Pre-dissolved Inorganic Carbon (DIC) for cultivation Chlorella sorokiniana MH923013, Coelastrella MH923011 and Coelastrella MH923012

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Abstract. A step to net-zero of carbon dioxide losses in the microalgae cultivation process was targeted in the current study. This research was carried out by using pre-dissolved inorganic carbon (DIC) as a source of carbon with two doses of twenty-five and fifty millilitres. C. sorokiniana MH923013, Coelastrella MH923011 and Coelastrella MH923012 strains were used in the present investigation. The experimental data emphasized the direct influence of carbonic solution on microalgal growth according to the fast adaption of algal cells and higher productivity compared to control and dilution cultures. It was observed that microalgae strains conduct a corresponding response associated with different dosing of the saturated carbonic solution. For instance, dosing of 50 ml carbon dioxide revealed fast performance to reach the stationary phase (23-25) day with clear growth improvement. In addition, 0.1633 day\(^{-1}\) as a maximum specific growth rate in the exponential phase was recorded with this dosing. While as there was another obvious growth enhancement with supplying 25 ml CO\(_2\) solution, but reached the stable phase after around (37-42) day from inoculation with a maximum specific growth rate 0.0987 day\(^{-1}\). These results demonstrate the potential of CO\(_2\) and HCO\(_3^-\) in control the CCM pathways, thus, another step in the development of the photobioreactor design.

Keyword: Carbon dioxide, Microalgae, Coelastrella sp., C. sorokiniana, CCM, Rubisco.

1. Introduction

The growth of photoautotrophic microalgae acts as an ideal model of recirculation of CO\(_2\) resulting from several industries such as power plant emissions [1]. However, the cost factor is still preventing this option of high potential for the production of sustainability microalgae biofuels and impedes it from being feasible commercially. This challenge is represented by the high cost of photobioreactor as well as the excessive energy requirements throughout microalgae cultivation [2]. Thereby, design a bioreactor with reduced energy cost and especially high mass transfer for either dissolution CO\(_2\) or removal O\(_2\) has become the bottleneck for enhancing microalgal productivity [3]. Virtually, an airlift bioreactors (ALBs) are significantly employed in microalgae cultivation. Although, there have been several types of
researches accomplished the efficiency of various ALBs; all of these studies were focused on the conventional methods for the gas supplying system. In addition, there are limited studies deal with the impact of microbubbles generation system on the performance of ALBs, such as DAF (dissolved air flotation), electrostatic, electro-flotation, spraying, as well as mechanical agitation, etc. They were not economically productive to be viable in bioprocesses, because of its high-cost demand for energy requirements [4]. In addition, some of them are characterized by hard operating conditions, which are sometimes not suitable for growing microorganisms. Besides, there was always limited CO2 mass transfer associated with the traditional bubble dosing. Hence, by this scenario of aeration, large quantities of CO2 were wasted as a result of poor mass transfer, in addition to the damaging of algal cells because of intensive agitation, that relate to the large energy consumption due to the longer time of dosing [5].

There are numerous attempts to investigate efficient approaches and techniques for carbon dioxide supplying. The goal of these systems is to increase the ratio of surface area to volume of sparging bubbles. Among these developments is the successive innovation of [5-7], that generating microbubbles of micron size by fluidic oscillation system, that relatively enhance mixing efficiency by increasing interfacial area, mass and heat transfer as well as the residence time of the gas. This technology performed low energy requirements in relation to other methods. [3] reported the comparison of microfluidic microbubbles to effect with fine bubbles with dosing (5% CO2 and 95% N2), which confirmed the enhancing of mass transfer for CO2 dissolution and O2 removing. The authors also proposed that increasing dosing flow rate would actually increase the wasted carbon dioxide due to a decrease in the efficiency of carbon capture, the results also revealed the promising of microbubble technology. On the other hand, a subsequent study was conducted on this technique by [8]. They used ALBs with a range of 50 to 1000 μm diameters of microbubbles by a simulation process with low aeration of air into the liquid (water). The results indicated that the presence of small microbubble diameter (50 – 100) μm may increase the mass transfer rate due to the high interfacial area with longer residence time. However, these sizes of bubbles obstructed the liquid recirculation in the downcomer as a result of the buoyancy force. Perhaps those sizes of bubbles have a more practical benefit for the separation and harvesting stages than for the cultivation stages, which requires a homogeneous distribution of living organisms to provide adequate opportunities to obtain the appropriate environment and food. Therefore, [9] investigated the harvesting method by using micro flotation for small microbubbles diameter (<50 μm) and reported that the recovery efficiency was affected by the bubble size due to the highly efficient separation with smaller particles which increased collision probability, on the other side, as there were no gas bubbles in downcomer area with diameters of (200 – 1000) μm because of higher buoyancy force, especially the bubbles of diameters of 200 and 400 provides lower obstruction of liquid circulation with better residence time due to increased interfacial surface, which leads to enhance mass transfer kinetics with relatively low energy requirements. Nevertheless, using these development approaches enhanced gas-liquid contacting and reduced the energy demand, there was high economic cost due to the large quantities of carbon dioxide still wasted to the atmosphere, as a result of the fact that the biological process is very slow.

