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Carbonic anhydrase (CA) activity by *Chlorella* sp. in immobilised matrix under carbon dioxide rich cultivation condition.

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Abstract: The continuous release of global CO_2 and greenhouse gases into the atmophere is considered one of the major contributors for global warming. Currently, microalgal biosequestration using enzyme carbonic anhydrase (CA) has been reported to be one approach that could be applied to overcome the issue. Eventhough this enzyme has been proven to show its potential to convert atmospheric CO_2 to bicarbonates, there are remaining issues related to its stability and production parameters that need to be addressed. In this study, the activity of CA produced by immobilized microalgae *Chlorella* sp. cultivated in a laboratory environment was investigated. For this study, the influence of cultivation conditions such as pH value ranging from 4.00 to 12.00, light intensity ranging from 330 lux to 1000 lux and CO_2 concentration ranging from 0.04% to 25% on CA activity were investigated. This present study indicates that the highest CA activity of 1.908 U/min was observed for the cultivation was performed using 15% CO_2 with a pH of 8.00 and a light intensity of 550 lux. This suggested that the entrapment of microalgal using a suitable matrix carrier could produce higher CA activity which could be further utilised for extended biomimetic CO_2 capture systems.

1. Introduction

Carbon dioxide (CO_2) emissions are one of the major environmental problems generated from human activity. Over the years, a subtantial amount of CO_2 gas is emitted from various activity such as; transportation, animal, energy and the coal industry. Gas produced from these sources heat up the

earth and enhance the global warming. Several carbon capture and storage (CCS) technologies that have captured CO_2 in the atmosphere and have been introduced to overcome this problem [1]. Technologies such as; physical processes (biochar burial, ocean storage, geological sequestration), chemical processes (mineral carbonization and chemical scrubber) and biological processes (reforestation, agriculture, photosynthetic microorganism) have been reported to have the potential to be applied in CCS [2].

Microbial CO_2 bio-sequestration is one of the most environmental friendly and cost effective methods to counteract CO_2 emissions [3]. Alternative approaches involve the utilization of CO_2 by photosynthesis microorganisms such as microalgae. Microalgae are unicellular groups that can be used to fix CO_2 emissions efficiently from different sources including the atmosphere, industrial exhaust gases, and soluble carbonate salts. These microorganisms are capable of utilizing CO_2 for its growth and at the same time producing a various valuable product [4, 5]. The benefits of using microalgae for environmental purposes are related to their growth rates and their tolerances regarding higher concentrations of CO_2.

The capability of microalgae to fix CO_2 is highly dependent on the special carbonic anhydrase (CA) enzyme which is key enzyme of the carbon concentrating mechanism (CCM) in algae [6, 7]. In general, the present of CO_2 concentration as an inorganic carbon in cultivation medium induces intra and extracellular carbonic anhydrase enzyme activity. The CO_2 essentials via the cell is transported into the cell by a hydration response depending upon the bicarbonate, CO_2 concentration inside and outside the cell. The transportation of CO_2 is through the transformation of carbon dioxide into bicarbonate and happens repeatedly in the cell. As a result, activation of CA activity under specific conditions increases the conversion rate of bicarbonate ions ($HCO3^-$) into CO_2 and subsequently promotes the uptake of CO_2 by the active transport into the microalgae cells [6]. According to Zeng et al., [8], microalgae have the ability to capture carbon dioxide gas, CO_2, in a single process and produce various valuable products at the same time.

The microbial carbon capture using CA from a free cell has been reported to show disadvantages such as difficult during its harvesting. Harvesting of the microalgae biomass, such as concentrating microscopic microalgae cells from dilute solutions of mass cultivation is an crucial step to secure high-quality effluents avoiding cell washout [9, 10]. On the other hand, in order to ensure the efficient CO_2 sequestration by microalgal CA, it is important to ensure the stability of enzymes and the microalgal growth. Therefore, immobilization of microalgae cells has been suggested to avoid harvesting issues. To date, there is little information on intracellular CAs activity produced by microalgae under immobilized conditions. Thus, this study is designed to investigate the CAs activity by *Chlorella* sp. in immobilized form cultivated under a CO_2 rich medium.

