**CO₂ capture using limestone for cultivation of the freshwater
microalga *Chlorella sorokiniana* PAZ and the cyanobacterium
Arthrospira sp. VSJ**

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Abstract

The present study reports a process wherein CO₂ is captured in the form of bicarbonates using calcium oxide and photosynthetically fixed into biomass. Microalgal cultures viz. *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ were grown in the medium containing bicarbonates. The rate of bicarbonate utilization by *C. sorokiniana* PAZ was higher when CO₂ trapped in the presence of 2.67 mM calcium oxide than in the presence of 10 mM sodium hydroxide and with direct addition of 10 mM sodium bicarbonate. For *Arthrospira* sp. VSJ the bicarbonate utilization was 92.37 %, 88.34 % and 59.23 % for the medium containing CaO, NaOH and NaHCO₃, respectively. Illumination of **photosynthetically active radiation (PAR) + ultraviolet A radiation (UVA)** enhanced the yield of *C. sorokiniana* PAZ and *Arthrospira* sp. VSJ by 1.3 and 1.8 folds, respectively. FTIR analysis revealed elevation in the biosynthesis of specific metabolites in response to the UVA exposure.

Keywords: CO₂ sequestration; bicarbonate; UVA; calcium oxide; cyanobacteria; microalgae.

1. Introduction

The **world** is facing the problem of global warming due to the increased CO₂ emissions. The reason behind the increased CO₂ emissions is an excessive burning of **fossil fuels** (de Morais and Costa, 2007). Therefore, it is necessary to take action to minimize anthropogenic contributions to atmospheric levels of CO₂. According to the observation of Mauna Loa Observatory, Hawaii, the concentration of CO₂ increased dramatically from 370 ppm in 2002 to 400 ppm in 2014. One of the potential ways of CO₂ mitigation is capturing effluent gas under pressure from a point source and storing the material below 1 km of the Earth's surface. However, this process is technically challenging and is currently prohibitively expensive at approximately US \$ 67 **per metric ton (MT)** of CO₂ (Chi et al., 2011).

Mixed strategies for reducing net CO₂ emissions may prove the most promising approach to the daunting overall challenge given the diversity of socio-economic circumstances. One strategy to CO₂ mitigation is providing it to algal cultures for photosynthetic capture into biomass. In future economies, this may prove useful if the biomass contains high-value products whose production otherwise depends on energy intensive processes. Normally, 54000 km² forest is required to assimilate 16.2 MT CO₂ per year while only 10,000 km² land is necessary for capturing 120 MT CO₂ per year via microalgae (Sudhakar et al., 2011). Typical algae farm is constructed on 100 ha land to consume around 31 kg of CO₂ m⁻² year⁻¹ (Sudhakar et al., 2011). Availability of such big part of land near the source of CO₂ emission is almost impossible. Therefore, this option generally requires transport of captured carbon through pipelines up to the targeted site. The cost for compressing and drying CO₂ was US\$8.48/MT CO₂ as well as US\$3.30/MT CO₂ for pipeline transportation for 100 km distance (Kadam, 1997). The high cost of the transportation makes this approach unsuitable for extensive operation. Therefore, it is necessary to find an alternative solution. The use of CO₂ by converting it to a biologically available form such as bicarbonates and carbonates can be an effective solution. This form of biological CO₂ is stable and storable with a cost of transportation ranging from US \$ 0.05 - 0.125 m⁻³ for 100 km distance (Zhou and Richard, 2005). This captured form of CO₂ may be subjected to long-term storage or used by photosynthetic organisms to minimize the level of CO₂ in the environment.

An additional obstacle is rooted in the physico-chemical nature of inorganic carbon transport in aqueous medium conversion of CO₂ to photosynthetic biomass may be inefficient due to the low dissolution rate of CO₂ into the water. Consequently, gassing of large-scale cultures is likely to result in the release of more CO₂ into the atmosphere than is fixed photosynthetically. The forward reaction rate constant for hydration of CO₂ in water is 6.2×10^{-3} s at 25 °C in the absence of a catalyst (Sullivan et al., 1993). Moreover, this

conversion is critical for biological fixation because microalgae and cyanobacteria largely depend upon relatively efficient systems for the uptake of bicarbonate. Consequently, the low rate of hydration of CO_2 dissolved in water often limits the growth of cyanobacteria and microalgae. At the same time, the equilibrium proportions of the different and interconvertible molecular forms of dissolved inorganic carbon changes as the pH of the system changes. When the pH of the water is acidic, the major form of inorganic carbon that is present in the water is dissolved CO_2 , while HCO_3^- (bicarbonate) is the dominant species at pH 8.0, and CO_3^{2-} (carbonate) is major species at pH 10. Accordingly, the limitations on capture by aquatic photosynthetic organisms could be mitigated under alkaline culture conditions. Use of NaOH and KOH for trapping CO_2 is an effective approach, but it results in unwanted effects on algal growth due to its high ionic strength and these substances are costly to use at a higher scale. Among all materials used for trapping CO_2 , limestone (CaO) has an enormous potential due to its availability at low cost. The waste water of alkaline pulp and paper industry can be the readily available source of Ca(OH)_2 for trapping emissions (Pérez-López et al., 2008). This process of capturing CO_2 is similar to the natural process that occurs through marine carbonate weathering (Murray and Wilson, 1997).

The main advantage of using photosynthetic microbes for CO_2 sequestration is that the consumption and conversion of CO_2 is powered by solar energy. After the complete utilization of bicarbonates, formed biomass is converted into useful products like biodiesel (Lam and Lee, 2013), polysaccharides (Chen et al., 2012) and phycocyanin (Zeng et al., 2012). When an organism utilizes dissolved CO_2 in the form of bicarbonate, it produces OH^- , which makes the water alkaline and again regenerates the substrate for CO_2 capture without spending any extra energy. In this study, a new chemo-photosynthetic approach is used for CO_2 sequestration. The given process addresses the challenge to overcome the high cost of CO_2 capture and transportation by capturing CO_2 at the point source and converting it into

utilizable form i.e. bicarbonates. Moreover this form of CO₂ can be stored during night when algae grow poorly. Cyanobacteria and microalgae utilize this form of CO₂ efficiently and help to mitigate the CO₂ from the environment. Further, the effect of UVA radiation on the utilization of bicarbonates by cyanobacteria and microalgae was assessed.