The hypothesis of this current study is using a pre-dissolved inorganic carbon as a source of carbon in microalgae improves productivity and reduces costs. This hypothesis will be tested via investigation of this source on general growth and specific growth rate on three microalgae strains: Chlorella sorokiniana MH923013, Coelastrella MH923011 and Coelastrella MH923012 which diagnosed, isolated from Iraqi environment, purified and registered in Gen Bank database.

2. Materials and methods
Three strains of microalgae species were used in this experiment: C. sorokiniana and two strains of Coelastrella species. These strains of microalgae were isolated from Iraq environment (Tigris River),
purified and registered in Gen Bank database (accession No. MH923013), (accession No. MH923011), and (accession No. MH923012) respectively via continuous dilution method [10].

2.1 The cultivation of microalgae species

The nutrient medium BG-11 was prepared according to the Guideline and recommendations given by (BioReady TM media, China). This medium was adjusted on initial pH (7±2) by using (0.1 N) HCl and NaOH. 1 ml stock culture of each strain was incubated in 500 ml conical flask with two replicators, to become three replicates for error bar purpose. All the experiments were operated under continuous illumination via illuminated incubator (Conviron Canada origin) with light intensity 168 μE m −2 s−1 and regular temperature (24±1 °C).

2.2 Preparation of dissolved inorganic carbon.

Dissolved inorganic carbon was prepared according to schematic diagram of the experimental setup in Figure 1. The system was operated by sparging carbon dioxide at a rate of 400 milliliters per minute to a vessel of 30 liters and a working volume of 20 liters of distilled water. At the beginning of the operation, carbon dioxide was pumped through the diffuser for the purpose of emptying the tank from the dissolved gases or from gases in the headspace. Thus, carbon dioxide will replace these removed gases (oxygen and nitrogen). The sparging time was approximately 30 minutes, which was determined by measuring the pH (pH-Basic 20, CRISON) and dissolved oxygen (OXI 45+, CRISON, Spain). All valves were then closed to prevent gas from escaping to/from the tank. The providing CO₂ to cultivations flasks was carried out via valve No. 5, while the generated vacuum pressure in the headspace was balanced by supplying low-pressure carbon dioxide reserve tank via valve No. 4.

2.3 The experimental setup and measurements

Each strain of microalgae stock was cultured in to five groups: (1) control (without any change), (2) dilution with 25 ml, (3) dilution with 50 ml, (4) adding of carbonic solution at 25 ml volume and (5) adding of carbonic source at 50 ml volume. Each single group was included 0.5 L flask with two duplicate which exposed identical environment to obtain more accurate results. In each flask, 1 ml of culture was added to 500 ml media solution (BG-11). After 24 hours, dilution and dosing carbon solution started and repeated three times a week. The shaking of the flasks manually during the growth was necessary to prevent microalgal cells agglomeration and to keep cellular multiplication. The pH value of carbonic solution was lower than 4 as a checking procedure before injecting. Before and after dosing carbonic source the measurements of pH values were also monitored. Samples of microalgal culture were measured by using UV spectrophotometer (GENESYS 10UV, USA) at wavelength 680 nm to observe the growth kinetics from lag phase (zero time), exponential phase and finally, stationary phase at which the experiment was stopped.