2. Material and Method

2.1 Microalgae strain and cultivation

Freshwater *Chlorella* sp. was used throughout the experiment. The culture was cultivated in an illuminator room. The mix was 0.3 L/min aired under light photons with an intensity of 1000 lux at 28-30C. The cultivation medium for the cultivation and seed preparation in this study consisted of 0.49 g/L magnesium sulfate ($MgSO_4.7H_2O$), 1.7 g/L sodium nitrate ($NaNO_3$), 0.14 g/L di-potassium phosphate (K_2HPO_4), and 0.03 g/L calcium chloride ($CaCl_2.2H_2O$) respectively.The growth of the culture was monitored daily by measuring its optical density and harvested during its late logarithmic growth phase prior to being subjected for the immobilization process.

2.2 Immobilization

The immobilization of *Chlorella* sp. was carried out using alginate as a immobilize matrix carrier. An active *Chlorella* sp. seed culture with an optical density of 680 nm (OD_{680}) of 1.0 was harvested by centrifugation at low speed (4500 rpm) for 10 minutes. Pellets rich with algal biomass was then washed and re-suspended in deionized water to form a concentrated algal suspension. The algal suspension was then mixed with a 4% sodium alginate solution in a 1:1 ratio in 4 mL of concentrated

algal and a 4 mL sodium alginate solution. The mixture was gently stirred until the algae was evenly distributed. The mixture was dropped into 6% calcium chloride using a syringe to form uniform algal beads. The beads were left in a crosslink solution ($CaCl_2$) for approximately 12 h for hardening. After the hardening was complete, beads were separated from the solution using a sterile strainer/filter. The beads were rinsed several times with deionized water to remove any remaining calcium chloride. Blank alginate beads were prepared in the same manner as the algal beads with the exception of using deionized water rather than a cell suspension.

2.3 Preliminary cultivation of carbonic anhydrase analysis

A study on the effects of cultivation including pH, light intensity and CO_2 concentration on CA activity by *Chlorella* sp. in a immobilised matrix was initially investigated using a one-variable at a time design (OVAT) method. The effect of CA activity at pH range (4 to 12), light intensity range 300 to 1000 lux and CO_2 concentration range 0.04 to 25% (v/v) was assessed in this study. After a cultivation period, the CA activity in a immobilised matrix was analyzed as discussed in the next section.

2.4 Response surface methodology

A Box Behnken Design (BBD) was used in order to determine the interactive effects of cultivation parameters such as pH, light intensity and CO_2 concentration regarding CA activity. A multi-level three parameter matrix was employed to determine the synergistic effects between three parameters and the optimal point value. This was analyzed by fitting the second order polynomial model. The statistical significance of each parameter was determined through P values, where smaller p-values indicate effective parameters. The contour plots were used to show the individual and cumulative effects of variables of CA activity. These graphical representation models were plotted as a function of two variables while keeping other variables at the central level.

2.5 Determination of carbonic anhydrase activity

CA activity was determined using a method describe by Komala and Kiium [11]. Approximately 100 microalgal beads were crushed using a mortar then centrifuged at 200 xg for 10 minutes. The sample was suspended with a 0.1 phosphate buffer. The enzyme activity was determined using a spectrophotometric method using a 0.2 mL extracted sample mixed with 1.0 mL 3mM p-nitrophenyl acetate and a 1.8 and 0.1 M phosphate buffer. One sample of the solution was taken and used to measured the absorbance reading using a spectrophotometer at 348 nm. The changes of the absorbance at 348nm was recorded over the first 5 minutes in order to estimate the amount of the p-nitrophenyl (p-NP) released. The non-enzymatic hydrolysis was substracted by using a blank without enzymes. One unit of the enzyme activity represents the amount of enzyme catalyzing to produce 1 unit of p-nitrophenol per minute under the assay conditions. All the processes were done in duplicate.

3. Results and Discussion

Production of CA and its activity by microalgae could be influence by several factors. In this study, the effects of pH, light intensity and CO_2 concentration of CA production and its activity by microalgae in a immobilised matrix were investigated.