2. Material and Methods

2.1. Isolation and culture maintenance

Strains were isolated from a unique aquatic ecosystem of the Lonar Lake, which is a soda lake situated in the Buldhana district of Maharashtra, India. Phytoplankton's-containing water sample were cultivated in BG-11 (Stanier et al., 1971) and Zarrouk's medium (Zarrouk, 1966) incubated at 28 ± 2 °C and $50 \mu\text{mole m}^{-2} \text{s}^{-1}$ light intensity with a 16 h light : 8 h dark photoperiod until visible growth was observed. Then, they were removed and serial dilutions were being made in respective medium. Samples were also streaked on Petri plate containing 1.5 % agar. All flask and plates were incubated under same condition up to 10 days. After incubation isolated colonies were picked up and inoculated in the medium. The purity of cultures was checked by repeated sub culturing and daily observation under the microscope (Nikon Eclipse 200).

Chlorella sorokiniana PAZ was maintained in BG-11 medium while *Arthrospira* sp. VSI was maintained in Zarrouk's medium. All the culture flasks were incubated at 28 ± 2 °C and $50 \mu\text{mole m}^{-2} \text{s}^{-1}$ light intensity with a 16 h light : 8 h dark photoperiod up to 10 days. Respective cells were harvested in the log phase of their growth and used as an inoculum for the further experiments.

2.2. Molecular identification

For molecular identification of isolates, genomic DNA was extracted and used for PCR amplification of the 16S rRNA gene from *Arthrospira* sp. VSJ using universal primers 8F 5' AGAGTTTGATCCTGGCTCAG 3' and 1492R 5' CGGTTACCTTGTTACGACTT 3' (Youssef et al., 2015). PCR amplification reaction was done in 50 µL of reaction mixture containing PCR buffer, 1X (Herculase II); dNTP mix, 0.25 mM; Herculase II fusion DNA polymerase, 1 µL; primer, 0.25 µM, DMSO, 0-8 % final concentration and template DNA, 100 ng. Amplification was carried out according to following condition: 5 min at 95 °C, then 35 cycles including 30 s at 95 °C, 45 s at 50 °C and 1 min 30 s at 72 °C, and final step of 15 min at 72 °C. The PCR product was purified from agarose electrophoresis gel using QIAquick® gel extraction kit (QIAGEN®, USA) and the mixtures containing the PCR product and primers were subjected to sequencing (Oklahoma State University Molecular Biology Core Facility). Sequencing and molecular identification of *Chlorella sorokiniana* PAZ was done at a commercial lab Triyat Scientific, Nagpur, India using primers 18S FP 5'GCCTGTCTCAAAGATTAAGCC 3' and 18S RP 5' CACCTACGGAGACTTTGTTAC 3'. PCR reaction was done in 20 µL of reaction mixture containing PCR buffer, 1X (Kappa, SA); MgCl₂, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U, primer, 1 picomol and template DNA, 50 ng. Amplification was carried out according to following condition: 2 min at 94 °C, then 30 cycles including 50 s at 94 °C, 30 s at 48 °C and 1 min 30 s at 72 °C, and final step of 6 min at 72 °C. Sequencing results for both species were then compared with the database of National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Isolated sequences with other known sequences from closely related species were used to construct a neighbor joining phylogenetic tree using MEGA 6 software (Felsenstein, 1985; Kimura, 1980; Saitou and Nei, 1987; Tamura et al., 2013).

2.3. Effect of pH

Effect of medium pH on growth of isolated cultures was assessed by cultivating *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ in 150 mL flasks containing 40 mL of BG-11 and Zarrouk's medium, respectively. The pH of growth media was adjusted using appropriate buffers in the range of 8 - 12 (Dayananda et al., 2007). All the culture flasks were incubated at 28 ± 2 °C with $50 \mu\text{mole m}^{-2} \text{s}^{-1}$ light intensity and 16 : 8 h as light to dark photoperiod. After three weeks of incubation, the cultures were harvested and analyzed for pigment content.

2.4. Effect of calcium

Tolerance of cultures to calcium was assayed using different concentrations ranging from 4 - 12 g L⁻¹ calcium in 150 mL flasks containing 40 mL of the respective medium at 28 ± 2 °C and $50 \mu\text{mole m}^{-2} \text{s}^{-1}$ light intensity with 16 : 8 h light : dark photoperiod. All the flasks were shook three times during light phase. After 15 days of incubation, the culture was harvested and analyzed for pigment content.

2.5. Effect of PAR + UVA radiation

To check the tolerance to ultraviolet radiation, the cultures were exposed to photosynthetic active radiation (PAR) + ultraviolet A radiation (UVA) illuminations. UVA exposure intensity was maintained $0.1 \mu\text{mol m}^{-2} \text{sec}^{-1}$ throughout 16 h light cycle using UVA tube (TL 20W/12, Philips, Holand) along with $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR from the fluorescent tube (TL 40W/ Philips, India) as mentioned earlier (Javalkote et al., 2015). The experiment was carried out in 150 mL borosilicate glass beakers with 40 mL of respective medium incubated at 28 ± 2 °C with 16 : 8 h light : dark photoperiod. On 5th and 10th day, culture was analyzed for pigment content.

