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Research Article

Isolation of Several Indigenous Microalgae from Kallar Kahar Lake, Chakwal Pakistan

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Background: Kallar Kahar lake, Punjab, Pakistan is a rich source of phytoplankton which can be used for biofuel production.
Objective: This study was conducted to investigate the presence of different microalgae species present in this lake and their possible utilization for bioenergy production.
Materials and Methods: The crude culture was examined under microscope. Isolation of the identified species was carried out by using serial dilution and colony picking methods. Isolated strains were evaluated by investigating their biomass productivity, salinity resistance and auto-flocculation ability.
Results: Four different microalgae species (*Chlorella, Scenedesmus, Oscillatoria* and *Spirulina*) were identified in the crude sample. The experimental results indicated that, among the four isolated strains, the *Oscillatoria* species showed highest biomass productivity (4.2 gL^{-1}) and *Scenedesmus* showed comparatively higher salt resistance. *Scenedesmus* also showed great potential of auto-flocculation as around 70 % of its cells sediment within 5 h without addition of any external flocculating agent. The lipid content in the isolated strains has also been carried out using Soxhlet extraction.
Conclusion: Four different microalgae strains have been found in Kallar Kahar lake that reflected good biomass productivity and are capable of auto-flocculation.
Key words: Auto-flocculation; Lipid content estimation; Microalgae; Salinity resistance

1. Background

Microalgae are unicellular photoautotrophic microorganisms (2-200 µm) in which both eukaryotic and prokaryotic species are included. Cyanobacteria are prokaryotic microalgae. Species of phyla prochlorophyta and cyanophyta are prokaryotic whereas species of phyla Chlorophyta, Euglenophyta, Glaucophyta, Cryptophyta, Chlorarachniophyta, Haptophyta, Heterokontophyta, Rhodophyta and Dinophyta are eukaryotic (1, 2). Microalgae has large diversity of habitat such as rivers, dams, lacustrine, hyper saline, freshwater, brackish wastewater maturation ponds, coastal and marine areas (3). For the growth of microalgae, appropriate pH, macronutrients (nitrates and phosphates), light, vitamins, suitable salinity, CO_2 and trace elements are required (4). Development of microalgal technology began in the middle of last century. Now, there are many commercial application of microalgae (5). Ecologically, microalgae tremendously influences the universal biogeochemical cycle because about 30 % worldwide CO_2 fixation per year is based on microalgae and it is also important part of marine food web (6). Microalgae also plays vital role in aquaculture (5). It was recognized that 50-60 species of microalgae out of 80,000 species can be used commercially as a good source for animal and human food due to the presence of high quality protein supplement (7). Economically, microalgae as nutraceuticals are potentially used for the production of cosmetics (8), pharmaceuticals, biofuel and for bioremediation (9, 10).

In this era of energy scarcity, microalgae evolved as an important source for biofuel production because natural sources of energy such as fossil fuels are limiting and depleting with the passage of time. Moreover, combustion of fossil fuels causes air pollution and global warming due to the emission of SO_X and CO_X (11). Microalgae is recognized as a good source of biodiesel production because it has rapid growing

ability as well as high amount of lipids are stored in it in the form of triglycerides. Microalgae require little amount of water for its growth and can be grown on unfertile land (12).

2. Objective

The objective of this study was to isolate and identify different species of microalgae collected from Kallar Kahar Lake Chakwal for their possible utilization in biofuel production. Different parameters such as pH, resistance towards salinity and auto-flocculation ability of microalgae were also optimized.

3. Materials and Methods

3.1 Sample Collection

Microalgal samples were collected from the water of Kallar Kahar Lake, Chakwal Pakistan. Monsoon climate is favorable for the growth of microalgae therefore microalgae samples were collected in August, 2017 when the rate of rainfall was sufficient. The samples were kept at 4 ℃. The crude culture was enriched with BG-11 medium which contains $NaNO_3$, $MgSO_4.7H_2O$, $K_2HPO_4.3H_2O$, $CaCl_2.2H_2O$, EDTA dinitrium-salt, citric acid, ferric chloride, NH_4Cl, Na_2CO_3, NaCl, the micronutrients and traces elements which include H_3BO_3, $ZnSO_4 \cdot 7H_2O$, $MnCl_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $NaMoO_4 \cdot 2H_2O$ and Co $(NO_3)_2 \cdot 6H_2O$. The pH was adjusted to neutral by using HCl or NaOH. Aeration was also provided to the crude culture.

3.2. Microscopic Analysis of Crude Culture

The enriched crude culture was examined under microscope to determine the number of strains present in crude culture. Isolation and identification of crude microalgal sample was monitored by using Euromex ISCOPE series (Holland). Microscopic images were taken with the help of Euromex Microscope Holland DC 5000 C CMEX 5 CAMERA.

3.3. Isolation of Strains

The isolation of microalgae strains from crude culture has been carried out by using two different methods which include colony picking and serial dilution methods. In colony picking method, petriplates were prepared by pouring agarized BG-11 medium under sterilized conditions. These petiplates were inoculated with a small colony of microalgae from crude culture with the help of sterilized inoculation loop (7). The inoculated petriplates were kept near window under sunlight. After the growth of microalgae culture in petriplate, a small colony was again picked up and transferred to freshly prepare agarized BG-11 medium containing petriplates. This method was repeated many times until the single strains of microalgae were obtained.