3.1Effect of pH

Figure 1 shows the effects of a pH value of cultivation medium on *Chlorella* sp.-CA activity in an immobilized matrix. Based on this study, the maximum CA activity of 1.02 U/min was obtained from the cultivation in a medium with an initial pH value of 7. It was found that a further increase of pH beyond pH 7 exhibited low CA activity.

It is known that pH value plays an important role in maintaining equilibrium of cultivation medium environments. Significant changes of pH values from 7 to 12 have reported to show a

noteable affects on CA activity. Similar observations were reported on CA activity of *B. mucilaginosus* KO2 exhibiting maximum CA activity at pH 5.5 to 6.6 [12]. This finding also has been supported by a previous study reporting that typical maximum CA activity can be obtained at pH range 5 to 10 [13]. Previous studies reported that in some cases, CA activity by *Chlorella* sp. induces when the cultivation was perfomed at alkaline conditions [14]. A study by Vaquero et al. [15] reported that total CA activity by *Cocomyxa* sp. increased with at higher pH values with a maxiumym CA activity obtained when the cultivation was carried out using pH 6. Under alkaline conditions, the cultivation medium was predominantly carbon in the form of bicarbonate. This required CA transport for this compund into a membrane cell [16]. This present study also showed that the microalgal could assist CO_2 sequestration via CA at this pH range which proves very usefull application which can be applied for CO_2 capture and alkaline wastewater treatment.

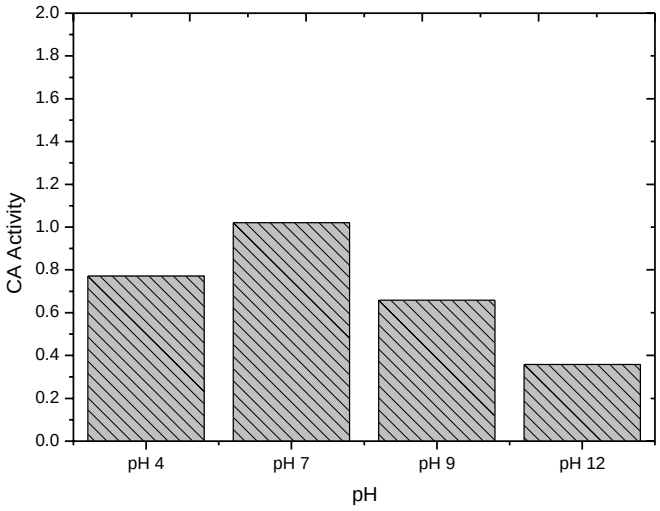

Figure 1. Effect of initial pH value on the CA activity in immobilised *Chlorella* sp.

3.2 Effect of light intensity

The influence of light intensity on the CA activity of *Chlorella* sp. in alginate polymer was also studied. Results are shown in Figure 2. The present study showed that exposure of immobilised *Chlorella* sp. could significantly influence the the CA activity. The maximum CA activity of 1.021 U/min was found when the cultivation was carried out using 550 lux. Further increase of light intensity and cultivation using low light could reduce CA activity. Results obtained from this study is in agreement with previous reports that suggest exposure of microalgae to higher light intensities could induce CA activity [17, 18]. Similar observations has also been reported on CA activity of *Tetraselmis gracilis*, in which 50% of CA activity loss was detected when cultivation was performed under low light conditions [19].

