DETERMINATION OF CARBONIC ANHYDRASE (CA) FROM SELECTED THREE MICROALGAL SPECIES ISOLATED FROM THERMAL POWER STATIONS IN INDIA.

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Abstract

Predominant microalgae, *Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus* were isolated from three selected Thermal power stations namely, Vallur Thermal power station (VT), Tamil Nadu, Kolaghat Thermal power station (KoT), West Bengal and Khapergada Thermal power station (KhP), Maharashtra, India. These strains were cultured in BBM medium. Gene specific primer for carbonic anhydrase gene in the genomic DNA of the three selected microalgae (*Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus*) were used and were also expressed in all the three organisms studied and which further confirms the presence of Carbonic anhydrase gene and the ability of those organisms to produce this particular enzyme which catalyzes the reversible conversion of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$) with very high efficiency. Amount of Carbonic anhydrase enzyme produced in media with and without 0.1M $NaHCO_3$ were analyzed. The enzyme was extracted from the harvested biomass of *Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus*. The molecular weight of the purified carbonic anhydrase was reported as 37 kDa and there were a band formations occurred at that particular range for all the three chosen microalgae.

Key Words: Microalgae, SDS-PAGE, CA Gene, Carbonic anhydrase

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Introduction

CO_2 concentration Mechanisms (CCM)

Photosynthetic organisms have developed special mechanisms for acclimating and adapting to changes in both CO_2 and O_2 concentrations, such mechanisms are called CO_2-concentrating mechanisms (CCM). During the low availability of CO_2 conditions *Chlamydomonas reinhardtii* and other microalgae can adapt to changes due to the induction of CCM. Algae have evolved carbon concentrating mechanisms (CCMs) during the imbalance between the high demand of inorganic carbon and low ambient CO_2

concentration. There are three major constituents of CCM namely, i) Active bicarbonate (HCO^-_3) uptake transporters, ii) Suite of Carbonic anhydrases (CAs) localized strategically within the cells, iii) Sub-cellular micro-compartment within which most RuBisCO is located (*i.e*., the pyrenoids within the chloroplasts) (Meyer and Griffiths 2013). The functions of this enzyme to take up inorganic carbon from the external environment into the cells and to elevate the CO_2 level around Rubisco.

Carbonic anhydrase (CA)

Carbonic anhydrase is an enzyme which catalyses the hydration and dehydration of CO_2 and plays a critical role in the physiology. The Carbonic anhydrases (CAs) are zinc-containing metallo-enzyme which is responsible to catalyze the reversible conversion of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$) with very high efficiency. Various one or more CA activity has been reported inside and outside of the cell in depending upon the microalgal strains. Many unicellular microalgae consist of abundant external CA and increase the replacement of high CO_2 from bicarbonate. The enzyme Carbonic anhydrase are found in animal cells, plant cells, algal cells, archaebacteria and eubacteria. They are isozymes and categorized into six families such as α, β, γ, δ, ε, and ζ respectively. The α type of CAs are widely identified in algae (Fujiwara et al., 1996), higher plants (Arabidopsis Genome Initiative, 2000), vertebrates (Meldrum and Roughton, 1933), and eubacteria (Chirica et al., 2001).

Materials and Methods

The isolated pure microalgal strains *Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus* from the selected three thermal power stations in India such as 1) Vallur Thermal power station (VT), Tamil Nadu, 2) Kolaghat Thermal power station (KoT), West Bengal and 3) Khapergada Thermal power station (KhP), Maharashtra. These strains were subjected to culture in BBM medium control and $NaHCO_3$ concentration for the identification of Carbonic anhydrase enzyme.

Determination of the carbonic anhydrase gene from selected microalgae

About 200µl of the microalgal samples were allowed to ground to fine powder with liquid nitrogen using a mortar and pestle. Exactly 2 ml of the solution-I was then added and grounded by complete homogenization to complete denaturation of protein. The whole content was subjected to thaw completely along with continuous grinding. Nuclease enzyme free water (800 µl) was added while grounding the mixture and transferred to 2 ml microcentrifuge tubes and kept undisturbed for 5 min. at room temperature. To the solution, 200 µl of chloroform was mixed and vortexed for some 5 to 10 sec. and kept at room temperature without disturbing. The solution was then allowed to centrifuge at 13,000 rpm for 10 min. at 4°C, the obtained upper phase was transferred into fresh 2 ml micro centrifuge tubes. About 0.6 volumes of isopropanol was served to it and vortexed briefly to 5 to 10 sec. Then the content was centrifuged after incubation for 10 min. at 13,000 rpm for 10 min. under 4°C, the obtained supernatant was discarded. The deposited pellet containing DNA was washed with 70% of ethanol; air dried and diluted with 50 µl of DEPC- treated water. The extracted and isolated DNA was then separated and analyzed using 0.8% Agarose gel using submarine agarose gel electrophoresis.

The isolated DNA was then amplified along with designed forward and reverse primer for carbonic anhydrase gene. About 20 µl of the total volume of the PCR reaction mixture constitutes 10 µl of master mixture, 1 µl of 1µM each of carbonic anhydrase forward and reverse primers CA-FP (5′ GAATGTGGTGCTGCGTGCTA 3′) and CA-RP (5′ GGCGTGTGGAAGTGGAACT3′) and 100 ng of template DNA. The PCR conditions were as follows: denaturation at 94°C for 5 min; 35 cycles of 94°C for 1 min, primer-specific annealing temperature at

49.7°C for 45 sec and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were then separated and resolved by using 1.2% agarose gel with 1X TAE buffer. The resulting gel was then pre-stained with 10 mg/ml of ethidium bromide.

Determination of the Carbonic anhydrase enzyme based on the molecular weight

The crude protein extract isolated from both the control and 0.1 M $NaHCO_3$ treated samples of the selected three microalgae *Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus* were separated in SDS-PAGE and the gel apparently demonstrating the different protein bands dragged based on the molecular weight.

Extraction of protein

About 15 ml of microalgal samples were centrifuged at 8000 rpm for 10 min. under 5°C the pellet was resuspended in PBS with 2 % triton – X (10 ml) and again centrifuged at 8000 rpm for 10 min. The greenish yellow supernatant was discarded and the pellet was again resuspended in PBS and centrifuged at 8000 rpm for 10 min. The pellet was then resuspended with extraction buffer (3.75 ml) with 1 aliquot of protease inhibitor cocktail and 10 ml of buffer. About 2 ml of acid washed glass beads was added and vortexed for 30 sec. and placed on ice for 30 sec. and procedure was repeated after 30 min. The whole suspension was the sonicated on ice bath for 5 min. and again vortexed for 5 min. the temperature was maintained below 5°C. Then revolved at 10,000 rpm for 5 min. the pellet was discarded and the obtained supernatant was conserved.

Two volumes of 10 % of TCA and two volumes of ice cold acetone containing 0.07 % DTT was mixed and incubated for overnight at -20°C. The whole content was then allowed to centrifuge at 10,000 rpm for 20 min. below 4°C the obtained pellet was conserved and the supernatant was discarded. About two volumes of 80 % of acetone and two volumes of ice cold acetone containing 0.07 % of DTT was added and kept undisturbed at -20°C for 30 min. Centrifuged at 10,000 rpm for 10 min. below 5°C and the whole procedure was repeated for three times until a blue green tinch of chlorophyll and other pigment was removed. At last, the obtained pellet was stored at -80°C and the extracted protein samples were allowed to separate using SDS-PAGE (Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis.

SDS-PAGE separation and determination of carbonic anhydrase enzyme

The two glass plates were spaced using 1 mm thickness spacers at all the margins except the top and sealed with the help of molten agar. Two iron clips were used to hold the plates without disturbing the seal. Then the seal was ensured with glass distilled water to check for any leakage. About 30 ml of 12%resolving gel was prepared which constitutes 30% acrylamide and bis-acrylamide mix, glass distilled water, 1.5 M Tris HCl (pH – 8.8),10% SDS, 10% APS and TEMED. The whole preparation was carefully allowed to pour in between the sealed glass plates. Intense care was taken to avoid the formation of air bubbles and kept undisturbed for 3 to 5 min. to polymerize the gel. The glass distilled water was added on top of the surface to avoid oxidation with the contact to air.

Then the 5% of stacking gel was prepared and the composition includes 30% acrylamide and bis-acrylamide mix, glass distilled water, 10 M Tris HCl (pH – 6.8), 10% SDS, 10% APS and TEMED. The remaining water present on the surface of the resolving gel was removed with the help of the Whatman No.1 filter paper. Then the 5% stacking gel was poured over the top of the resolving gel and the 1 mm thick comb was used to form wells on the gel and the whole set up was undisturbed for 5 min. The spacer placed at the bottom of the gel was removed before placing the gel plate to the unit. The comb was removed cautiously

without disturbing the well's after casting and glass distilled water was poured to all the wells to avoid oxidation. After removing water from all the wells, protein ladder (5 µl) and samples (20 µl) were slowly loaded to the wells using 10 µl micro-pipette. Tris-Glycine (tank buffer) buffer was then subjected to pour to each of the tank and the cathode and anode electrodes were fixed to their respective slots of their respective tanks. Initially 100 V of electric current was fixed with the help of the power pack and gradually increased to 120 V for the separation of protein. After the incubation period of 5 h. for the separation of the protein, the gel was carefully taken out from the glass plates and washed twice with Milli-Q water.

Silver staining of gel

The obtained gel was treated with the Hypo solution for 1 min. and again washed twice with Milli-Q water. Silver nitrate solution was added to the gel in a tray and kept in a gel shaker for 20 min. in dark to prevent oxidation. After incubation with silver nitrate the gel was washed with developers until the protein bands were obviously visible. The developers were drained followed by the addition of stop solution and stored. The gel image with blatant protein bands were clearly photographed and recorded.

Results and Discussion

Carbon dioxide (CO_2) emissions from electricity generation totaled 1,925 million metric tons in 2015, the lowest since 1993 and 21% below their 2005 level. A shift on the electricity generation mix, with generation from natural gas and renewables displacing coal-fired power, drove the reductions in emissions. Though there was a slight reduction, still the thermal power stations are the important source of emission of carbon dioxide. Rankings of the 4,000 electric power companies in the world shows the biggest carbon polluters, globally, nationally, and at sub-national levels. Power generation accounts for about one-quarter of total emissions of CO_2, the main culprit in global warming. But, until now, people concerned about climate change lacked information about the emissions of particular power plants and the identities of the companies that own them. Alarming issues associated with this high emission of carbon dioxide is urging the environmentalists to find an ecofriendly and sustainable solution for these problems. We have isolated microalgae from the thermal power stations 'effluent and found out predominant microalgae and further which was used to determine whether the algae possess the gene to produce carbonic anhydrase enzyme because which was found to be more effective in carbon sequestration mechanisms.

Determination of the carbonic anhydrase gene from selected microalgae

One of the more attractive features of algal biomass production is the potential to trap gaseous CO_2 generated from point sources in ponds as bicarbonate. Cyanobacteria and eukaryotic algae transport and use bicarbonate as a source of carbon dioxide (Spalding 2008, Jansson and Northen 2010). At pHs between 6.4 and 10.3, the dominant (> 50%) chemical species of CO_2 in water is bicarbonate, a nongaseous form of CO_2. This transiently captured carbon is pumped into algal cells by bicarbonate transporters present in both the plasma membrane and in the chloroplast envelope of eukaryotic algae (reviewed in Spalding 2008). Inside the chloroplast, bicarbonate is converted into CO_2 that can be fixed by rubisco (ribulose bisphosphate carboxylase oxygenase) to produce two molecules of 3-phosphoglycerate. Through a series of reactions these three carbon organic acids are reduced to the sugars that are substrates for starch and oil production. However, oxygen can compete with CO_2 for fixation by rubisco. The products of the oxygenase reaction are 3-phosphoglycerate and 2-phosphoglycolate. The phosphoglycolate is subsequently metabolized to glycine, which, when condensed with another glycine molecule to produce serine, results in the loss of CO_2. This carbon loss (one carbon per two molecules of glycine) diminishes the ability of the Calvin cycle to regenerate the five-carbon sugar substrate ribulose bisphosphate—required for CO_2 fixation by rubisco—further reducing the efficiency of photosynthesis. This overall process is known as

photorespiration because it occurs largely in the presence of light. The process of photorespiration reduces photosynthetic carbon fixation efficiency by 20% to 30% (Zhu *et al*. 2008). To reduce the competitive inhibition of oxygen on carbon fixation by rubisco, algae actively pump sufficient bicarbonate into cells to elevate internal CO_2 concentrations to levels above those achievable by equilibrium with air, and competitively inhibit photorespiration (Badger and Price 1994).Photosynthetic organisms have developed special mechanisms for acclimating and adapting to changes in both CO_2 and O_2 concentrations, such mechanisms are called CO_2-concentrating mechanisms (CCM). During the low availability of CO_2 conditions *Chlamydomonas reinhardtii* and other microalgae can adapt to changes due to the induction of CCM. Algae have evolved carbon concentrating mechanisms (CCMs) during the imbalance between the high demand of inorganic carbon and low ambient CO_2 concentration. There are three major constituents of CCM namely, i) Active bicarbonate (HCO^-_3) uptake transporters, ii) Suite of Carbonic anhydrases (CAs) localized strategically within the cells, iii) Sub-cellular micro-compartment within which most RuBisCO is located (*i.e*., the pyrenoids within the chloroplasts) (Meyer and Griffiths 2013). The functions of this enzyme to take up inorganic carbon from the external environment into the cells and to elevate the CO_2 level around Rubisco. The most studied component of the CCM in *C. reinhardtii* is the extracellular carbonic anhydrase CAH1 and α-carbonic anhydrase that is encoded by the gene Cah1 (Fukuzawa *et al*., 1990).

The transport of the Ci result in the accumulation of intercellular bicarbonate, so the CCMs are also the co-localization of Carbonic anhydrase (CA) activity near to the Rubisco enzyme to catalyse the dehydration of bicarbonate and to provide saturating CO_2 concentration for carboxylation of RuBP (Badger *et al*., 1980; Spalding *et al*., 1983a, b; Moroney *et al*., 1987). Aquatic photosynthetic microorganisms are able to concentrate dissolved inorganic carbon (DIC) intracellularly, permit to rapid growth despite low CO_2 availability externally (Kaplan and Reinhold, 1999). The carbon concentrating mechanism (CCM) shows acclimation to external DIC to optimize CO_2 fixation efficiency (Badger, Kaplan, and Berry, 1980). In the present study, the ability of the three microalgae which could able to express CA gene and subsequently to produce the enzyme was examined and agarose gel image **(Fig. 1)** showing the genomic DNA bands belongs to the three microalgae *Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus*.

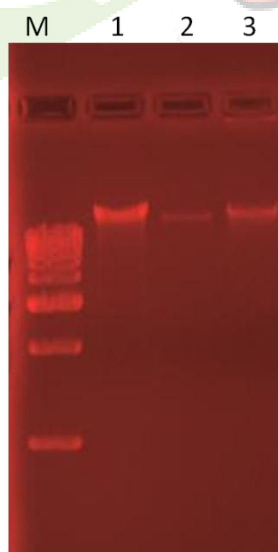

Fig.1: Genomic DNA extracted from microalgal samples. M - 1kb DNA ladder (Genei); 1 - *Chlorella vulgaris; 2- Acutodesmus nygaardii; 3- Scenedesmus armatus*

The Microalgal and Cyanobacterial Carbon Concentrated Mechanisms (CCM) are the use of energy dependent active Ci transport to increase the concentration of Carbon dioxide (CO_2) at the site of Rubisco for the photosynthetic CO_2 fixation while the low concentration of external Ci (Badger *et al*., 1980). In much number of unicellular microalgae consist of abundant external CA to increase the replacement of high CO_2 from bicarbonate and the CO_2 cross into the plasmalemma. There are multiple isoforms of CA even in unicellular micro-algae. In microalgae, the expression of CA is influenced by the environmental level of CO2 and in some cases the presence of alternate carbon sources (Villarejo, Orus & Martinez 1997). The eukaryotic alga in which the function of CAs has been most extensively studied is the green microalgae *C. reinhardtii*. *C. reinhardtii* contains at least five genes coding for CAs, 3 a-CAs and two b-CAs. Two of the a-CA proteins are located in the periplasmic space (Fujiwara *et al*. 1990; Rawat & Moroney 1991), and one a- CA is located in the thylakoid lumen (Karlsson *et al*. 1998). The two b-CA proteins are almost identical and are located in the mitochondrial matrix (Eriksson *et al*. 1996; Geraghty & Spalding 1996). Our present study confirms the presence of carbonic anhydrase gene in the genomic DNA of the three selected microalgae (*Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus*) which was determined by using a specific short primer of CA gene from *Chlorella sorokiniana*. The occurrence of the CA gene in the three microalgal strains *Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus* was evidently found in the agarose gel image **(Fig. 2)** at 500 bp.