The second method used for isolation of microalgae strains from crude culture was serial dilution method in which liquid form of crude culture was transferred to BG-11 medium. The diltution of crude culture for isolation of microalgae strains was carried out in two different ways. In the first method, the crude culture was diluted ten times and this diluted culture was used for inoculation in petriplates containing liquid BG-11 media (without agar). This process was repeated many times for the isolation of single strain of microalgae (13). In second method, the enriched culture was diluted many times (> 20 times) until to observe a single cell under microscope. The single cell containing slide was then shifted to the petriplate containing liquid BG-11 medium (14). The petriplate was placed near window until to achieve growth in petriplate.

3.4. Microscopic Identification of Isolated Strains

Isolated species were identified based on their morphology and cell size measured through microscope.

3.5. Molecular Identification of Isolated Strains

Molecular identification of different microscopically identified microalgal strains was carried out. For this purpose, the microalgae strains were provided to Plant Molecular Virology and Gene silencing Lab, Agricultural Biotechnology Division (ABD), National Institute for Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan.

Isolated microalgae strains were stored at -80°C before DNA extraction. Sampling, DNA extraction, amplification, sequencing and classification of microscopically identified microalgae strains was carried out by following the procedure described by Alonso et al., 2012 (15). In DNA extraction, total genomic DNA was extracted by taking batch of cells of 20µL from respective microalgae strains using Nucleo Spin plant II (Macherey-Nagel). The corresponding microalgae samples were amplified by using 16s rRNA and 18s rRNA molecular markers. Later on, sequencing of obtained products was carried out in order to get the results (15).

3.6. Different Properties of Isolated Microalgae

pH of growth media plays an important role for microalgae cultivation; therefore, experiment was designed to evaluate the effect of pH the on growth of isolated strains by varying the pH of BG-11 growth media from 3-11.

For the optimization of flocculent dose to harvest

microalgae from the liquid culture, Potassium Aluminum Sulfate was used as flocculent in different concentrations ranging from 0.01-1 %.

Biomass productivity of the isolated strains was estimated by measuring the weight of the dried biomass obtained from 1 L liquid culture through centrifugation.

Resistance of isolated strains against salinity was determined by using different concentrations of NaCl in BG-11 medium (1 %, 4 %, 7 % and 10 %). 200 mL of each of these solutions was added to conical flasks and in one conical flask BG-11 medium was added without NaCl for the use as control. All these flasks were inoculated with 1 mL culture of isolated strains. Growth of isolated strains was monitored in each flask to determine the ability of strains to grow in saline environment.

Auto-flocculating ability of isolated strains was determined by putting specific volume of liquid culture of each isolated strain in conical flasks. The conical flasks containing isolated strains of microalgae were allowed to settle. Further, settling time was noted to determine the auto-flocculating ability of isolated strains.

Lipid content in microalgae strains was determined using solvent extraction which was carried out in an In-line extraction unit from Behr Labor-Technik, Germany.

4. Results

4.1. Isolation of Microalgae

Microscopic study revealed that five different strains were present in the crude culture which can be seen in **Figure 1.** These strains showed resemblance with *Chlorella, Scenedesmus, Oscillatoria and Spirulina* species. For the isolation of strains, the serial dilution method was found to be most efficient as compared to the colony picking method. One strain (*Oscillatoria* sp.) was successfully isolated through colony picking method. As dilution process of isolation was carried out in two different ways, the *Chlorella* was successfully isolated through dilution method in which the culture was diluted many times until a single cell on slide while *Scenedesmus* and *Spirulina* were isolated through serial dilution of crude culture in which the culture was diluted ten times and the process was repeated for five times.