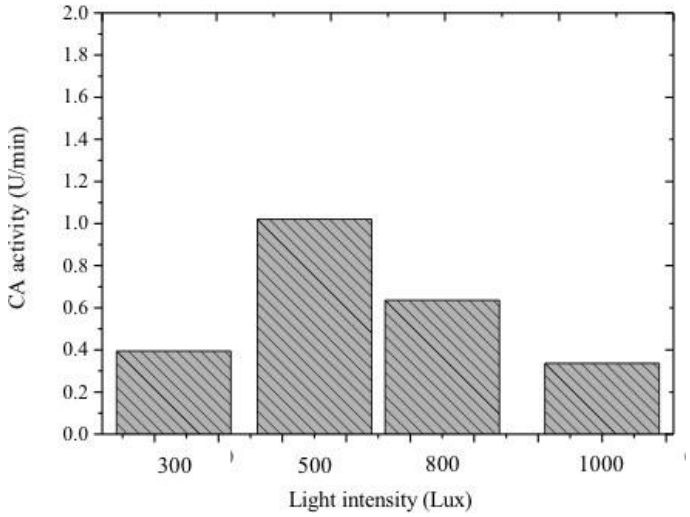

Figure 2. Effect of light intensity on the CA activity in immobilised *Chlorella* sp.

3.3 Effect of CO_2

CO_2 concentration plays an important role in microalgae growth and its carbon capture activity [20, 21]. In this study, the effects of CO_2 concentration on CA activity in an immobilised matrix was evaluated using CO_2 concentration ranging from 0.04 to 25% (v/v). Results are presented in Figure 3. Based on this study, it was clearly indicated that higher CA activity was observed for the cultivation using a higher CO_2 concentration compared to normal air. It was found that maximum CA activity in an immobilised matrix of 1.721 U/min can be obtained when the cultivation was performed using 15% CO_2. An increase of CO_2 concentration from normal air to 15% CO_2 could enhance the CA activity up to 41%. However, cultivation of microalgae using CO_2 concentrations up to 25% CO_2 could reduce CA activity.

Varied observations have been reported on the effects of CA activity by different microalgae species [19, 22]. Studies indicate that CA activity by *Tetraselmis gracilis* increased when the CO_2 level decreased suggesting that CO_2 was used by the microalgal via assitant of the CA enzyme. According to Xia et al. [23], reports that higher CA activity by *Chlorella pyrenoidosa* and *Chlamydomonas reihardtii* were observed when the cultivation was perfomed at low CO_2 concentrations. In contrast, CA activity by *Scenedesmus obliqus* cultivated using different CO_2 concentrations showed the highest CA activity when this strain was supplied with 35% CO_2 after 15 days of cultivation [24]. High CA activity reported in this study represents internal and external CA activity. These results therefore imply that exposure to suitable CO_2 concentrations could favour CA activity by *Chlorella* sp. in a immobilised matrix. Differences in CA activity reported in this study also showed that CA activity by the microalgae is species dependent.

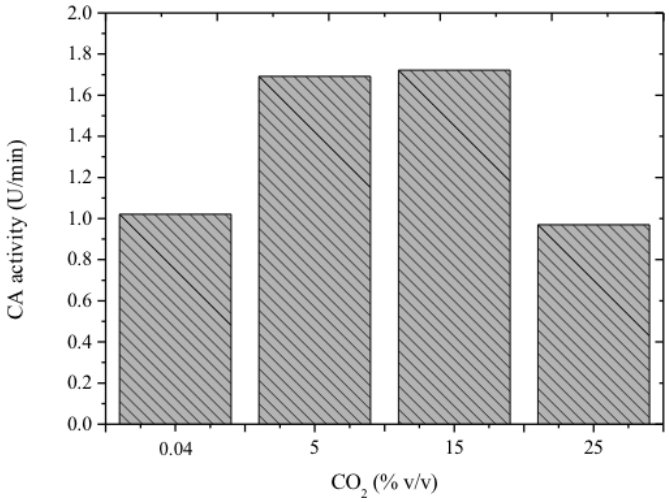

Figure 3. Effect of CO_2 concentration on the CA activity in immobilised *Chlorella* sp.

3.4 Effect of cultivation on enzyme activity

Further studies on the effects of cultivation conditions such as pH, light intensity, and CO_2 concentration for CA activity were carried out using the Box Behnken response surface design (BBD). Based on the present study, the quadratic model was found to be the most suitable explanation to for the interaction of cultivation parameters on CA activity in alginate polymer with P > F value of < 0.05 (Table 1). The coefficient of determination (R^2) of the regression equation for CA activity was 0.97 (Adj = 0.92). The R^2 value was close to 1 indicating that the predictability of the value is at 95% confidence level and the response function for the predicted value agreed well with the experimental data.