The growth curves of algae biomass density versus time carried out by estimation of specific growth rate. While the calculation of specific growth rate (\( S_g, \text{day}^{-1} \)) was evaluated from the following straight line formula [11]:

\[
\ln F_m - S_g \Delta t + \ln I_m
\]

\[
S_g = \ln F_m - \ln I_m / \Delta t
\]

Where \( F_m \) and \( I_m \) were the final and initial concentrations of algal biomass respectively, \( \Delta t \) was the duration of active exponential phase of cultivation process in day [12]. Therby, the generation time (\( \tau_g, \text{day} \)) of the cell population was estimated from the below relation [13]:

\[
\tau_g = ln2 / S_g
\]
3. Results and Discussions

The pathways of the biological reactions of the metabolic processes are determined by the quantity and quality of nutrients, as well as the operating conditions of the cultivation stage. However, the cellulosic change of microalgae is more affected by the change of the main elements involved in biological reactions compared to the change of operational conditions. With this context, the focus on those materials may be necessary to increase bio-mass resulting from micro-algae. In fact, the apparent effect of the carbon source came as a result of the main biological reaction in the microalgae cell with water to form glucose when the light was available as a biocatalyst. However, the element of carbon is related to changing operational conditions, which caused the determination of ways to provide this element to the microalgae cell. The current study used pre-dissolved carbon dioxide with its ions as carbon source provider for the metabolic processes. The experiments also were done by using three sorts of microalgae species (C. sorokiniana MH923013, Coelastrella MH923011 and Coelastrella MH923012) which have the ability to grow in Iraqi environment. The main experiment was split into five groups for each single strain: dosing 50 ml carbonic solution as well as 50 ml dilution of algal culture, in addition to 25 ml aqueous carbon dioxide and 25 ml dilution flasks. The groups of same additional volume have identical experimental finish time, while the fifth group was the control culture (without any change for comparison purpose) carried on until the experiment stopped, so the finish time for each group differed according to the period until the carbonic flasks reached the stationary phase. Figure 2 shows the conduct of the experiment from flasks culture to biomass drying till obtaining the dry weight of microalgal biomass.
Figure 2. The experimental steps for each sample flask culture.

The addition of 50 ml CO₂ solution into different microalgal strains showed obviously growth increment. On the contrary, there is a slow enhancement with control and dilution groups as illustrated in Figure 3. Moreover, it can be seen that Coelastrella MH923012 exhibited the maximum optical density (1.913) recorded among the other strains, reached the peak after 25 day of experimental cultivation period followed by (1.487) and (1.416) for C. sorokiniana MH923013 and Coelastrella MH923011 respectively, which both entry the stationary phase after 23 day of algal inoculation.

While as, Figure 4 shows the response of applying 25 ml of carbon dioxide solution for three strains cultures. Generally, there was a significant difference between the response of the two carbonic volumes (25 and 50 ml). The three microalgal strains of 25 ml carbonic source reached the stable phase after longer time than 50 ml groups.

After (37, 39, 42) day, optical density registered (1.930, 1.955, 1.976) for C. sorokiniana MH923013, Coelastrella MH923011 and Coelastrella MH923012 respectively. In addition, it can be observed that microalgal strains conduct a corresponding response in this addition (i.e. 25 ml), since the cell absorbance readings were almost the same for all strains when reached to a stable phase, which did not recognize in applying (50 ml) of carbon source. Thus, both patterns of carbonic sources have a positive attitude limited
by the quantity of carbonic dose beside the type of algal culture as illustrated in Figure 5. All 50 ml carbonic dosing exposed fast performance (23-25) day to reach the plateau state in comparable with 25 ml carbon dioxide solution groups, which took (37-42) day for the adopted strains. The final productivity of 25 ml CO$_2$ solution dosing has a higher registered optical density with (2.138) (2.043) in comparable with (1.697) and (1.294) in supplying 50 ml carbonic solution for both $C$. *sorokiniana* MH923013 and *Coelastrella* MH923011 respectively, but still adding 50 ml CO$_2$ solution is the most preferable, since it achieved the higher specific growth rate in relatively short time. While as *Coelastrella* MH923012 recorded the best performance among the adopted strains in responding to the supplying of inorganic carbon solution, which registered almost identical final productivity (2.037- with 25 ml CO$_2$) and (2.248- with 50 ml CO$_2$).

![Figure 4](image.png)

**Figure 4.** The response of applying 25 ml aqueous carbon dioxide in to different microalgal cultures in comparable with control and dilution groups.

On the other hand, the control as well as dilution flasks revealed slow and fluctuate growing for the various strains because of ceaseless loss of nutritional elements that revealed the evidence of carbonic source for microalgal biomass growth. Indeed, they need a longer time to grow as indicated in previous studies [14, 15]. The obtained data confirmed the results accomplished with Razooki et al. [16], which scoped that the growth of microalgae can be enhanced with direct additions of CO$_2$ solution, and approved that the concentration of 25 ml CO$_2$ solution had a positive action towered growth progression of *Coelastrella* MH923012, as demonstrated in Figure 4.

