Bioresource Technology 271 (2019) 316–324

Contents lists available at ScienceDirect

Bioresource Technology

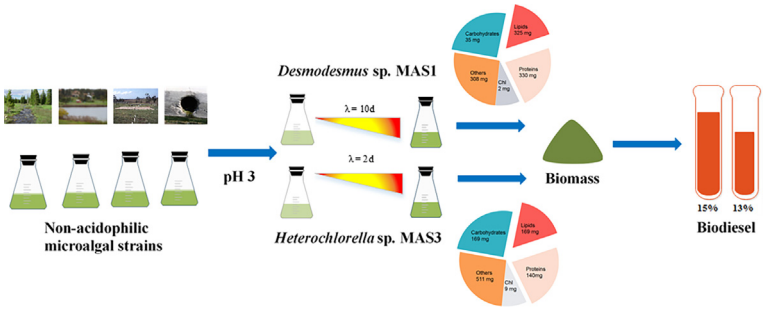
journal homepage: www.elsevier.com/locate/biortech

Sustainable production of biomass and biodiesel by acclimation of non-acidophilic microalgae to acidic conditions

Sudharsanam Abinandan[a], Suresh R. Subashchandrabose[a,c], Nicole Cole[b], Rajarathnam Dharmarajan[a,c], Kadiyala Venkateswarlu[d], Mallavarapu Megharaj[a,c,*]

[a] Global Centre for Environmental Remediation (GCER), Faculty of Science, ATC Building, University of Newcastle, Callaghan, NSW 2308, Australia
[b] Analytical and Biomolecular Research Facility (ABRF), University of Newcastle, Callaghan, NSW 2308, Australia
[c] Cooperative Research Centre for Contamination Assessment and Remediation of Environment (CRC CARE), University of Newcastle, ATC Building, Callaghan, NSW 2308, Australia
[d] Formerly Department of Microbiology, Sri Krishnadevaraya University, Anantapur 515055, India

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Microalgae
Non-acidophiles
Acclimation
Biochemical response
Lipids
Biodiesel

ABSTRACT

The overwhelming response towards algal biodiesel production has been well-recognized recently as a sustainable alternative to conventional fuels. Most microalgae cannot grow well at acidic pH. The present study, therefore, investigated whether non-acidophilic microalgae *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3 can be acclimated to extreme-acidic pH for sustainable production of biomass and biodiesel. Growth analysis indicated that both the microalgal strains possessed a passive uptake of CO_2 at pH 3.0 with biomass production of 0.25 g dry wt. L^{-1} in *Desmodemus* sp. and 0.45 g dry wt. L^{-1} in *Heterochlorella* sp.. Flow-cytometry analysis for reactive oxygen species, membrane permeability and neutral-lipids revealed the capabilities of both strains to adapt to the stress imposed by acidic pH. Lipid production was doubled in both the strains when grown at pH 3.0. *In-situ* transesterification of biomass resulted in 13–15% FAME yield in the selected microalgae, indicating their great potential in biofuel production.

1. Introduction

Microalgae significantly contribute to the environment through CO_2 fixation, contaminant reduction and production of biomass as a promising feedstock for biofuel. As ubiquitous primary producers, microalgae are crucial to the ecological biota. Recent research widely

* Corresponding author at: Global Centre for Environmental Remediation (GCER), Cooperative Research Centre for Contamination Assessment and Remediation of Environment (CRC CARE), Faculty of Science, The University of Newcastle, ATC Building, University Drive, Callaghan, NSW 2308, Australia.
E-mail address: megh.mallavarapu@newcastle.edu.au (M. Megharaj).

https://doi.org/10.1016/j.biortech.2018.09.140
Received 16 August 2018; Received in revised form 27 September 2018; Accepted 28 September 2018
Available online 29 September 2018
0960-8524/

acknowledged the influence of extreme environments such as ocean acidification and acid mine drainage on microalgal communities (Sassenhagen et al., 2015; Abinandan et al., 2018a). The most common phenomenon is the extent of pH that plays a critical role in algal growth dynamics. Several studies combined the effect of pH, nutrient starvation or cultivation modes for enhancing algal biomass preferably for increased biofuel production (Abinandan et al., 2018b). For instance, the addition of molasses (9.82 g L^{-1}) to serve as an organic carbon source at pH 6.7 resulted in higher yield (2 g L^{-1}) of microalgal biomass (Kose Engin et al., 2018). Cheirsilp and Torpee (2012) observed increased lipid content at a circumneutral pH upon exposure of microalgae to higher concentrations of glucose. Huang et al. (2017) demonstrated that microalgae turned the medium acidic with glucose when ammonium was predominantly present.