Table 1: Analysis of variance (ANOVA) for CA activity by immobilized *Chlorella* sp.

Source	**SS**	**Df**	**MS**	**F value**	**Prob > F**
Model	5.08	9	0.56	6.60	0.0105
CO_2	0.25	1	0.25	2.95	0.1296
pH	0.12	1	0.12	1.42	0.2718
Light intensity (LI)	0.38	1	0.38	4.44	0.0730
CO_2 x pH	0.28	1	0.28	3.31	0.1118
CO_2 x LI	0.22	1	0.22	2.53	0.1556
pH x LI	0.029	1	0.029	0.34	0.5761
CO_2x CO_2	2.52	1	2.52	20.42	0.0010
pH x pH	0.27	1	0.27	3.14	0.1195
LI x LI	0.70	1	0.70	8.21	0.0241
Residual	0.60	7	0.086		
Lack of fit	0.44	3	0.15	3.79	0.1152
Pure error	0.16	4	0.039		
R2	0.9654				
Adj-R2	0.9210				

DF: degree of freedom of variance source, SS: sum of squares, MS: mean of squares.

A three dimensional (3D) response surface plot analysis was performed to determine the interaction and the most suitable condition for total CA activity in the immobilised matrix with the response of chosen parameters. Figure 4 shows the 3D contour plots for the effects of CO_2 concentration (%) and initial pH on carbonic anhydrase (CA) activity (U/min). From this figure, it shows that the increase of CO_2 concentration (%) and pH will increase CA activity. Further cultivation beyond CO_2 concentration and pH will reduce CA activity of *Chlorella* sp. in an immobilized matrix. The decreasing of CA activity was due to the use of a higher CO_2 level resulting the low in pH. This is because the microalgae *Chlorella* sp. showed maximum biomass productivity at pH 6.5. It has been reported that the decreasing of pH will reduce the activity of CA and inhibit cell growth. Therefore, both interactions play important roles to the production of CA activity.

Figure 4B reveals the 3D contour plots and the interaction effects of CO_2 concentration (%) and light intensity (lux) on carbonic anhydrase (CA) activity (U/min). The increase of CO_2 concentration (%) from 0.04% to 15% significantly increased CA activity. However, further cultivation beyond this value slowly reduced CA activity. For various light intensities (lux), the cultivation of high light intensity from 330 lux to 550 lux significantly increased CA activity. However, further cultivation beyond this value slightly decreased CA activity. According to Solovchenko et al., [25], the higher the light intensity the more reduction in growth rate resulting damage to the cells. Previous studies found that microalgae showed higher activity and growth at a light intensity of 3000 lux but the evaporation rate of the medium increased and most of the cells dried in the flask when illuminated to direct sunlight. Therefore, selecting a suitable CO_2 concentration and light intensity is important in order to produce high CA activity.

Figure 4C shows the interaction of an initial pH and light intensity (lux) on carbonic anhydrase (CA) activity (U/min) by microalgae in the alginate matrix. The present study indicated that the increase in pH value from 4.00 to 8.00 increases CA activity. Further cultivation using a high alkaline medium with a pH value of 12.00 slightly reduced CA activity. Generally, the maximum CA activity from microalgae is induced at alkaline condition. Previous study has reported that the maximum extracellular CA activity of *Chlorella* sp. is at pH 7.5 and 8.5 [26]. For the light intensity (lux), an increase of light intensity from 330 to 550 lux increasec CA activity. However, for the cultivation of a high light intensity slowly decreased CA activity. Therefore, the interaction between pH and light intensity did not really effect the production of CA activity.