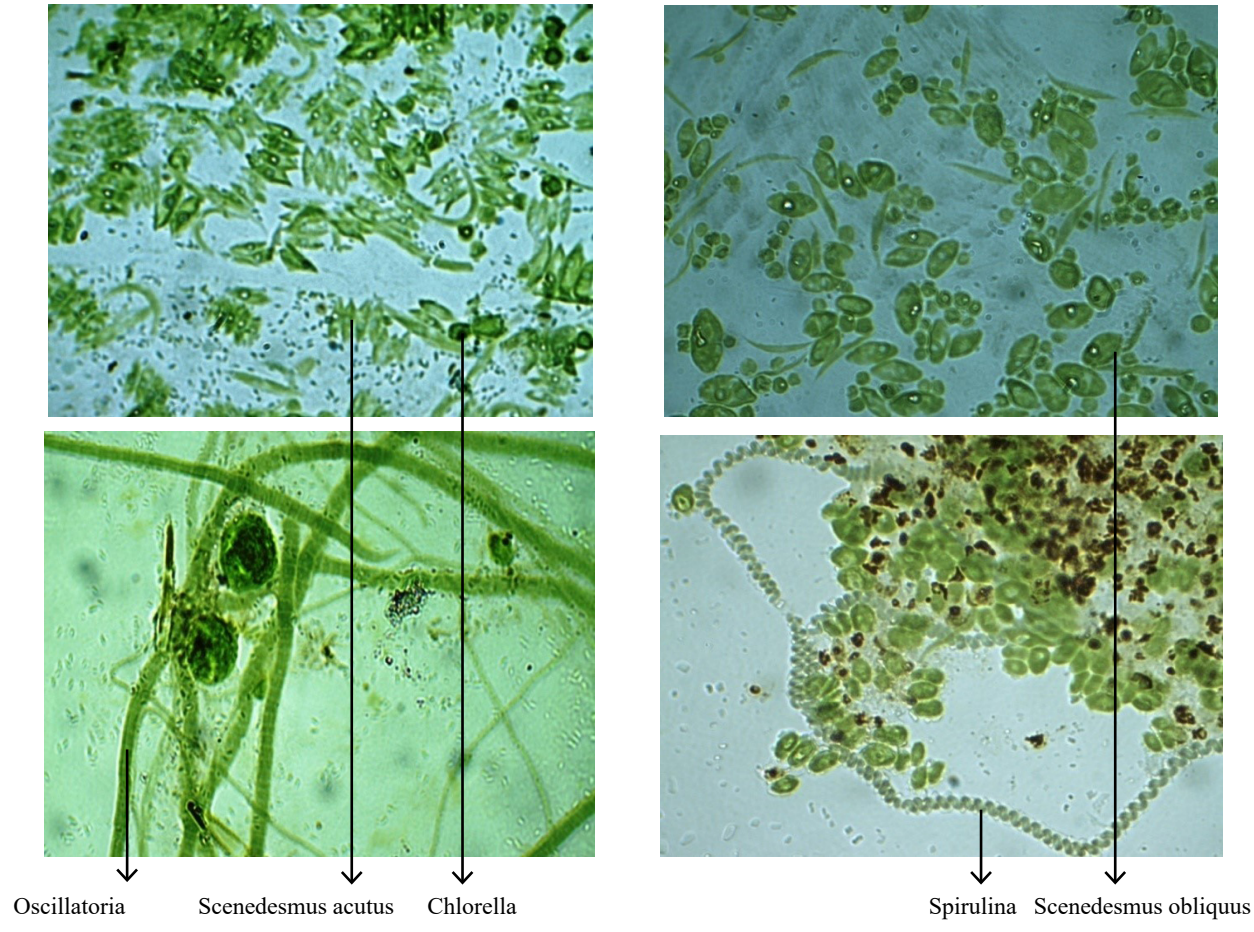


Figure 1. Microscopic images of crude sample showing the presence of different microalgae strains. All these four panels are showing the crude cultures of microalgae collected randomly from different locations of kallar kahar lake Chakwal.

4.2. Identification of Isolated Strains

The isolated microalgae strains were identified based on their morphology (cell structure and cell size). The first isolated strain was round in shape (**Fig. 2A**). The average cell size was found to be 2.7 µm. Overall, the cell size of randomly selected cells was observed to vary from 2 µm to 3 µm. The cell size measurement and cell shape showed that this isolated strain belongs to *Chlorella sp*. This cell size also is also similar to the cell size reported in literature (16).

The second isolated strain was oval in shape (**Fig. 2B**). The average cell length was found to be 6 µm and average width was 3.9 µm. Minimum cell length was observed to be 5 µm and maximum cell length was 7 µm. The width was found to be in the range of 3 µm to 6 µm. Oval shape of cells and cell size of this strain show its close resemblance with *Scenedesmus sp*. This measured cell size resembles to the cell size of the *Scenedesmus sp.* reported in the literature (17).

The third isolated strain consists of small cells stacked on each other to form a long thread-like structure which has close resemblance with *Oscillatoria sp* (Fig. 2C). Average cell length was found to be 2 µm and 1 µm was the average width. Cell length varied from 1 µm to 3 µm. This cell length resembles the cell length given in literature (18).

The forth isolated strain was in the form of long spiral thread which has close resemblance with *Spirulina sp*. (**Fig. 2D**). Average width of these filaments is 1.8 µm which varied from 1 µm to 2 µm which is similar to the filament width given in literature (19). Distance between two turn of spiral filamentous structure was also measured. In this regard, 1.5 µm was the average distance between two turn which varied from 1 µm to 2 µm.