Most of the microalgae cannot survive at low pH (< 6.0) as the transporters become inactive (Perez-Garcia et al., 2011). Several studies indicated that exogenous supply of pure CO_2 or flue gas to enhance microalgal biomass productivity is favorable only at controlled pH maintained with bicarbonate availability (Ma et al., 2017). Jiang et al. (2012) reported that microalgae non-adapted to acidic conditions could not survive at pH 3.0, indicating that acid adaptation is imminent for survival. Also, microalgae, when grown at pH 4.0, exhibited a drastic decline in the biomass, suggesting the significant toxicity of pH (Khalil et al., 2010). Interestingly, even pH 4.5 inhibited 50% of growth in acid-tolerant microalgae (Nalewajko et al., 1997). El-Ansari and Colman (2015) also reported that acid-tolerant microalgae could not grow at pH 3.0 due to a decrease in intracellular pH. Thus, acid-tolerant microalgae are also sensitive to low pH, implying that only acidophiles are capable of growth under such extreme conditions due to the gene inheritance through evolutionary response (Hirooka et al., 2017). Sassenhagen et al. (2015) noted that microalgae could grow under a wide range of environmental conditions due to high phenotypic plasticity. An exogenous supply of carbon source (organic or inorganic) may be imminent for biofuel production (Kose Engin et al., 2018). Ma et al. (2017) suggested that pH of the medium (irrespective of carbon source) should be maintained at near neutral for microalgal cultivation. But, addition of hydroxides is required for maintenance of neutral pH and is not cost-effective (Abinandan et al., 2018b). However, available information suggests that non-acidophilic microalgae can withstand naturally-occurring acidic events such as ocean acidification by expressing high phenotypic plasticity or through adaptation process (Jiang et al., 2012).

While perusing the literature on remediation of acid mine drainage (AMD) by microalgae–bacteria biofilms, it was hypothesized that acclimation of non-acidophilic microalgae to acidic conditions might be a better option than applying acidophilic counterparts for reclamation of AMDs (Abinandan et al., 2018a). This is because under different environmental pressures such as acidic conditions, only limited strains of non-acidophilic microalgae could phenotypically adjust to thrive and grow (Abinandan et al., 2018a). To validate this hypothesis, four microalgae isolated from natural habitats of soil and lake waters with near neutrality exposed to pH 3.0 to investigate the microalgal growth response to acclimation at this acidic condition. Subsequently, two microalgal strains capable of growth at pH 3.0 were selected to assess the potential for sustained production of biomass under the environmental pressures imposed by extreme acidic conditions following flow cytometry, and yield of biodiesel following FTIR-based microalgal fatty acid methyl esters (FAME) analysis. The present study reports for the first time on acclimation of non-acidophilic microalgae to extreme acidic pH for the sustainable production of biomass and biodiesel.

2. Materials and methods

2.1. Microalgal strains and determination of growth rate

Microalgae were isolated from local soil and lake water samples by streaking onto agar with modified Bold's basal medium (BBM) with low phosphate. Cell sorting (BD FACSAria IIu) was done to obtain axenic cultures of the isolates. Briefly, log phase cells were sampled to measure chlorophyll dependent autofluorescence (FL3, 670 nm LP). The channel estimates at log scale and the sensitivity was set at 300 mV. Measurements of 10,000 events and 10^5 cells were sorted in sterile BBM and plated subsequently. The cells took nearly two weeks to develop axenic colonies. These isolates were grown at pH 3.0 (experimental) and pH 6.7 (control) in 30 mL BBM contained in 100 mL conical flasks under continuous illumination of 60 µmol m^{-2} s^{-1} at 23 ± 1 °C with 100 rpm shaking. The pH of the culture medium was monitored using LAQUA PC1100 pH meter (Horiba scientific, Japan).