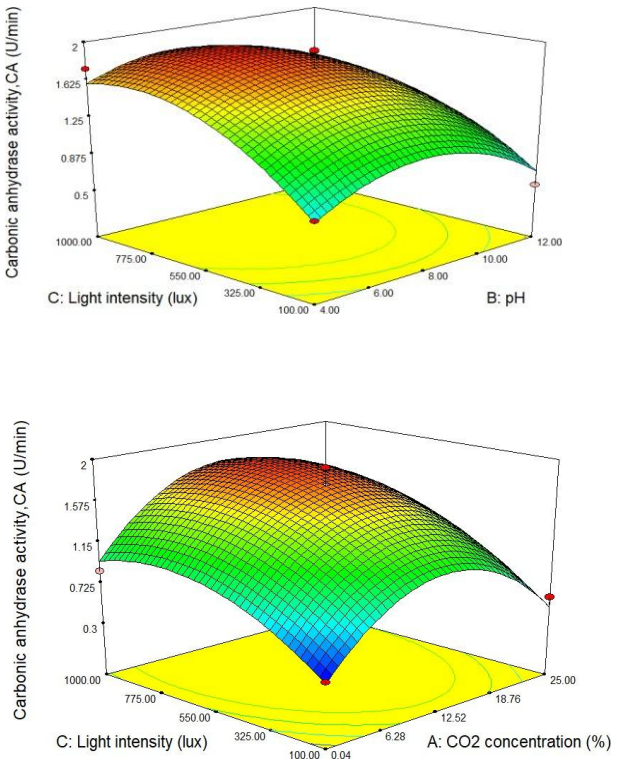

Figure 4. 3D plot on the effect of cultivation condition on the CA activity (a) effect of pH and light intensity (b) effect of pH and CO_2 concentration and (c) effect of light intensity and CO_2 concentration

4. Conclusion

In conclusion, this work showed that the cultivation condition parameters including the initial cultivation pH, light intensity and CO_2 are important variables that may be required to produce maximum CA activity by *Chlorella* sp. in an alginate polymer as a immobilised matrix carrier. The maximum CA activity of 1.908 U/min was observed when the cultivation was conducted using 15% of CO2 , with a pH of 8.00 and a light intensity of 550 lux. This present study also demonstrated that a microalgae biomass has great potential as a source of a carbonic anhydrase enzyme. The information generated from this study is crucial to determine the most suitable strategy for to be applied for CO_2 sequestration using CA enzyme.Adapting the most stable condition could play an important role to ensure the reduction of CO_2 and keeping environmental sustainability. On the other hand, results generated could assist in understanding the biomineralization by microalgae under an intergrated cultivation system, especially involves indutsrial processing plant.