2.6. Trapping of CO₂ and formation of bicarbonates

Various concentrations of calcium hydroxide i.e. 0.89 mM, 1.78 mM, 2.67 mM, 3.57 mM as the source of alkalinity necessary for trapping of gaseous carbon dioxide was added separately in the growth medium (BG-11 and Zarrouk's medium with omission of Na₂CO₃ and NaHCO₃ respectively). In each carbon dioxide trapping flask, the compressed carbon dioxide gas (99.9 %) was passed at a flow rate of 100 mL min⁻¹ until the pH of the medium dropped to 6.0 ± 0.1. The medium was filtered through Whatman's No. 1 filter paper to remove the lime left undissolved. For comparison to bicarbonate formed due to trapping of gaseous carbon dioxide in alkaline calcium hydroxide solution, sodium hydroxide (10 mM) flushed with CO₂ compressed gas until pH dropped to 6.0 ± 0.1 and powdered sodium bicarbonate (10 mM) as a direct bicarbonate source were used in the culture medium. Cultures were grown in conical flasks of 2 L capacity with a working volume of 1.5 L (Aishvarya et al., 2012). All the flasks were inoculated either with the log phase culture of *Chlorella sorokiniana* PAZ or *Arthrospira* sp. VSJ on the basis of an equal dry weight of inoculum.

2.7. Effect of PAR + UVA on bicarbonate utilization

Culture flasks were illuminated under PAR+UVA radiation to check the efficacy of UVA on the growth of microalgae and their bicarbonate utilization profile. In this experiment, bicarbonate trapped medium was equally inoculated with log phase of algal growth and incubated at 28 ± 2 °C and illuminated with PAR and PAR+UVA as 16 : 8 h light : dark photoperiod. Other conditions of culturing were similar as stated earlier.

2.8. Growth measurement

Cultures were grown in conical flasks of 2 L capacity with a working volume of 1.5 L (Aishvarya et al., 2012). After every 24 h 10 mL of samples were drawn aseptically and checked for pH and optical density at 750 nm (Hille et al., 2013) using spectrophotometer (Shimadzu UV mini-1240). A calibration curve between absorbance and dry weight of cells was made prior to experiments and used in the calculation. The correlation is shown as follow:

For *Chlorella sorokiniana* PAZ (1),

$$\text{Dry weight (g L}^{-1}\text{)} = 0.3524 \times \text{OD}_{750 \text{ nm}} + 0.1875, \quad R^2 = 0.9834 \quad (1)$$

For *Arthrospira* sp. VSJ (2),

$$\text{Dry weight (g L}^{-1}\text{)} = 2.1702 \times \text{OD}_{750 \text{ nm}} + 0.3397, \quad R^2 = 0.9724 \quad (2)$$

2.9. Measurement of bicarbonate utilization by cultures

For calculation of bicarbonate, after every 24 h, 25 mL sample was drawn aseptically and centrifuged to remove cell biomass and the supernatant was used for the titration. Supernatant was titrated against 0.005 N H₂SO₄ with phenolphthalein indicator to get colorless as the first end point and later with methyl orange indicator to get the pink color as the second end point. First end point corresponds to total alkalinity (TA) while the second end point corresponds to bicarbonate alkalinity. K_2 measures second dissociation constant of carbonic acid.

Calculations were made as follows:

$$\text{Bicarbonate alkalinity (CaCO}_3 \text{ mg L}^{-1}\text{)} = 50000 (2\text{TA} - 10^{-14+\text{pH}})/(1 + 2K_2 10^{\text{pH}})$$

$$\% \text{ Utilization of bicarbonates} = 100 (\text{Total HCO}_3^- - \text{Residual HCO}_3^-)/\text{Total HCO}_3^-$$

2.10. Small scale study

The small scale study was carried out for the maximum biomass production through chemo-photosynthetic approach. A 50 L rectangular glass fish tank was used for microalgal cultivation fed with growth medium prepared using **CO₂-captured** Ca(OH)₂ solution containing 2.67 mM CaO. CO₂ was trapped from the CO₂ cylinder (99.9 % purity) in sequentially arranged solutions of Ca(OH)₂ as shown in Fig. 1. The flow rate of CO₂ was maintained approximately at 100 mL min⁻¹ and continued until the pH decreased to 6.0 ± 0.1. Further, captured CO₂ in the form of bicarbonates (Section 2.5) was provided to the culture to convert it into biomass.

2.11. Biochemical analysis through FTIR spectroscopy

Fourier transform infrared (FTIR) spectral analysis was done to analyze the biochemical composition (lipids, proteins, amide, carbonate, phospholipids, nucleotides, chlorophyll pigments etc.) of algal cells exposed to various illumination conditions and grown with chemically trapped CO₂ (PerkinElmer Spectrum Version 10.03.06). At the end of experiment, 2 mg aliquot of dry algal biomass mixed with 98 mg of IR grade potassium bromide and KBr pellets containing dry algal biomass were prepared. FTIR spectra were recorded in absorbance mode (4000 to 400 cm⁻¹) with 100 scans per sample at the spatial resolution of 2 cm⁻¹.

2.12. Statistical analysis

Laboratory methods and all growth and biochemical analysis were carried out with three independent experiments. Results are expressed as mean values ± standard deviation. All the graphs were generated using KaleidaGraphTM version 3.5.

3. Results and Discussion

3.1. Molecular identification of strains

Two newly described photosynthetic strains were isolated from the alkaline lake of Lonar Crater Lake (19°58'36" N 76°30'30" E) and employed for this study. A green alga, *Chlorella sorokiniana* PAZ, was deposited at National repository for Cyanobacteria and Microgreen algae (Freshwater), DBT-ISBD, Imphal, Manipur, India with accession number BTA9030. For identification of taxonomic affiliation, the 16S rRNA and 18S rRNA genes, were amplified and sequenced for *Arthrospira* sp. VSJ and *Chlorella sorokiniana* PAZ, respectively, resulting sequence was submitted to NCBI with accession number (*Arthrospira* sp. KT779290 and *Chlorella sorokiniana* PAZ KT779289). BLAST analysis of sequence showed 98 % sequence similarity with *Arthrospira* sp. and 97 % sequence similarity with *Chlorella sorokiniana* strain HTA1-65. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.43018702 and 0.61902894 for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ, respectively (Fig. 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replications) are shown next to the branches (Felsenstein, 1985).). Higher value indicates confidence of the branching with its closest relative. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 147 and 731 positions for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ, respectively in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

3.2. Effect of pH

The sample enrichment and cultivation in broth and solid media resulted in the isolation of 30 cultures of photosynthetic microalgae. All isolated cultures were screened for their tolerance to pH of ranging from 8 to 12. *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ the two species out of 30 cultures tested which showed tolerance to the higher alkaline pH with a production of higher amount of pigments (data not shown). *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ showed tolerance to pH 10 with the maximum content of chlorophyll *a* as $51.22 \pm 0.14 \text{ mg L}^{-1}$ and $67.58 \pm 0.23 \text{ mg L}^{-1}$, respectively (Table 1). It can also be seen that the initial pH of the culture was changed during growth and it was found stabilized around pH 10 ± 0.3 . In order to thrive at high pH, culture possesses various mechanisms through which they maintain pH homeostasis. One of the mechanisms to maintain pH homeostasis is the increased expression and activity of monovalent cation/proton antiporters (Giraldez-Ruiz et al., 1997). These transmembrane proteins maintain the intracellular pH through the uptake of protons, utilizing outward monovalent cation gradients. Multiple cation/proton antiporters have been identified in cyanobacteria and their involvement in pH homeostasis was studied, this includes the Na^+/H^+ and K^+/H^+ antiporters, and H^+ pumps (Giraldez-Ruiz et al., 1997). Similarly, $\text{Ca}^{2+}/\text{H}^+$ antiporter located on plasma membrane of cell transports Ca^{2+} out (and H^+ in) to maintain pH homeostasis and an optimal Ca^{2+} concentration inside the cell (Yates and Robbins, 2001). At an alkaline pH, readily available form of inorganic carbon i.e. HCO_3^- present at the highest concentration (Basu et al., 2014). Algal cells utilize this form efficiently and ultimately increases the growth. Similar results were observed in the present study.

Microalgal cultures tolerating high alkaline pH can be advantageous for the development of CO_2 sequestration process, especially under open culture conditions. Higher alkaline pH

itself prevents the growth of undesirable microbes, particularly bacterial contaminants, in microalgal culture systems (Chi et al., 2011). Alkaline pH also favors stable sequestration of gaseous CO₂ and makes it available to the culture system. Maximum tolerance and optimum growth of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ at higher alkaline pH can be attributed to the positive feedback mechanism that makes maximum carbon accessible for the production of biomass i.e. at alkaline pH, solution absorbs more CO₂ from the environment and **makes** it available for culture growth (Talling and Talling, 1965). As CO₂ is absorbed into the medium it makes pH of medium acidic, **this increases the availability** of micronutrient to the culture.

3.3. Effect of calcium

Cultures were assessed for their tolerance capacity to calcium. The data is summarized in terms of the chlorophyll *a* content after 10 days of culturing (Table 2). It can be seen that the growth of both cultures was decreased at increased concentrations of calcium. *Chlorella sorokiniana* PAZ tolerated calcium concentrations up to 0.3 M, **whereas** the growth of *Arthrospira* sp. VSJ was inhibited at 0.1 M calcium. Chlorophyll *a* content at highest tolerating concentration for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ was $5.70 \pm 0.39 \text{ mg L}^{-1}$ and $9.73 \pm 0.38 \text{ mg L}^{-1}$, respectively (Table 2).

Carneiro et al. (2011) postulated the inhibitory effect of calcium on cyanobacteria *Cylindrospermopsis raciborskii* due to extra energetic demand required for Ca²⁺ efflux mechanism. High concentration of calcium in the medium causes the toxicity to the culture. Higher calcium carbonate alkalinity of Lonar lake might imply a unique characteristic of higher CO₂ mitigation through calcification. As calcification is well-recognized source of atmospheric CO₂ it increases the CO₂ uptake mechanism (Frankignoulle and Canon, 1994).

3.4. Effect of PAR + UVA radiation

The purpose behind illumination cultures with UVA radiation in combination with PAR is to enhance bicarbonate utilization. Solar spectrum contains UVA radiation, along with PAR, which drives the photosynthetic carbon fixation. Photosynthetic growth was not inhibited in either species by the addition of UVA radiation (Table 2), which is presumably due to the action of photoprotective and photorepair mechanisms (Mengelt and Prézelin, 2005). Indeed, the addition of UVA improved the carbon fixation process for both species at these moderate doses. Chlorophyll *a* content on 10th day of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ was $21.59 \pm 0.77 \text{ mg L}^{-1}$ and $31.49 \pm 0.84 \text{ mg L}^{-1}$, respectively. The increased growth of microalgal cultures upon PAR+UVA exposure in comparison with exposed to PAR alone can be attributed to the enhancement in photosynthetic carbon fixation and pigment production due to UVA exposure (Gao et al., 2009). UVA radiation increases the O₂ evolution rate in algae **likely** due to the presence of the absorption tail of chlorophyll *a* (Gao et al., 2009; Jeffrey et al., 1997).

3.5. Trapping of CO₂ and formation of bicarbonates

To determine the optimum concentration of alkali for efficient scrubbing of gaseous CO₂ and formation of bicarbonates, various concentrations of calcium oxide were tested. Fig. 3 shows the growth performance of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ when cultivated using the medium enriched with bicarbonates formed after trapping of gaseous CO₂ in calcium hydroxide alkali. Fig. 3 also shows the change in pH of culture with simultaneous utilization of bicarbonates, by *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ. CO₂ gassing was continued in the alkaline solutions till the pH decreased to 6.0 ± 0.1 , thus the initial pH in all culture flasks was the same. However, the amounts of bicarbonates formed found to be dependent on the concentration of calcium hydroxide used in the

medium. Highest bicarbonate formation in the range of 850 - 960 mg L⁻¹ was obtained at 3.57 mM concentration of calcium oxide. Growth of both cultures was found to be higher with increasing initial concentrations of bicarbonates in the medium. It can be noticed that the highest biomass was obtained in the medium containing 2.67 mM concentration of calcium oxide. The highest dry weight obtained for *Chlorella sorokiniana* PAZ was 0.52 ± 0.023 g L⁻¹ on the 6th day of incubation; while in case of *Arthrospira* sp. VSJ it was 1.27 ± 0.012 g L⁻¹ on the 10th day, both at 2.67 mM CaO. Growth of each alkaliphilic photosynthetic organism resulted in the rise of pH of the medium, which reached pH 10.3 on 6th and 10th days of culturing of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ, respectively. Highest productivity (as increment in dry weight) was 47.17 ± 0.74 mg L⁻¹ day⁻¹ was obtained for *Chlorella sorokiniana* PAZ when grown in the medium flushed with CO₂ in the presence of 2.67 mM CaO, which was 3-fold higher as compared to the control medium lacking calcium. Highest specific growth rates were 0.56 ± 0.01 day⁻¹ and 0.11 ± 0.00 day⁻¹ for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ, respectively and were obtained when each strain was grown at 2.67 mM CaO (Table 3).

In this method, carbon dioxide is captured in the form of bicarbonate using calcium oxide. The following reactions take place:

Calcium oxide on dissolution produces the solution of calcium hydroxide, which can be the effective absorbing material for CO₂. It can be understood from the above equations that, when CO₂ is flushed into calcium hydroxide medium (3), it first forms calcium carbonate precipitate (4) and, when further flushed with excess CO₂, colorless liquid in the form of calcium bicarbonate is formed (5). So aqueous solution contains calcium, bicarbonate and

carbonate ions together with dissolved carbon dioxide. The reaction of CO₂ with calcium hydroxide boosts the absorption of CO₂ into the medium and also enhances the ability of the liquid solution to dissolve more solute molecule (Velts et al., 2011). No other external carbon source was added to the medium. This whole solution can be used as the medium for algae. Algal cultures can efficiently utilize this solution rich in bicarbonate content and enhance the carbon uptake and thus leads to the overall biomass production. In dark conditions pH usually tends to drop as result of the respiration activity (Touloupakis et al., 2016). But this drop was not sudden and next day culture again utilizes bicarbonate. A stable bicarbonate water solution can be stored during night without CO₂ loss back to the environment. Results for the productivity and specific growth rate are almost the same as in Aishvarya et al. (2012) i.e. $47.17 \pm 0.74 \text{ mg L}^{-1} \text{ day}^{-1}$ for *Chlorella sorokiniana* PAZ, while for *Arthrospira* sp. VSJ it is 9-fold more as compared to control conditions i.e. $116.4 \pm 0.62 \text{ mg L}^{-1} \text{ day}^{-1}$.

3.6. Utilization of bicarbonates formed in calcium hydroxide solution

Trapping of CO₂ in alkaline solution results in the formation of soluble bicarbonates. The increase in the bicarbonate content was found associated with increased concentrations of Ca(OH)₂ as the source of alkalinity. Maximum bicarbonate ($957.57 \pm 0.25 \text{ mg L}^{-1}$) was formed in the solution containing 3.57 mM CaO (Table 3). Growth of cultures in normal BG-11 and Zarrouk's medium resulted in $58.96 \pm 17.02 \text{ mg L}^{-1}$ ($77.60 \pm 0.89 \%$) and $2253.55 \pm 17.42 \text{ mg L}^{-1}$ ($49.34 \pm 0.65 \%$) utilization of bicarbonate, respectively. However, the maximum utilization of bicarbonate percentage was found in CO₂ trapped medium with 2.67 mM CaO. This was about $766.74 \pm 19.32 \text{ mg L}^{-1}$ ($97.30 \pm 0.21 \%$) and $737.78 \pm 18.76 \text{ mg L}^{-1}$ ($92.37 \pm 0.21 \%$) for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ, respectively, in the medium prepared by trapping CO₂ in alkali with 2.67 mM CaO (Fig.3). The culture

performance also compared with known culture *Chlorella pyrenoidosa* NCIM 2734 and data are presented in Fig. S1 (Supplementary material).

At an alkaline pH, $\text{Ca}^{2+}/\text{H}^{2+}$ antiporter transports Ca^{2+} out of the cell. Export of Ca^{2+} enhances the concentration of Ca^{2+} outside the cell and precipitate CaCO_3 (calcification) (Jansson and Northen, 2010). Cyanobacteria and microalgae have ability to transport HCO_3^- inside the cell through transporters present on the cell membrane (Price, 2011). Carbonic anhydrase enzyme present on the cell membrane enhance the local levels of HCO_3^- and CO_3^{2-} from incoming CO_2 or CO_2 that is leaked out of cytosol (Price et al., 2002). Cyanobacteria accumulate HCO_3^- inside the cell, where the carboxysomes enzyme carbonic anhydrase (CA) convert HCO_3^- to CO_2 near the site of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) (Velea et al., 2009), while, in microalgae, carbonic anhydrase (CA) converts HCO_3^- to CO_2 inside the pyrenoid body. Decreased bicarbonates from the medium correspond to the accumulation of inorganic carbon inside the cell and its subsequent conversion to organic carbon through the process of photosynthesis.

3.7. Comparison of calcium hydroxide with other trapping materials

3.7.1. Productivity of cultures in various trapping materials

To determine a suitable medium for maximizing absorption of CO_2 and conversion into biomass, the solutions containing 2.67 mM CaO , 10 mM NaOH (both flushed with CO_2) and 10 mM NaHCO_3 (inorganic source of carbon) were compared. The comparative data of three systems are presented in Fig. 4. For *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ maximum change in pH and dry weight was observed at the condition of 2.67 mM CaO , while, the lowest change was observed at 10 mM of NaHCO_3 . Maximum dry weight for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ was $0.52 \pm 0.01 \text{ g L}^{-1}$ and $1.55 \pm 0.01 \text{ g L}^{-1}$ respectively at 2.67 mM of CaO .

Productivity for *Chlorella sorokiniana* PAZ in 2.67 mM CaO was similar to that in 10 mM NaOH. In contrast, productivity of cells in medium 10 mM NaHCO₃ was 1.72 times lower than other trapping media (Table 3). For *Arthrospira* sp. VSJ productivity in medium with 2.67 mM CaO was 1.18 times high as compared to 10 mM NaOH medium. In fact, for *Arthrospira* sp. VSJ productivity in 10 mM NaHCO₃ was 1.55 times lower than in 2.67 mM CaO.(Table 3).

Ca(OH)₂, NaOH (both flushed with CO₂) and NaHCO₃ were used as a source of carbon for growing an algal culture. When NaOH and CaO were used as a trapping material, a system contains OH⁻. These OH⁻ reacts with CO₂ to form HCO₃⁻. While NaHCO₃ is used as standard carbon source for culture. Hence, at all conditions HCO₃⁻ is the only primary source of carbon available to the culture. An increased bicarbonate formation and its utilization resulted in higher growth rates of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ when calcium hydroxide was used as the CO₂ absorbing solution, which validates earlier reports on CO₂ sequestration. However, use of these carbon sources by algal culture is strain-dependent and its habitat from where it was isolated. As per earlier reports (Lam and Lee, 2013; Murray et al., 2012), when the air is supplemented with different concentrations of CO₂ generates almost two times higher biomass compared with NaHCO₃ as a carbon source, these results are comparable with the results of experiments conducted in this study. While using air enriched with CO₂ as a carbon source when the concentration of CO₂ increased, most of the CO₂ gets released into the earth's atmosphere due to its lower dissolution capacity in water. In the present study, the growth of both cultures *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ in media 2.67 mM CaO and 10 mM NaOH was almost double than 10 mM NaHCO₃ containing medium. Higher biomass in both media reflects more dissolution of CO₂ and its higher availability to the cultures. Xie and Wu, (2013) reported that the dissolved limestone enhances the microalgal growth whereas microalgal growth can also promote limestone

dissolution. They also observed that, the use of limestone increased productivity, which was nine times higher than that of the medium without limestone. Dissolved CaCO_3 could promote the change of the aqueous bicarbonate–carbonate system. As a result, the continuous increase in algal biomass was found in medium with CaCO_3 (Zhao et al., 2016). The advantage of the use of calcium oxide over NaOH as trapping material is its ready availability in the form of effluent from paper and pulp industry. Although concentrations used of calcium oxide (2.67 mM) is much less in this study as compared to NaOH but still it gives a yield similar to that of NaOH and hence the use of calcium oxide as trapping material will be more economical for the carbon sequestration approach.

3.7.2. Utilization of bicarbonates formed in various trapping materials

Maximum bicarbonate was formed at 10 mM NaOH i.e. $806.76 \pm 6.28 \text{ mg L}^{-1}$ but utilization of formed bicarbonate is $722.51 \pm 26.86 \text{ mg L}^{-1}$ ($85.55 \pm 0.63 \%$) by *Chlorella sorokiniana* PAZ. In 2.67 mM CaO medium $788.00 \pm 6.83 \text{ mg L}^{-1}$ of bicarbonate ions were formed and about $766.74 \pm 19.32 \text{ mg L}^{-1}$ ($97.3 \pm 0.36 \%$) were utilized, i.e. the rate of bicarbonate utilization was higher in the system containing 2.67 mM CaO (Table 3). For *Arthrospira* sp. VSJ, it was found that bicarbonate utilization was higher in the system containing 2.67 mM CaO i.e. $737.78 \pm 18.76 \text{ mg L}^{-1}$ ($92.37 \pm 0.36 \%$) as compared to 10 mM NaOH i.e. $801.93 \pm 16.50 \text{ mg L}^{-1}$ ($88.34 \pm 0.34 \%$) (Table 3).

Interestingly, critical concentration of CaO found in these experiments showed comparable results with Aishvarya et al. (2012). The concentration used by them was 10 mM NaOH and concentration used in this study was 2.67 mM CaO, which was far less than the NaOH concentration, still it exhibited almost similar specific growth rate and productivity. Based on this, NaOH source could be replaced with CaO, which is cheaply available from paper industry.

3.8. Effect of UVA on algal growth and bicarbonate utilization

3.8.1. Productivity of cultures under PAR and PAR+UVA

To determine the combine effect of PAR+UVA radiation on bicarbonate utilization, the culture was exposed to PAR+UVA. Initial studies as described above (Section 3.3) indicated that supplementation of standard laboratory illumination with UVA did not inhibit growth, but actually resulted in modest increases in growth that are likely attributed to an enhancement of PAR due to spectral overlap by the UVA light source. Because the experimental system employs the exposure of photosynthetic cells to relatively high concentrations of calcium, we sought to extend these tests by exploring the impact of UVA under these high calcium conditions. So from initial experiments 2.67 mM CaO was only used for this study. It was observed that *Chlorella sorokiniana* PAZ exposed to PAR+UVA showed increased dry weight (Fig. 5b) and bicarbonate utilization (Fig. 5c) as compared to *Chlorella sorokiniana* PAZ exposed to PAR only. This might be due to the increased ability of culture to utilize HCO_3^- . The pH of the culture medium also increased with the increase in growth (Fig. 5a). Similar results were found with *Arthrospira* sp. VSJ exposed to PAR+UVA radiation (Fig. 5d,e,f).

For *Chlorella sorokiniana* PAZ specific growth rate and productivity were 1.2 and 1.3 times, respectively, higher than same cultures exposed to PAR radiation only (Table 3). While for *Arthrospira* sp. VSJ specific growth rate and productivity were 1.3 and 1.8 times, respectively, higher than PAR exposed cultures (Table 3). Productivity for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ grown under PAR+UVA was $72.28 \pm 0.29 \text{ mg L}^{-1} \text{ d}^{-1}$ and $157.84 \pm 0.72 \text{ mg L}^{-1} \text{ d}^{-1}$, respectively. Thus, it concludes that the potential of UVA light supplementation shows a modest increase in growth rates that, again, is likely to be to spectral overlap of the UVA source with the absorption bands of the photosynthetically active pigments of the test strains.