Genomic DNA from algal strains was isolated using microbial DNA isolation kit (Mo Bio Laboratories, Inc.) as per the instructions provided. The DNA was amplified with 18S universal primers, the amplicons were cleaned using PCR and Gel kit (Bioline Laboratories, Inc.), and sequenced at Ramaciotti Centre, UNSW, Australia. The preliminary sequence identification was carried out for three isolates of microalgae using the NCBI Blast nucleotide search tool and a phylogenetic tree was constructed using MEGA 5.0 (Kumar et al., 2016). Phylogenetic analysis obtained from 1000 replicates as per the bootstrap test of clustal muscle alignment indicated that two of the microalgal isolates belong to the genus, *Desmodesmus*, with a slight difference of 3% similarity among nucleotides and hence designated as *Desmodesmus* sp. MAS1 and *Desmodesmus* sp. MAS2 (Fig. 1a). Since the third isolate is closely related to the genus, *Heterochlorella*, it has been designated as *Heterochlorella* sp. MAS3 (Fig. 1b). A well-studied *Chlorella* sp. MM3 (Ramadass et al., 2017; Subashchandrabose et al., 2017a,b; Ganeshkumar et al., 2018), obtained from in-house Phycology laboratory, was used in the present study as a reference microalga.

Microalgal growth, in terms of cell density, was determined in triplicate samples every alternate day using Neubauer hemocytometer (Bright line, Hausser Scientific, USA) under a light microscope (Olympus CX31, Japan). The growth rate was calculated using data at the exponential phase following the equation:

$$\mu = \frac{\ln N_1 - \ln N_0}{T_1 - T_0}$$

where N_1, N_0 are the final and initial cell densities, and T_1, T_0 are the times taken, in days.

2.2. Determination of growth response

Triplicate samples from microalgal cultures were withdrawn every week for determining the activity of carbonic anhydrase (CA), chlorophyll, biomass and metabolic biomarkers such as carbohydrates, proteins and total lipids. After sonicating the microalgal cell suspension, the activity of CA was measured in terms of esterase activity (Ores et al., 2016), and expressed as U L^{-1}. One unit (U) of enzyme activity is defined as the quantity of enzyme needed to release 1 µmol of *p*-nitrophenol min^{-1} in the assay conditions. Total chlorophyll and carbohydrates were estimated after methanol extraction (Chen and Vaidyanathan, 2013). Bradford bioassay was carried out to determine proteins using Bio-Rad kit (Bio-Rad Protein Assay Dye Reagent Concentration; Protein Standard II), and the color intensity was read in a spectrophotometer (Orion AquaMate 7000, Thermofisher Scientific, USA). Chloroform from the extracts was dried before gravimetric analysis of total lipids. Chlorophyll, carbohydrates and total lipids are expressed as mg g^{-1} dry wt. respectively. Microalgal biomass, in triplicate samples, was determined by the gravimetric method and expressed as g dry wt. L^{-1}.

2.3. Assay of reactive oxygen species (ROS), membrane permeability and neutral lipids

Aqueous stock solution (0.5 mg mL^{-1}) of DCFH-DA (Sigma, USA)