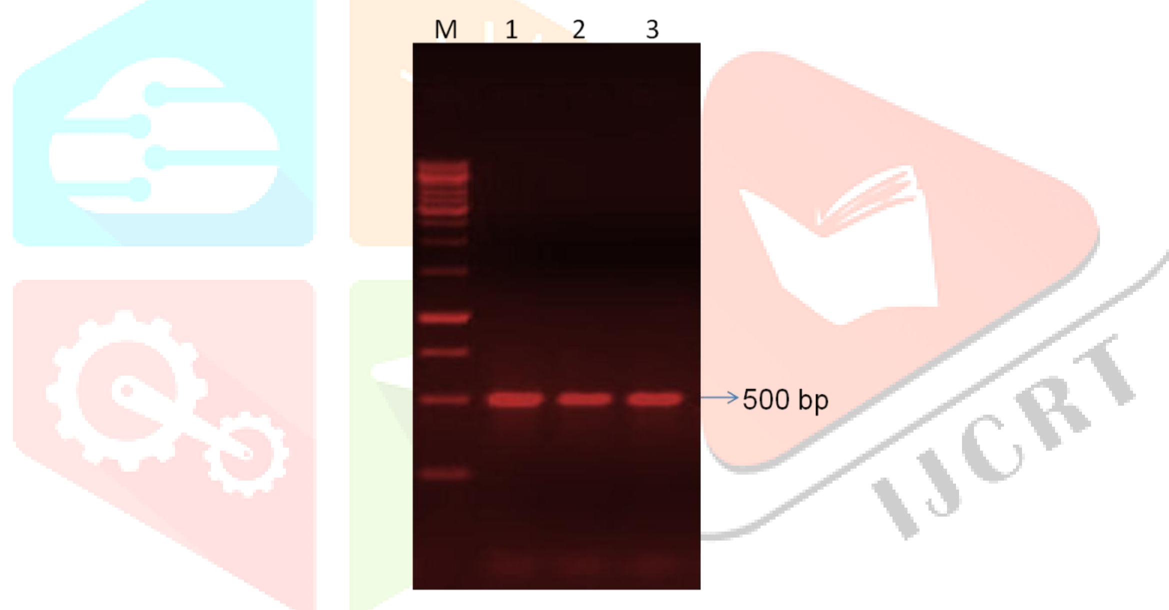

Fig. 2: PCR amplified gene encoding carbonic anhydrase from microalgal samples. M- 1kb DNA ladder (Fermentas); 1- *Chlorella vulgaris; 2- Acutodesmus nygaardii; 3- Scenedesmus armatus*

Determination of the Carbonic anhydrase enzyme based on the molecular weight

The amino acid sequences of CAs are categorized into six independent families such as α, β, γ, δ, ε, and ζ (Moroney, Bartlett, and Samuelsson, 2001), these are isozymes localized in plasmalemma (Badger and Price, 1994), cytoplasm (Hiltonen *et al*., 1998), chloroplast (Husic and Markus, 1994), and mitochondria (Eriksson *et al*., 1996). These enzymes may express in different forms including structures and molecular weights (Trachtenberg, *et al*., 1999). The molecular weight of crbonic anhydrase was reported as 30 kDa (Lindskog *et al.* 1971). The crude protein extract isolated from both the control and 0.1 M $NaHCO_3$ treated samples of the selected three microalgae (*Chlorella vulgaris*, *Acutodesmus nygaardii* and *Scenedesmus armatus*) were separated in SDS-PAGE and the gel apparently demonstrating the different protein bands dragged based on the molecular weight **(Fig. 3).** In which, the specific band at 37 kDa represents the presence of the enzyme Carbonic anhydrase. The band at 37 kDa was obviously dark in lane 3 which represents the carbonic anhydrase enzyme from 0.1 M $NaHCO_3$ treatment of the microalgal

strain *Chlorella vulgaris*. But the band intensity was seemingly low in lane 2 represents the CA of *Chlorella vulgaris* (Control). At the same point of view, the band intensity of lane 5 was low in the case of *Acutodesmus nygaardii* (0.1 M $NaHCO_3$) when compared with lane 4 represents the *Acutodesmus nygaardii* (Control) at 37 kDa. The CA enzyme band intensity at 37 kDa was found high in the lane 7 when compared with the lane 6 which represent the *Scenedesmus armatus* 0.1 M $NaHCO_3$ treatment and *Scenedesmu armatus* control respectively **(Fig. 3).**