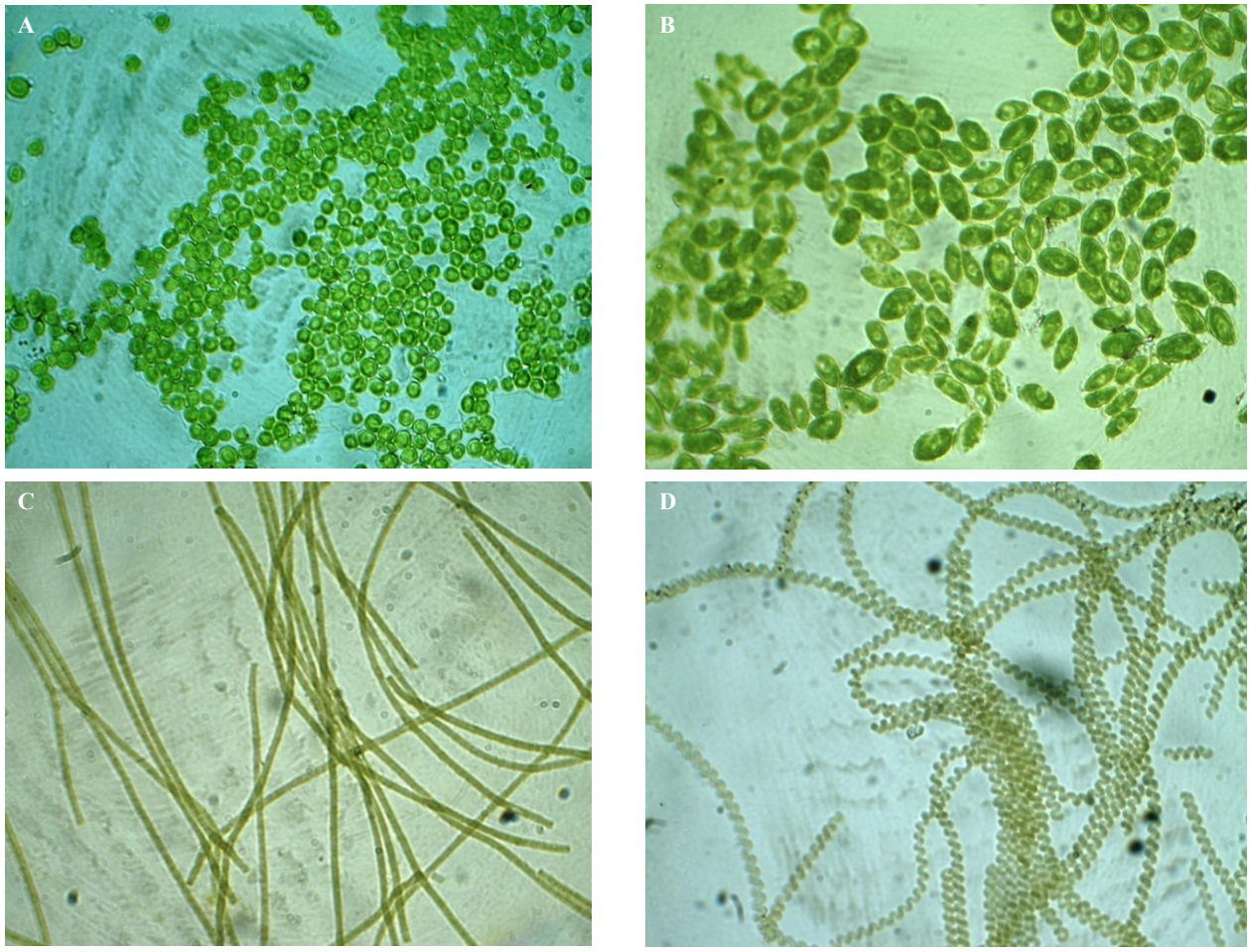


Figure 2. Microscopic images of isolated strain **(A)** Chlorella **(B)** Scenedesmus **(C)** Oscillatoria **(D)** Spirulina

4.3 Molecular Identification of Isolated Strains

Microscopically identified microalgae strains such as *Chlorella, Scenedesmus, Oscillatoria and Spirulina* were subjected to DNA extraction. Molecular characterization of microalgae samples was carried out by using molecular markers *i.e.* 16s rRNA and 18s rRNA. The 16s rRNA and 18s rRNA were amplified from algae samples and amplification size was adjusted

according to Alonso et al., 2012 (4) (**Fig. 3**).

The molecular identification of different isolated microalgae strains based on 16s rRNA and 18s rRNA is given in Table 1. According to literature, classification based on 16 s rRNA is suitable for prokaryotes and 18s rRNA is preferable for eukaryotes.

Microscopic images predicted that cells of sample 1 are round in shape pointing towards its resemblance with *Chlorella sp*. It is stated that *Chlorella* species are green eukaryotic microorganisms belongs to phylum *Chlorophyta*, family *Chlorellaceae* and genus *Chlorella*. The molecular identification of *Chlorella sp*. was confirmed by 18s rRNA sequencing as shown in Table 1. Molecular studies followed by amplification and sequencing of L1 kb 18s-rDNA ladder fragment confirmed the microscopic identification of the sample 1. Sequence obtained from sample 1 showed very close resemblance with two *Chlorella* species (*Chlorella sp.* KR869729.1 and *Chlorella sp.* GQ122349.1) available in NCBI database. Hence, sample 1 isolate was identified as *Chlorella sp.*

According to microscopic study cell size and shape, sample 2 resembles with *Scenedesmus sp.* Microscopic images showed that sample 2 cells are oval in shape due to close resemblance with *Scenedesmus sp.* Moreover, it belongs to phylum *Chlorophyta*, class *Chlorophyceae* and genus *Scenedesmus*. The identification of *Scenedesmus* was confirmed by 18s rRNA sequencing as shown in table. Molecular studies followed by amplification and sequencing of L1 kb 18s-rDNA ladder fragment confirmed the microscopic identification of the sample 2. Sequence obtained from sample 2 showed similarity with two *Scenedesmus* species (*Scenedesmus sp.* MN604371.1 and *Scenedesmus sp.* KY268297.1) available in NCBI database. Hence, sample 2 isolate was identified as *Scenedesmus sp.*

Microscopic study regarding sample 3 concluded the very close resemblance of isolate with *Oscillatoria*. In addition to this, taxonomic keys pointed its belonging with genus *Oscillatoria*, class cyanophyceae and phylum *cynaobacteria*. Microscopic images of *Oscillatoria* showed its filamentous nature where in small cells stacked on each other forming a thread like structure. The identification of *Oscillatoria* was confirmed by 16s rRNA sequencing as shown in table. Molecular studies followed by amplification and sequencing of L1 kb 16s-rDNA ladder fragment confirmed the microscopic identification of the sample 3. Sequence obtained from sample 3 showed similarity with two *Oscillatoria* sequences (*O. earlei* KF487294.1 and *O. earlei* KF487295.1) available in NCBI database. Hence, sample 3 isolate was identified as *Oscillatoria earlei.*