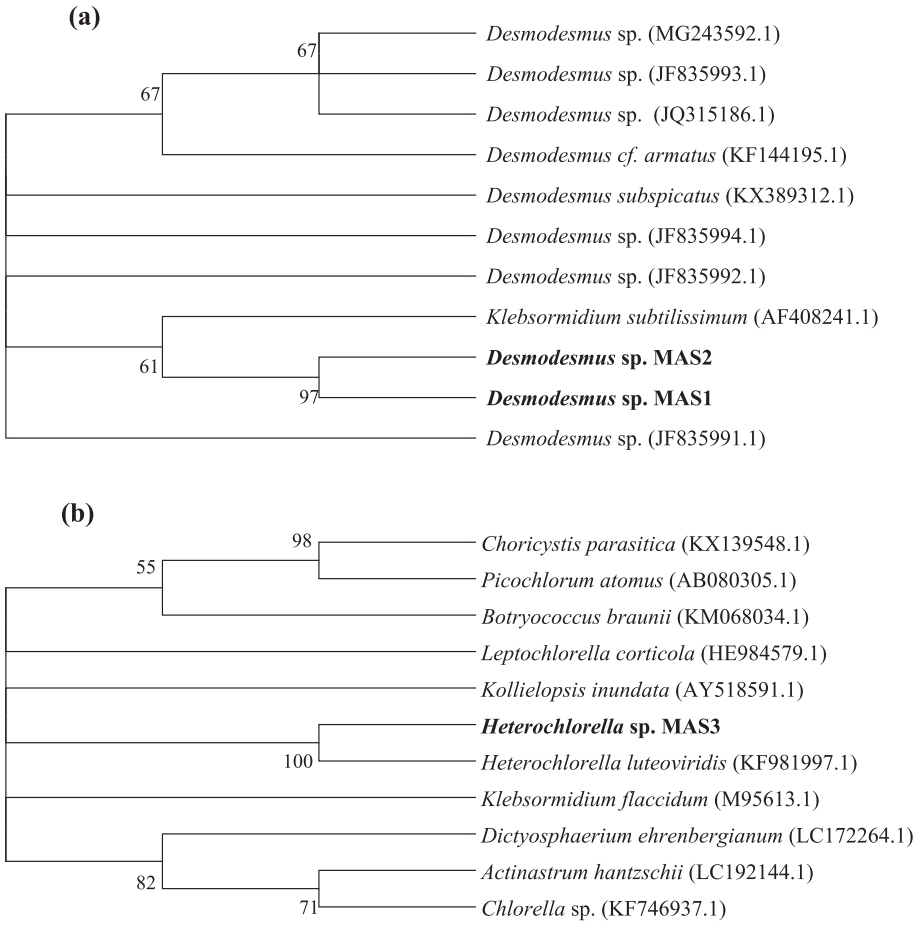


Fig. 1. Bootstrap consensus phylogenetic trees showing the evolutionary relationships of (a) *Desmodesmus* sp. MAS1 and *Desmodesmus* sp. MAS2, and (b) *Heterochlorella* sp. MAS3.

was used to determine ROS as described by Yilancioglu et al. (2014). Briefly, to 1 mL microalgal cell suspension, in triplicates withdrawn at desired intervals, 5 µL of dye solution was added and incubated for 20 min in the dark prior analysis. DCFH-DA, which is nonfluorescent, would pass inside the cells and converted into dichlorodihydrofluorescein (DCF) due to the activity of cellular esterase. Fluorescence of DCF formed was measured using a 488 nm laser and a 556LP 585/42 filter set on a BD FACSCanto Flow Cytometer.

Membrane permeability was measured using the staining dye, fluorescein diacetate (Sigma Aldrich, St. Louis, MO, USA), dissolved in acetone (1000 ppm, w/v) as per the method described by Chae et al. (2016). Intensities of fluorescein were measured in a BD FACSCanto Flow Cytometer fitted with a FITC filter (530/30), and the values were used to quantify cell permeability of each algal species. Non-stained cells were used as a negative control in both the channels to get the images for the samples stained. Data were evaluated using FlowJo Ver. 7.6.1 (Tree Star, Inc.).

Harvested microalgal suspensions were stained with 15 mM Nile Red (Sigma) to determine neutral lipids following a modified protocol of Dempster and Sommerfeld (1998). Aliquots of 50 µL solution of NR in acetone (0.1 mg mL^{-1}) were added to 1.0 mL suspension with gentle vortexing and incubated for 10 min at 37 °C in the dark. The uptake of NR in triplicate samples was monitored using a BD FACSCanto Flow Cytometer (Becton Dickinson Instruments) equipped with a 488 nm argon laser. The optical system used in the flow cytometer collects yellow and orange light (560–640 nm) that corresponds to neutral lipids. For the flow cytometry analysis, a positive control (heat-killed algal cells treated with the dye) and a negative control (untreated algal cells) were used, and the data were expressed in terms of fluorescence intensity as well as cell count.