3.8.2. Utilization of bicarbonate under PAR and PAR+UVA

Increased biomass production is due to efficient utilization of bicarbonate ions present in the medium. Bicarbonate utilization in *Chlorella sorokiniana* PAZ was slightly higher in PAR+UVA exposed culture than the only PAR exposed culture. Cultures of *Arthrospira* sp. VSJ showed about 98 % utilization of bicarbonate in PAR+UVA exposed culture as compared to PAR-only exposed culture, which showed only 92 % of bicarbonate utilization. Fig.5c and Fig.5f gives a representation of the difference in bicarbonate utilization in *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ, respectively. Overall, the results show that utilization of bicarbonates in medium exposed to PAR+UVA was higher i.e. around 98 % for both the cultures. Bicarbonate produced in the system was used rapidly by *Chlorella sorokiniana* PAZ with concentration dropping precipitously below 300 mg L⁻¹ within the first day followed by a more gradual decline during the ensuing days. Within 6 days of cultivation, it was observed that all bicarbonates present in the medium were depleted. The media of *Arthrospira* sp. VSJ also showed a large early decline, but after two days lag. Because the increase in biomass does not follow the rapid uptake, it appears that these strains are capable of rapid internalization and storage of inorganic carbon. Further physiological studies would be required to evaluate this interesting phenomenon.

3.9. Small scale study

Based on the observations of the laboratory study, the CO₂ mitigation was checked in 5 L of trapping medium. CO₂ from the cylinder (99.99 %) was captured in a solution of 2.67 mM CaO in a sequential manner. Use of sequential bioreactor for trapping CO₂ avoids its loss back to the environment (Lam and Lee, 2013). The capacity of each 2.67 mM CaO container was 5 L. CO₂ was flushed through the sequential bioreactor until its pH dropped to the 6.0 ± 0.1. Part of CO₂, which is not trapped in the first container, was passed to a second container

of 2.67 mM CaO and so on. This process increased the contact time between the dissolution of CO₂ in the limestone water and thus avoided its loss back to the environment. Later this captured form of CO₂ was used for the cultivation of algae in a fish tank. When algae grew, they started using bicarbonates in the medium and producing biomass. After harvesting culture, same medium was used again for capturing another stream of CO₂ with or without addition of extra calcium hydroxide solution. This practice not only helped to reuse water but also improved the biomass yield. Lam and Lee (2013) reported that the use of recycled water for subsequent cultivation improves biomass productivity and CO₂ removal efficiency 8 times higher than in original cultivation. The reason behind this improved yield was the increased adaptability of culture to the media conditions and once fresh nutrient source was added cells reproduced readily avoiding lag phase.

For *Chlorella sorokiniana* PAZ cultured in 2.67 mM CaO and exposed to PAR obtained 0.37 ± 0.01 g of biomass per g of HCO₃⁻ utilized, while it was 0.56 ± 0.01 g of biomass per g of HCO₃⁻ utilized under PAR+UVA exposure. In case of *Arthrospira* sp. VSJ, growth yield of 1.57 ± 0.02 and 2.01 ± 0.03 g g⁻¹ HCO₃⁻ was obtained when illuminated with PAR and PAR+UVA, respectively.

This study has not experimentally considered nor discussed the utilization of the resultant biomass, it only speculates that this strategy may be employed in a range of practices from the adaptation of traditional practices of utilizing algae as fertilizer to support of algal cultures producing high-value commodities.

3.10. Biochemical analysis through FTIR spectroscopy

FTIR spectra of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ cultivated in medium containing 2.67 mM CaO and exposed to PAR and PAR+UVA are depicted in Fig. S2 (Supplementary material). There was no significant change in the biochemical

composition of culture when it was exposed to UVA, but increased absorption supports the higher growth and accumulation of metabolites in the organism. Change in integrated peak intensities of FTIR bands enlisted in Table S1 (Supplementary material).

Six distinct absorption peaks are seen for *Chlorella sorokiniana* PAZ. Absorption peak at $\sim 2926\text{ cm}^{-1}$ represents CH_2 stretching of lipids and proteins (Kim and Jeune, 2009) and $\sim 1650\text{ cm}^{-1}$ can be assigned to amide I group of proteins (Stehfest et al., 2005). The peak at $\sim 1424\text{ cm}^{-1}$ corresponds to $\text{C}=\text{O}$ stretching of carbonate ions. This indicates that HCO_3^- might be accumulated and stored in the chloroplast stroma (Basu et al., 2013; Ponnuswamy et al., 2013). The peak at ~ 1053 is a peak in the range of 1,020-1,085 and can be assigned to $\text{P}=\text{O}$ group of phospholipids, DNA and RNA (Kim and Jeune, 2009). The peaks at ~ 874 and $\sim 712\text{ cm}^{-1}$ are the characteristic peaks of stable calcite (Reig et al., 2002).

In case of *Arthrospira* sp. VSJ, the number of distinct peaks suggested that PAR+UVA exposure resulted in a synthesis of several metabolites. The absorption peak at $\sim 2928\text{ cm}^{-1}$ represents CH_2 stretching of lipids and proteins. The peak at ~ 1656 and $\sim 1538\text{ cm}^{-1}$ can be assigned to amide I and II of proteins, respectively (Kim and Jeune, 2009; Mahapatra et al., 2013; Ponnuswamy et al., 2013; Stehfest et al., 2005). Peak at ~ 1384 and $\sim 1040\text{ cm}^{-1}$ can be assigned to CH_2 and CH_3 bending of methyl and $(\text{C}-\text{O})$ stretching of COO^- and carbohydrate $(\text{C}-\text{O}-\text{C})$ of polysaccharides (Mahapatra et al., 2013; Ponnuswamy et al., 2013). While, peaks at ~ 1270 and ~ 838 represents $\text{C}-\text{O}-\text{H}$ group of carbohydrates, protein, DNA, RNA and $\text{C}=\text{N}$ (ring) of nucleotides, chlorophyll pigments respectively (Kim and Jeune, 2009).