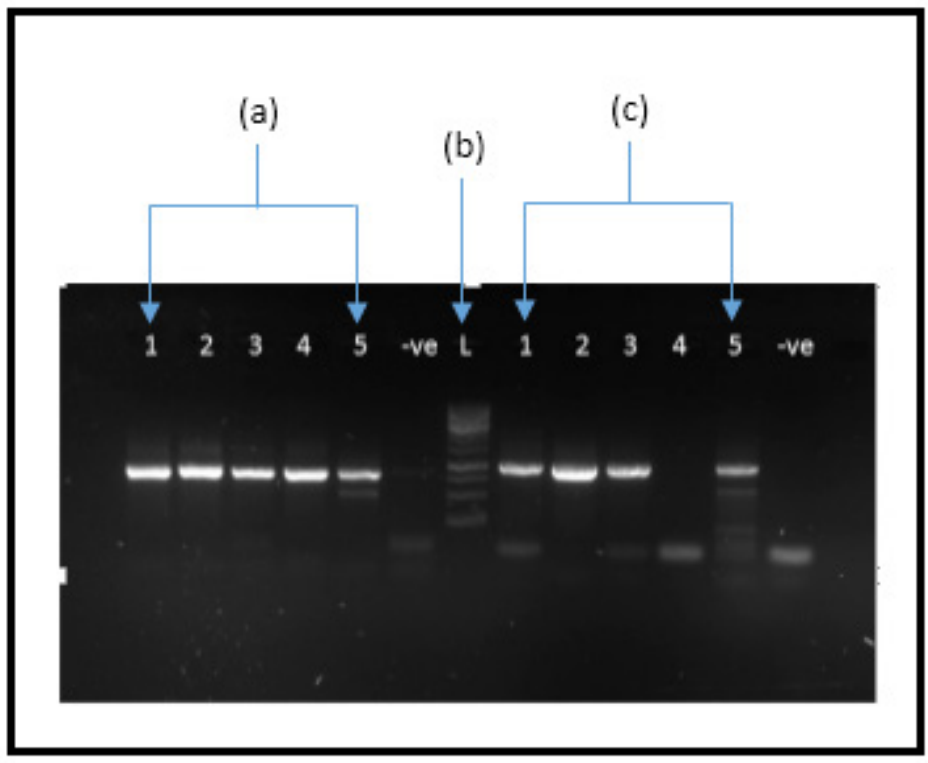


Figure 3. PCR amplification for five isolated strains of microalgae (a) Molecular identity according to 16 s (b) 1 kb DNA ladder (c) Molecular identity according to 18 s

Morphological features of sample 4 analyzed at microscopic level revealed that it has resemblance with *spirulina*. Furthermore, taxonomic keys investigated that it belongs to the genus *spirulina*, class *cyanophyceae* and phylum *cyanobacteria*. Microscopic images of *spirulina subsalsa* showed filamentous coiled and spiral appearance (16). The identification of *spirulina subsalsa* was confirmed by 16s rRNA sequencing as shown in table. Molecular studies followed by amplification and sequencing of L1 kb 16s-rDNA ladder fragment confirmed the microscopic identification of the sample 4. Sequence obtained from sample 4 showed similarity with two *spirulina* sequences (*S. subsalsa* AY575935.1 and *S. subsalsa* LC215280.1) available in NCBI database. Hence, sample 4 isolate was identified as *Spirulina subsalsa*.

4.4. Optimization of pH and Flocculent Dose

Growth of microalgae can be affected by change in pH in different ways. pH can alter the availability of carbon, essential nutrients and trace metals. For the optimization of pH, experiment was conducted by varying pH of growth media from 3-12. It was found that 7 pH is more suitable for the growth of *Chlorella*, 7.5 for *Scenedesmus*, 10 for *Oscillatoria* and 9 for *Spirulina* (**Fig. 4**).

High energy input for the harvesting of microalgae is one of the bottlenecks for its commercialization. This is because of their colloidal stability of suspension, lower cell density (less than 1 g.L) and small cell size (2-20 µm) in culture medium. In this study, potassium aluminum sulphate (alum) was used as flocculent for rapid harvesting of microalgal biomass. Dose of flocculent fluctuated from 0.01-1 % for its optimization. It was observed that 0.1 % flocculent is sufficient for the harvesting of *Chlorella* in 5 min and 0.05 % dose is enough for the harvesting of *Scenedesmus*, *Oscillatoria* and *Spirulina* but *Scenedesmus* takes 5 min for its complete flocculation while *Oscillatoria* and *Spirulina* take 15 min for their complete harvesting.