2.4. FAME analysis

Analysis of FTIR-based FAME (Mathimani et al., 2015) from microalgal biomass was carried out by *in-situ* transesterification (Laurens et al., 2015) followed by gravimetric quantification. In brief, algal biomass in triplicates, harvested after three weeks was dried overnight at 40 °C and transesterified using 0.3 mL of HCl-MeOH blend (5%, v/v) for one h at 85 °C. The mixture was washed with water and chloroform to allow debris to be separated in the methanol layer and biodiesel in chloroform layer. Controls were maintained without the algal biomass. FT-IR spectroscopy was employed through Agilent Technologies Cary 660 FT-IR system working in mid-IR energy range (4000–400 cm^{-1}) in ATR mode to determine FAME. All the measurements were made through multi-bounce ZnSE ATR prism by placing FAME dissolved in chloroform. Solvent alone served as control. A total of 16 scans obtained for each sample were co-averaged to improve signal-to-noise ratio at a resolution of 8 cm^{-1} using air-cooled DTGS detector. All the spectra acquired are processed through Agilent IR Resolutions Pro software. Simultaneously, triplicate samples were analyzed for the yield of total lipids to make a comparison with the FAME yield.

2.5. Statistical analysis

The averages and standard deviations of the experimental data were identified using Graphpad Prism 7 software, and the statistical significance of means was determined by *t*-test using IBM SPSS Statistical Software (ver.24).

3. Results and discussion

3.1. Growth response of non-acidophilic microalgae to acidic pH

Initially, three different cell densities (1×10^4, 1×10^5, 5×10^5 cells mL^{-1}) of all the four microalgal strains were used to screen for assessment of tolerance and growth at pH 3.0. All the four microalgae at both the cell densities of 1×10^4 and 1×10^5 cells mL^{-1} could not survive after 12 days (Supplementary Data). However, *Desmodesmus* sp. MAS1, *Desmodesmus* sp. MAS2 and *Heterochlorella* sp. MAS3 at the cell density of 5×10^5 cells mL^{-1} grew well during this incubation period. Interestingly, *Chlorella* sp. MM3 that was used as a control did not survive even at this higher cell density. Thus, these results clearly indicate that *Desmodesmus* sp. MAS1, *Desmodesmus* sp. MAS2 and *Heterochlorella* sp. MAS3 with good phenotypic plasticity could withstand acidic conditions (Sassenhagen et al., 2015). Such a tolerance in non-acidophiles to stress mediated by acidic conditions is unique since most of the microalgae are reported to be neutrophils as they grow ideally at circumneutral pH (Hirooka et al., 2017). Furthermore, *Desmodesmus* sp. MAS2 exhibited more than ten days of lag phase (data not shown) when compared to other two microalgae, confirming that the strain MAS2 is distinct from MAS1 (Fig. 1b). Based on these observations, only two cultures, viz., *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3, were used for further studies on growth and metabolic activities.

The maximum specific growth rates for *Heterochlorella* sp. MAS3 and *Desmodesmus* sp. MAS1 at pH 6.7 were 0.20 and 0.15 day^{-1}, and 0.19 and 0.13 day^{-1} at pH 3.0, respectively (Fig. 2). Similarly, an acidophilic microalga, *Coccomyxa onubensis*, achieved an approximate growth rate of 0.16 day^{-1} at pH 2.5 under light intensity of 50 µmol m^{-2} s^{-1} (Vaquero et al., 2014). On the other hand, the doubling times when grown at pH 6.7 for *Heterochlorella* sp. MAS3 and *Desmodesmus* sp. MAS1 were 3.4 and 4.6 days, respectively, and the corresponding values at pH 3.0 were 3.6 and 5.5 days. Thus, the growth rates observed for both the microalgal strains are much lower than those reported for other microalgae found growing abundantly in acidic pH environment (Sassenhagen et al., 2015), suggesting that the microalgae used in the present study are non-acidophiles that grow normally at circumneutral conditions. The possible reason for the lower growth rate in non-acidophilic microalgae could be due to culture conditions and low-level expression of proteins under acidic conditions (Hirooka et al., 2017). Since microbial acid tolerance is indicative of an adaptive response that results in enhanced tolerance to pH 3.0, both the microalgae used in the present study can be considered as 'acid-tolerant' strains. