and *Acutodesmus* sp. versus NCBI database sequences demonstrated 99% identity to *Scenedesmus* sp. and 97% identity to *Acutodesmus obliquus* respectively.

Accession Numbers and Phylogeny

The ITS1-5.8S-ITS2 gene complex sequences of algal isolates were submitted to the GenBank database of NCBI with the following accession numbers, JX519261.1 (*Scenedesmus* sp. PSV1) and JX519262.1 (*Acutodesmuus obliquus* strain PSV2). *Scenedesmus* sp. PSV1 and *Acutodesmuus obliquus* strain PSV2 have also been deposited at the Microalgal Culture Collection (MCC), IARI, New Delhi, India and obtained the MCC numbers as *Scenedesmus* sp. MCC26 and *Acutodesmus* sp. MCC33.

Identification of the microalgal cultures were also supported by sequence-based phylogenetic analysis. A phylogenetic tree was constructed by comparing other published microalgal 18S rRNA gene sequences from Genbank by using the Neighbor Joining method in Mega 5.0 software (Saitou and Nei, 1987). The two strains investigated in this study, are tagged as *Scenedesmus* sp. PSV1 and *Acutodesmus obliquus* strain PSV2 by NCBI. Fig 4, represents the Neighbour-Joining showing phylogentic position of strains and related taxa based on 18S rRNA sequence comparisons. Bootstrap values are indicated at nodes. Only bootstrap value > 50% are shown. Scale bar, 0.05% sequence dissimilarity (0.5 substitutions per 100 nt). In the phylogram analysis of the microlagal sequences ties the isolates PSV1 to *Scenedesmus* sp. and and PSV2 to *Acutodesmus obliquus*.

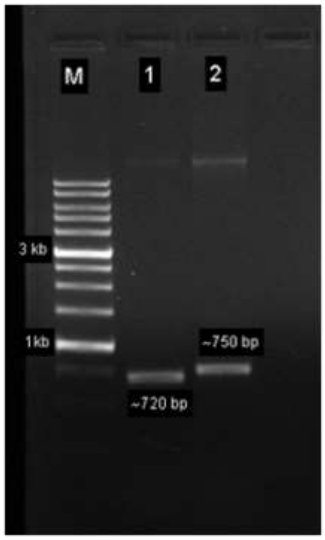

Fig. 3: Electrophoresis separation of the PCR products (about 750bp) amplified from microalgal culture using primers (ITS1-ITS4): (lane 1) 1 kb Marker (Fragments sizes (kb)10.0,8.0,6.0,5.0,4.0,3.0x2,2.5,1.5,1.0x2,0.75,0.5,0.25 (lane 2) *Acutodesmus* sp. and (lane 3) *Scenedesmus* sp.

Growth Rate of Microalgal Strains

The growth pattern of *Scenedesmus* sp. and *Acutodesmus* sp. in BG-11 medium was monitored by calculating total chlorophyll *a* content from day of inoculation to the next 8 days. Both microalgal strains showed a typical sigmoid pattern of growth (Fig 5 & Fig 6). The growth rate with the initial inoculums of 0.1µg ml^{-1} chlorophyll *a* showed a lag phase (2 days) followed by an exponential phase extending till 6^{th} days, a slower late log phase (2 days) and a stationary phase. The specific growth rate of *Scenedesmus* sp. MCC26 and *Acutodesmus* sp.MCC33 was found to be 0.839 day^{-1} and 0.654 day^{-1} respectively. The growth pattern of algal strains are in accordance to the findings of Mohamed and Markert (2006) and Abou-Shanab *et al*. (2011) who described the growth rate analysis and characterization of microalgae.