At last, the two microalgal strains except *Acutodesmus nygaardii* evidently confirm the enhancement of the enzyme carbonic anhydrase during the 0.1 M $NaHCO_3$ treatment. But, the crude protein extract yielded from the 0.1 M $NaHCO_3$of *Acutodesmus nygaardii* was comparatively low in comparison with the control (*Acutodesmus nygaardi*). It shows that the microalgal strain *Acutodesmus nygaardii* was under enormous stress condition which succumb the proliferation of cells.

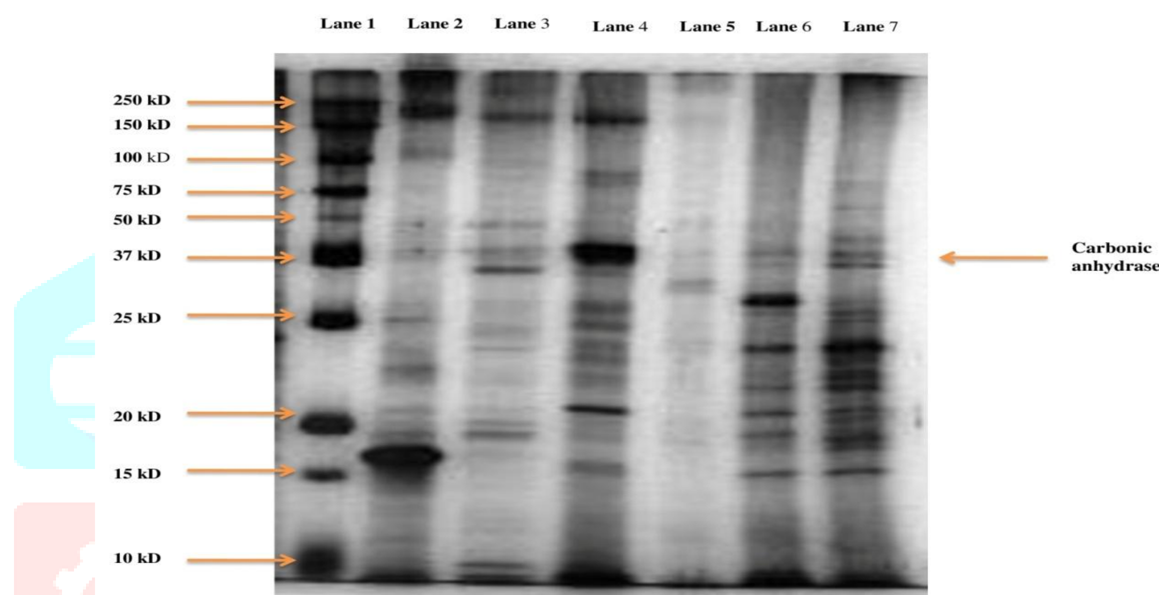

Fig.3: SDS-PAGE gel image showing different protein bands visible where carbonic anhydrase was determined based on the molecular weight, Lane 1: Protein ladder; Lane 2: *Chlorella vulgaris* (Control); Lane 3: *Chlorella vulgaris* (0.1 M. $NaHCO_3$ treated); Lane 4: *Acutodesmus nygaardii* (Control); Lane 5: *Acutodesmus nygaardii* (0.1 M $NaHCO_3$ treatment); Lane 6: *Scenedesmus armatus* (Control) and Lane 6: *Scenedesmus armatus* (0.1 M $NaHCO_3$)

CONCLUSION:

Chlorella vulgaris, *Acutodesmus nygaardii* and *Scenedesmus armatus* which were isolated from three selected Thermal power stations namely, Vallur Thermal power station (VT), Tamil Nadu, Kolaghat Thermal power station (KoT), West Bengal and Khapergada Thermal power station (KhP), Maharashtra, India has the ability to express Carbonic anhydrase gene and subsequently produces the enzyme. Hence these indigenous microalgae can serve as potent candidates to combat the issues associated with elevated levels of carbon dioxide emitted from the thermal power stations.

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