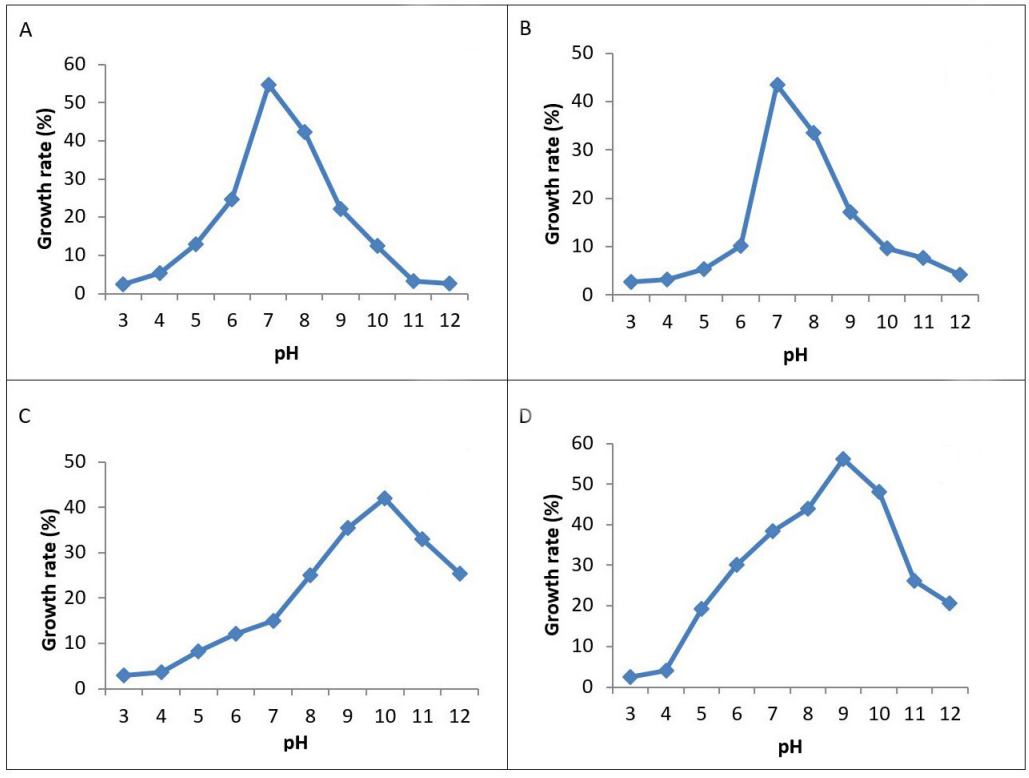


Figure 4. Optimization of pH for growth of **(A)** *Chlorella sp.* **(B)** *Scenedesmus sp.* **(C)** *Oscillatoria sp.* **(D)** *Spirulina sp.* (Growth media volume= 200mL; Inoculation size=1mL)

4.5. Different Properties of Isolated Microalgae

Biomass productivity of isolated strains was found to investigate their possible utilization for different purposes. It was found that the biomass productivity of *Chlorella, Scenedesmus, Oscillatoria* and *Spirulina* is 0.94 g, 0.58 g, 4.2 g and 3.45 g per liter respectively. *Oscillatoria* has maximum biomass productivity.

4.5.1. Salinity Resistance

Resistance of these strains against salinity was also

determined to find out that either these strains are able to grow on saline soil or not. As the percentage of salinity increased from 1 % to 10 %, the percentage of growth rate was decreased. This decrease in growth rate for *Chlorella* is from 36 % to 4.9 %, in *Scenedesmus* from 48 % to 12 %, in *Oscillatoria* from 21 % to 4.1 % and in *Spirulina* 48 % to 7.5 % (**Fig. 5**).

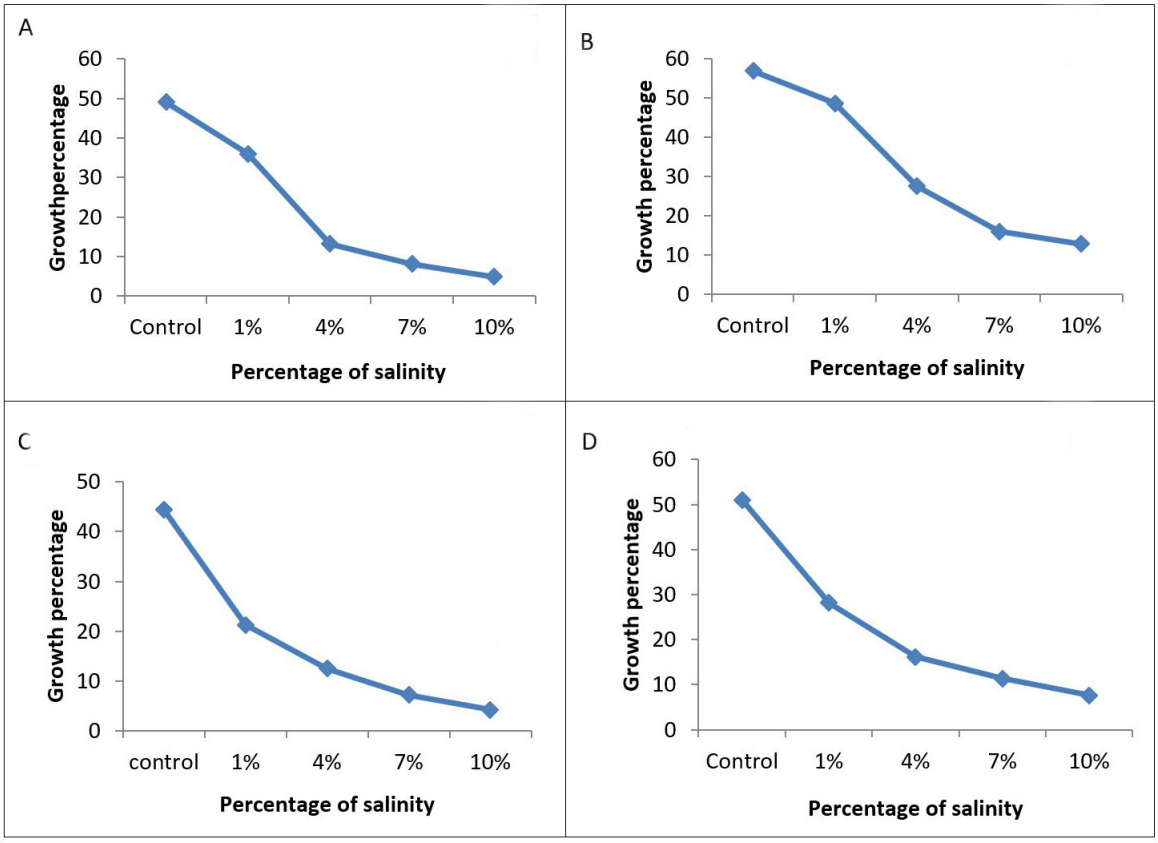


Figure 5. Effect on growth of different isolated strains **(A)** Chlorella **(B)** Scenedesmus **(C)** Oscillatoria **(D)** Spirulina against saline conditions