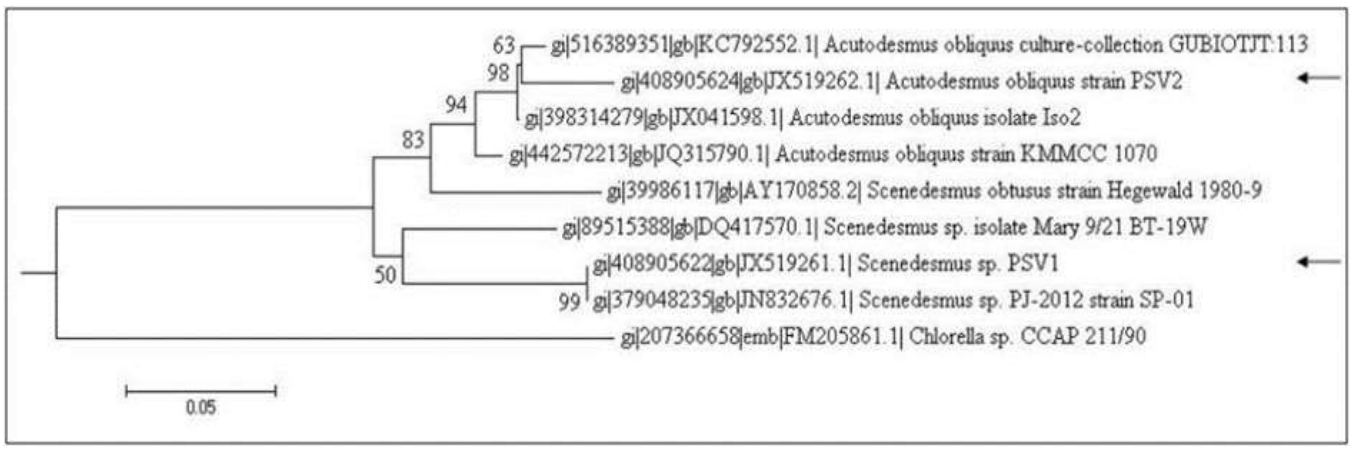

Fig. 4: Phylogenetic tree of *Scenedesmus* sp. PSV1and *Acutodesmus obliquus* strain PSV 2 (represented by arrows) including sequences from NCBI data base

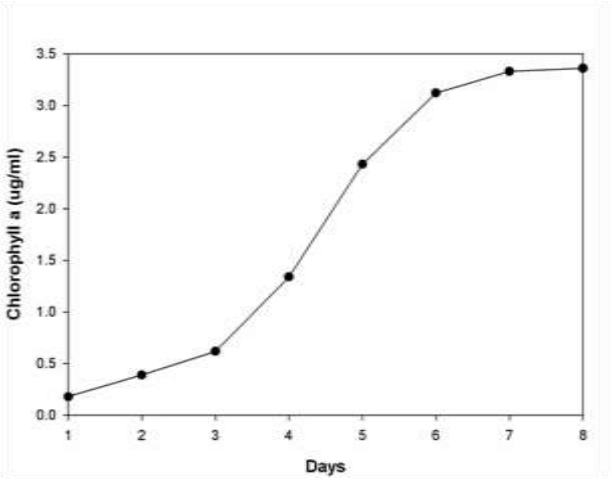

Fig. 5: Growth curve of *Scenedesmus* sp. (Chlorophyll a estimation)

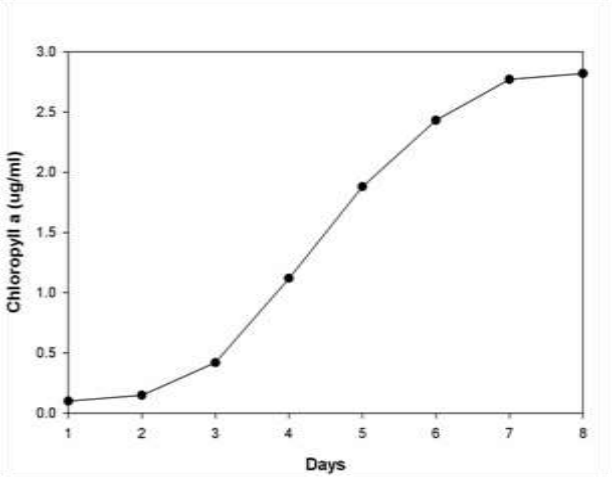

Fig. 6: Growth curve of *Acutodesmus* sp. (Chlorophyll a estimation)

In literature, many researchers placed *Scenedesmus* and *Acutodesmus* as one genus (Kessler *et al*., 1997; van Hannen *et al*., 2002) due to their much similarity in morphological features but in recent years, on the basis of rRNA sequence analysis, researchers have segregated *Acutodesmus* sp. from *Scenedesmus* sp. (Krienitz and Book, 2012; Elias *et al*., 2010; Hegewald and Wolf, 2003; Lang *et al*., 2011). In the present investigation, we also observed distinct variation in 18S rRNA sequences of both the microalgal strains and thus the present findings are in agreement with reported literature and isolated microalgae can be classified in genus Scenedesmus and genus Acutodesmus.

Conclusion

Two microalgal strains isolated from industrial contaminated site of Jaipur, were identified as *Scenedesmus* sp. MCC26 and *Acutodesmus obliquus* MCC33 based on their morphological features and 18S rRNA gene sequence analysis. Microalgal strains are observed to be round and fusiform in shape and in cellular suspensions one to two celled coenobia were more frequent. *Scenedesmus* sp. and *Acutodesmus obliquus* showed high cell growth (μ_{max} 0.839 day^{-1} and 0.654 day^{-1} respectively). Phylogenetic relationship based on 18S rRNA gene sequences demonstrated that both strains belongs to the family Chlorophyceae.

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