The calculations of specific growth rate and generation time were also estimated at this work. It demonstrated the importance of carbonic source for the applied microalgal strains, that clearly evident with increasing the specific growth rate accompanies with decreasing the generation time, when the amount of carbonic dosing increased. Thus, again *Coelastrella* MH923012 recorded the maximum specific growth rate with minimum generation time over other strains in all the experimental groups, which observed in the addition of 50 ml CO$_2$ solution with (0.1633 day$^{-1}$, 4.24 day), which was around twice the specific growth rate and half generation time (0.0946 day$^{-1}$, 7.33 day) for the same strain (*Coelastrella* MH 923012) in dosing 25 ml carbonic solution, while the other two strains registered (0.1379, 0.1486) day$^{-1}$ and (5.026, 4.66) day in supplying 50 ml CO$_2$ solution, whilst in 25 ml aqueous carbon dioxide recorded (0.0939, 0.0987) day$^{-1}$ with (7.38, 7.023) day for both *Coelastrella* MH923011...
and *C. sorokiniana* MH923013 respectively. On the other hand, the limitation in carbon source amount within control and dilution solution made the values of specific growth rate and generation time for the three strains were almost close, which registered lower range of specific growth rate values about (0.0611-0.0789) day⁻¹, and thereby higher range of generation time values around (11.35-8.778) day.

**Figure 5.** The impact of carbonic source addition for (a): *Chlorella sorokiniana* MH923013; (b): *Coelastrella* MH923011; (c): *Coelastrella* MH923012.
Table 1 shows the amount of improvement that occurred in microalgae farms in previous studies, regardless the growth conditions and type of algal strain. While Table 2 shows the improvement in the current farms and for the three selected strains as a result of using these doses. With a simple comparison, the effect of the carbon source on three strains microalgae growth becomes evident than in previous studies. It can be noticed that supplying 50 ml improved the growth more than twice that of their 25 ml groups, which their enhancement was (3.7), (2.4), (4) based on control as well as (3), (3.3), (3) over dilution cultures for Coelastrella MH923011, C sorokiniana MH923013 and Coelastrella MH923012 sequentially. While as the enhancement of growth rate by using carbon dioxide solution (50 ml CO2 solution) was about (8.5), (6), (3.7) times the control groups and more than (7.32), (4.6), (4.2) over dilution solution. Within Coelastrella MH923012, C. sorokiniana MH 923013 and Coelastrella MH 923011 respectively. Eventually, there is remarkable reduction in the cost function with regard the current work, stemmed from using relatively small quantities of carbon dioxide as saturated solution, which led to enhance the production with prevent returning to atmosphere as much as possible. Thereby environmentally and economically interest.

Table 1. The efficiency of growth rate (X factor) of previous researches.

| Microalgae type         | CO2  | X factor | References |
|-------------------------|------|----------|------------|
| Coelastrella sp. MH 923012 | 15 ml | 3.462    | [16]       |
| Coelastrella sp. MH 923012 | 20 ml | 4.04     | [16]       |
| Coelastrella sp. MH 923012 | 25 ml | 5.01     | [16]       |
| Chlorella vulgaris      | 5%   | 3.64     | [17]       |
| Chlorella PY-ZU1        | 15%  | 1.249    | [18]       |
| Nannochloropsis sp.     | 10%  | 1.0631   | [19]       |
| Nannochloropsis sp.     | 15%  | 1.321    | [19]       |
| Chlorococcum littorale  | 5%   | 0.83     | [20]       |
| Neochloris oleoabundans| 6%   | 4.12     | [21]       |

The current study based on using the inoculation size of (1 ml) relied on previous studies [15, 22, 23]. While as studies of [16, 17, 21] accomplished on using (2, 10, 20) ml respectively as a dosage of inoculation, which focused on carbon source as limiting factor for microalgal biomass growth, and leads to enhance the growth rate by many times by employment of different microalgae cultures, as can be seen in Table 1. Nevertheless, the proposed inoculation size plays a prominent role in the assessment of comparison, since the growth rate as well as the final culture productivity can be enhanced with increase inoculation dosage [24, 25]. This striking footprint reflected especially with Razooki et al. [16], as it is based on interplay conditions with the present work, which is the deployment of Coelastrella MH923012 in BG-11 media with dosing 25 ml of carbonic, as observed in Table 1, achieving growth enhancement (X factor) of (5 times) in relative to (4 times) of this current study (see Table 2).