4.5.2. Auto-Flocculation Ability

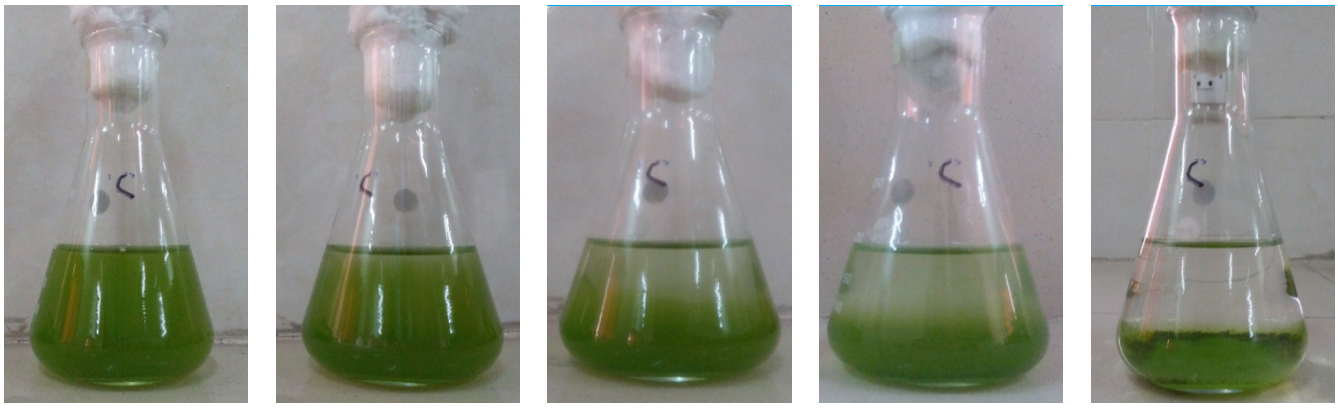
Auto-flocculation is a reliable technique for the harvesting of microalgae because no flocculent is required in this technique. Auto-flocculation ability of these isolated strains was also determined to find out that either these strains can be harvested without the addition of flocculent or not. It was observed that *Scenedesmus* have higher auto-flocculating ability which is 96.60 % in 24 hours while *Chlorella, Oscillatoria* and *Spirulina* have 94.49 %, 91.15 % and 87.72 % flocculation in 24 hours respectively. From this experiment it can be concluded that these strains can be harvested without the addition of flocculating agent within 24 hours (**Fig. 6**).

4.5.3. Estimation of Lipid Content

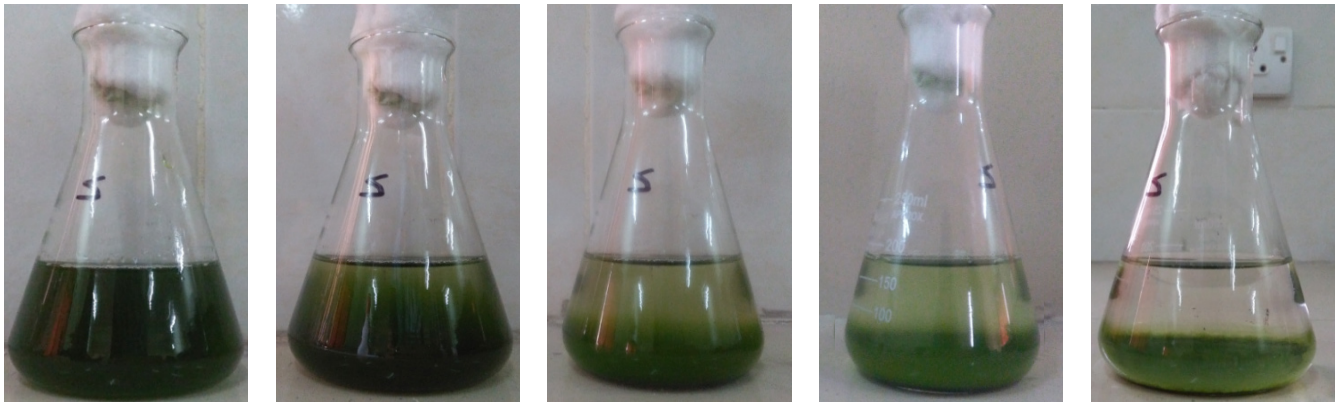
The conventional Soxhlet extraction was used for estimation of lipid contents in isolated strains. The lipid extraction was carried out with n-hexane and it was observed that *Scenedesmus* strain has higher lipid content (~ 4 %) as compared to other isolated strains. By using Soxhlet extraction method, the amount of lipid in *Chlorella* strain was found to be 2 % while negligible amount of lipid was observed in *Oscillatoria* and *Spirulina*.

5. Discussion

Dilution method was found more efficient for the isolation of strains then the colony picking method. The time required for isolation of strains in colony picking method is more than dilution method. Different researchers have used the colony picking method for the isolation of microalgae strains (3, 7). They have also used the colony picking method to get isolated microalgae strains and also to remove protists from the crude culture. Dilution method was also used by different scientists to isolate microalgae strains. Wu and co-workers diluted wastewater from 10 to 10^5 times, collected from Jimei wastewater plant (China) for the isolation of microalgae and isolated nine different microalgae species by this method (20). In another study, Abou-Shanab and his co-

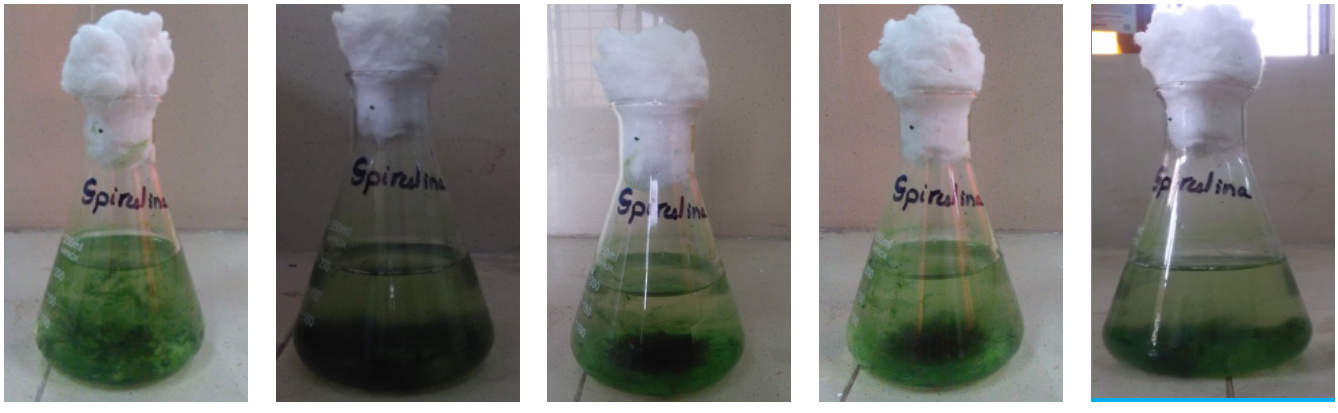


Autoflocculation of Chlorella sp.



Autoflocculation of Scedesmus sp.

Autoflocculation of Oscillatoria sp



Autoflocculation of Spirullina sp

Figure 6. Auto-flocculation of different isolated strains (Fully grown culture volume=200 mL)

worker isolated 45 algal species from freshwater lake by using dilution method (21).

Microscopy is a simple and quick method for identification of unknown strains. The first isolated strain showed resemblance with *Chlorella sp.* (22). The cell size measurement and cell shape show that this isolated strain belongs to *Chlorella sp*. The reported cell size in literature for *Chlorella* is 2-4 µm (23). Illman *et al.,* 2000 reported that diameter of *Chlorella vulgaris* ranges from 2-10 µm (17). Oval shape of cells and cell size of this strain show its close resemblance with *Scenedesmus sp.* (18). The third isolated strain consists of small cells stacked on each other to form long thread like structure which has close resemblance with *Oscillatoria sp* . *Oscillatoria* has been observed in sea waters by different researchers and it exists in the form of dense bloom (24, 25). The forth isolated strain was *Spirulina sp*. *Spirulin*a *sp*. belonging to the family of oscillatoriaecae. Helical (spiral) shape of filaments is the characteristic of this genus, but the parameters such as dimensions and pitch length of this helix vary from species to species (26). Growth parameters such as temperature also affect the dimension of this helix. In solid media helical shape changed to true spirals (27).

It is essential to determine the optimum pH for the growth of microalgae to enhance its growth rate and lipid content (28). Physiological effects potentially occur at extreme pH (29). Different researchers worked on the optimization of media pH for these microalgae species and our results are in accordance with their findings (30-32).

In microalgae, there is no aggregation due to the negatively charged surface (33). This negative charge can be neutralized by adding different flocculants such as alum, ferric chloride and ferric sulfate (34). These flocculants increase particle size by the aggregation of microalgal cells which assist the process of flocculation. Flocculent should be less toxic, inexpensive and effective at lower concentration (35). The study results revealed that very little amount of alum is required for harvesting these strains which show their good self-flocculation ability. Chemical flocculent such as aluminum affect the composition of fatty acid methyl ester, use of its biomass for animal feed and cells of microalgae also damaged by aluminum salts. Moreover, these flocculent also remain present in the lipid extracted from microalgal biomass and interfere with the process of trans-esterification.

The isolated strains depicted good biomass productivity especially *Oscillatoria* which is an important nitrogen fixer, good food source for zooplanktons and also meet oxygen demand of fish in ponds with their high biomass productivity (36, 37).

The amount of lipid in isolated strains was not found so high for use in biofuel production. In this study, lipid content was estimated using Soxhlet extraction with n-hexane. As microalgae cell was is rigid so some other cell disruption techniques might increase the chance of lipid release from microalgae cells for their possible use in bioenergy (38, 39).

6. Conclusion

Kallar Kahar lake Chakwal is the potential source of different useful microalgae strains. Four species of microalgae were successfully isolated from crude culture and identified on the basis of their morphology and molecular identification. Serial dilution method proved an efficient method for the isolation of different microalgae strains. It was found that from these species *Oscillatoria* and *Spirulina* require alkaline pH for their maximum growth. Maximum biomass can be obtained from *Oscillatoria sp*. due to its high biomass productivity and *Scenedesmus sp*. have high resistance against salinity as well as auto-flocculation ability as compared to other isolated strains.

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