References

[1] Araújo OdQF, and de Medeiros JL 2017 Carbon capture and storage technologies: present scenario and drivers of innovation *Current Opinion in Chemical Engineering*, **17** 22- 34.
[2] Grover M 2015 Elevated CO2: Plant associated microorganisms and carbon sequestration *Applied Soil Ecology* **95** 73-85.
[3] Prabhu C, 2009 Immobilization of carbonic anhydrase enriched microorganism on biopolymer based materials *Journal of Molecular Catalysis B: Enzymatic* **60** 13-21.
[4] Kassim MA and Meng TK 2017 Carbon dioxide (CO_2) biofixation by microalgae and its potential for biorefinery and biofuel production *Science of The Total Environment* **584-585** 1121-1129.
[5] Chen CY, Kao PC, Tsai CJ, Lee DJ, Chang JS 2013 Engineering strategies for simultaneous enhancement of C-phycocyanin production and CO_2 fixation with *Spirulina platensis Bioresource Technology* **145** 307-312.
[6] Tang D, Han W, Li P, Miao X, Zhong J 2011 CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. *Bioresource Technology* **102** 3071-3076.
[7]Boone CD, Gill S, Habibzadegan A, McKenna R 2013 Carbonic anhydrase: An efficient enzyme with possible global implications *International Journal of Chemical Engineering* **2013**: p. 6.
[8] Zeng, X., Danquah MK, Zhang S, Zhang X, Wu M, Chen XD, Ng IS, Jing K, Lu Y 2012 Autotrophic cultivation of *Spirulina platensis* for CO_2 fixation and phycocyanin production *Chemical Engineering Journal* **183** 192-197.
[9] Richmond A, Cheng-Wu Z, Zarmi Y 2003 Efficient use of strong light for high photosynthetic productivity: interrelationships between the optical path, the optimal population density and cell-growth inhibition *Biomolecular Engineering* **20**(4) 229-236.
[10] Muñoz R, and Guieysse, B 2006 Algal–bacterial processes for the treatment of hazardous contaminants: A review *Water Research* **40**(15) 2799-2815.
[11] Komala T, and Khun TC 2014 Biological carbon dioxide sequestration potential of *Bacillus pumilus.* Sains Malaysiana **43** 1149-1156.
[12] Zhang Z, Lian B, Hou W, Chen M, Li X, Shen W and Li Y 2011 Optimization of nutritional constituents for carbonic anhydrase production by *Bacillus mucilaginosus* K02 *African Journal of Biotechnology* **10**(42) 8403-8413.
[13] Beuf L, Kurano N, and Miyachi S 2000 Effect of external pH on inorganic carbon assimilation in unicellular marine green algae *Phycological Research* **48**(1) 47-54.
[14] Vaquero I, Vazquez M, Ruiz-Dominguez MC, Vilchez C 2014 Enhanced production of a lutein-rich acidic environment microalga *Journal of Applied Microbiology* **116**(4) 839-850.
[15] Shiraiwa Y, and Miyachi S, 1985 Effects of temperature and CO_2 concentration on induction of carbonic anhydrase and changes in efficiency of photosynthesis in *Chlorella vulgaris* 11h *Plant and Cell Physiology* **26**(3) 543-549.
[16] Ores, JC, De Amarante MCA, Fernandes SS, Kalil SJ 2016 Production of carbonic anhydrase by marine and freshwater microalgae *Biocatalysis and Biotransformation* **34**(2) 57-65.
[17] Dionisio ML, Tsuzuki, M, and Miyachi S 1989 Light requirement for carbonic anhydrase induction in *Chlamydomonas reinhardtii Plant and Cell Physiology* **30**(2) 207-213.
[18] Borodin VB, 2008 Effect of red and blue light on acclimation of *Chlamydomonas reinhardtii* to CO_2-limiting conditions *Russian Journal of Plant Physiology* **55**(4) 441.

[19] Rigobello-Masini M, Aidar E, and Masini JC 2003 Extra and intracelular activities of carbonic anhydrase of the marine microalga *Tetraselmis gracilis* (Chlorophyta) *Brazilian Journal of Microbiology* **34** 267-272.
[20] Almomani F, Ketife AA, Judd S, Shurair M, Bhosale RR, Znad H, Tawalbeh 2019 Impact of CO_2 concentration and ambient conditions on microalgal growth and nutrient removal from wastewater by a photobioreactor. *Science of The Total Environment* 662 662-671.
[21] Neves FdF, Hoinaski L, Rorig LR, Derner RB, Lisboa HdM 2018 Carbon biofixation and lipid composition of an acidophilic microalga cultivated on treated wastewater supplied with different CO_2 levels. *Environmental Technology* **40**(25) 3308-3317.
[22] Nimer NA, Brownlee C, and Merrett MJ 1999 Extracellular carbonic anhydrase facilitates carbon dioxide availability for photosynthesis in the marine dinoflagellate *Prorocentrum micans Plant physiology* **120**(1) 105-112.
[23] Xia JR, and Gao KS 2005 Impacts of elevated CO_2 concentration on biochemical composition, carbonic anhydrase, and nitrate reductase activity of freshwater green algae *Journal of Integrative Plant Biology* **47**(6) 668-675.
[24] Basu S, Roy AS, Mohanty K, Ghoshal AK 2014 CO_2 biofixation and carbonic anhydrase activity in *Scenedesmus obliquus* SA1 cultivated in large scale open system *Bioresource Technology* **164** 323-330.
[25] Solovchenko AE, Khozin-Golderg I, Didi-Cohen S, Cohen Z, Merzlyak MN 2008 Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incisa Journal of Applied Phycology* **20**(3) 245-251.
[26] Li L, Fu ML, Zhao YH, Zhu YT 2012 Characterization of carbonic anhydrase II from *Chlorella vulgaris* in bio-CO_2 capture *Environmental Science and Pollution Research* **19**(9) 4227-4232.

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