However still dosing 50 ml inorganic carbon solution for Coelastrella MH923012 registered the highest enhancement (8.5), although the inoculation size is (1 ml) only. Besides, it is worthy to mention the influence of dosing situations on the adaption and the quantities of carbonic solution used, dosing in
three times a week of this present work, can offer more time for conforming and to be familiar with the subsequent dosing, this can be more priority than dosing five times a week with Razooki et al. [16].

Table 2. The efficiency of growth rate (X factor) of current study.

| DIC dosing | Microalgae strain | X factor based Dilution | X factor based Control |
|------------|-------------------|-------------------------|------------------------|
| 25 ml      | C. sorokiniana MH 923013 | 3.3 | 2.4 |
|            | Coelastrella MH 923011   | 3  | 3.7 |
|            | Coelastrella MH 923012   | 3  | 4  |
| 50 ml      | C. sorokiniana MH 923013 | 4.6 | 6  |
|            | Coelastrella MH 923011   | 4.2 | 3.7 |
|            | Coelastrella MH 923012   | 7.32 | 8.5 |

Regardless of the method used to provide the carbon source for microalgae, the relationship of that source with the growth rate is restricted by the carbon dioxide –concentrating mechanism (CCM) pathways. Figure 6 illustrates the conventional stages of carbon dioxide transport from the gas bubbles to the Pyrenoid region inside the cell. The use of gases as a source of carbon may be one of the restrictions on the activation of these pathways, as they are governed by the surface area between the gas and the culture medium phase as well as the contact time. This is followed by the liquid film in which the chemical reaction occurs between carbon dioxide and water to produce dissolved CO₂ and its ions, as shown in Figure 6. Actually, acquiring CO₂ by microalgae faces several challenges, in particular, CO₂ have low solubility in water. Therefore, the slow diffusion of this gas in the aquatic environment, which is slower about 10^4 times CO₂ diffusion in the air [26], makes the photosynthesis in higher plants relied on directly CO₂ diffusion to obtain their sources of inorganic carbon (Ci), rather than organisms of microalgae and cyanobacteria. That possesses an important function (effectively inorganic carbon (Ci) uptake system CCM) to overcome these challenges [27]. These challenges represented by, firstly their ability to capture CO₂ must be quickly as much as possible. Secondly, the characteristic of Rubisco (ribulose bisphosphate carboxylase/oxygenase), which have very low apparent affinity of Rubisco with CO₂ [28]. This poor performance of RUBISCO towered fixing CO₂, is due to its dual role [29], as it catalyzes the oxygenation, which leads to divert carbon as wasteful in the pathway of photorespiration. Therefore, effect intracellular system increases the apparent ratio CO₂/O₂ specificity on the site of RUBISCO [30], and also relatively owing to the high concentration of oxygen which is in competition with CO₂. As result the activity of photosynthesis and carbon dioxide capturing is based on the action of oxidation and carboxylation.

The current apparent increase in the growth rate of microalgae (with 25 ml or 50 ml of carbon solution) is the result of shortening the first transition phase and the reactions phase. These two processes have been accomplished in advance before starting the experiments. This method increased the presence of CO₂ and HCO₃⁻ close to the cell surface (the fifth stage in Figure 6. And through passive transport, the balance between carbon dioxide and its ions is occurred by the enzyme carbon anhydrase (CAH). Thus more driving force can be achieved into followed stages.
Figure 6. Schematic model of components associated in the operation of the inorganic carbon concentrating mechanism in eukaryotic microalgae.

4. Conclusion
This study reported the successful cultivation of three strains of microalgae: *C. sorokiniana* MH923013, *Coelastrella* MH923011, and *Coelastrella* MH923012 in a rich-carbon environment. The results confirmed the direct effect of carbon, due to the remarkable growth with volumetric dosing (25 and 50) ml compared with control and dilution groups for variable adopted strains, which revealed slow and fluctuate
growing. It can speculate that providing 50 ml of inorganic carbon solution reflects maximum productivity of microalgal biomass, and represented the best response, that observed among the other experimental cultures, rather than the addition of 25 ml CO₂ solution. Especially with *Coelastrella MH923012* of 50 ml CO₂ solution, which investigated the highest growth enhancement of (8.5) times control flasks. In addition, the CCM of microalgae gives the path of inorganic carbon solution, which accelerated by more than one step via applying the aqueous carbon dioxide directly to the internal cellular culture. Thus, leads to fast carbon fixation, in response to increasing the dissolved inorganic carbon to the vicinity of Rubisco sites.

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