4. Conclusion

A practical method of CO_2 sequestration that relies upon absorption of carbon dioxide into calcium hydroxide followed by conversion to biomass using photosynthetic organisms has been tested. These studies illustrate how locally available strains of cyanobacteria and

eukaryotic algae may be suitable for the incorporation into CO₂ mitigation strategies that could prove practical without huge capital investments. PAR radiation along with UVA radiation improves the CO₂ mitigation. In future, this study can lead to the path for possible use of calcium hydroxide from paper industry waste as a low cost and readily available source.

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Table 1

Chlorophyll *a* content for *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ and associated pH changes

pH of medium	<i>Chlorella sorokiniana</i> PAZ		<i>Arthrospira</i> sp. VSJ	
	Chlorophyll <i>a</i> , mg L ⁻¹	Final pH of medium	Chlorophyll <i>a</i> , mg L ⁻¹	Final pH of medium
7	35.97 ± 0.41	9.5 ± 0.10	39.14 ± 0.46	9.1 ± 0.04
8	20.09 ± 0.38	8.7 ± 0.13	40.53 ± 0.18	9.2 ± 0.14
9	47.43 ± 0.14	10.3 ± 0.17	55.12 ± 0.36	9.6 ± 0.05
10	51.22 ± 0.14	10.2 ± 0.9	67.58 ± 0.23	10.3 ± 0.15
11	46.11 ± 0.26	10.2 ± 0.22	49.75 ± 0.15	10.5 ± 0.17
12	30.14 ± 0.33	10.5 ± 0.06	32.45 ± 0.40	11.2 ± 0.31

Values are mean ± SD, n = 3 per treatment group.

738 **Table 2**
739 **Effect of calcium and UVA radiation on Chlorophyll *a* content of *Arthrospira* sp. VSJ and**
740 ***Chlorella sorokiniana* PAZ**

	Chlorophyll <i>a</i> (mg L ⁻¹)	
	<i>Chlorella sorokiniana</i> PAZ	<i>Arthrospira</i> sp. VSJ
Control	16.54 ± 1.00	18.98 ± 0.77
Ca, 0.1 M as CaCl ₂	12.44 ± 0.87	9.73 ± 0.38
Ca, 0.2 M as CaCl ₂	8.34 ± 0.50	ND
Ca, 0.3 M as CaCl ₂	5.70 ± 0.39	ND
PAR+UVA (5 days)	15.55 ± 0.50	22.68 ± 0.41
PAR+UVA (10 days)	21.59 ± 0.77	31.49 ± 0.84

741 ND, Not Detectable

742 Values are mean ± SD, n = 3 per treatment group.

743

744 **Table 3**

745 Specific growth rate, productivity and bicarbonate utilization rate of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ grown under different
746 culture conditions

Culture condition	Specific growth rate ($\mu \text{ d}^{-1}$)		Productivity ($\text{mg L}^{-1} \text{ d}^{-1}$)		Bicarbonate utilization (mg L^{-1})	
	<i>Chlorella sorokiniana</i> PAZ	<i>Arthrospira</i> sp. VSJ	<i>Chlorella sorokiniana</i> PAZ	<i>Arthrospira</i> sp. VSJ	<i>Chlorella sorokiniana</i> PAZ	<i>Arthrospira</i> sp. VSJ
Control	0.18 ± 0.00	0.04 ± 0.00	12.89 ± 0.11	12.09 ± 0.18	146.82 ± 13.50	160.90 ± 14.51
Normal medium	0.09 ± 0.00	0.10 ± 0.01	9.89 ± 0.06	101.00 ± 0.45	58.96 ± 17.02	2253.55 ± 17.42
CaO, 2.67 mM	0.56 ± 0.01	0.11 ± 0.00	47.17 ± 0.74	116.4 ± 0.62	766.74 ± 19.32	737.78 ± 18.76
CaO, 3.57 mM	0.53 ± 0.01	0.10 ± 0.01	44.11 ± 0.13	103.45 ± 0.44	912.21 ± 16.73	777.79 ± 17.85
NaOH, 10 mM	0.55 ± 0.01	0.10 ± 0.01	46.50 ± 0.28	97.98 ± 0.22	722.51 ± 26.86	801.93 ± 16.50
NaHCO ₃ , 10 mM	0.31 ± 0.01	0.08 ± 0.00	26.99 ± 0.62	49.25 ± 0.32	416.70 ± 22.27	726.15 ± 24.14
CaO, 2.67 mM	0.72 ± 0.00	0.12 ± 0.00	72.28 ± 0.29	157.84 ± 0.72	778.33 ± 17.24	783.26 ± 20.44
PAR+UVA exposure						

747 Values are mean $\pm$ SD, n = 3 per treatment group.
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Legends to the figures:

Fig. 1. Scale up system for CO₂ mitigation.

Fig. 2. Evolutionary relationships of taxa.

Fig. 3. Growth of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ utilizing bicarbonates formed on trapping of CO₂ in presence of varying concentrations of calcium; (a) pH of the culture (b) dry weight of culture (c) bicarbonate utilization profile. All data represent means $\pm$ SD from three independent experiments.

Fig. 4. Growth of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ utilizing bicarbonates formed on CO₂ trapping in the presence of calcium hydroxide and sodium hydroxide; (a) pH of the culture (b) dry weight of culture (c) bicarbonate utilization profile. All data represent means $\pm$ SD from three independent experiments.

Fig. 5. Effect of UVA radiation on growth performance of *Chlorella sorokiniana* PAZ and *Arthrospira* sp. VSJ; (a) pH of the culture (b) dry weight of culture (c) bicarbonate utilization profile. All data represent means $\pm$ SD from three independent experiments.

Fig. 1.

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Fig. 2.

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781 **Fig. 4.**

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Diesel generator exhaust as an example of flue gases point source

Sequential trapping of CO₂ in limestone

CO₂ trapped in Ca(OH)₂ water to form bicarbonates

Algal culture grown in bicarbonate rich water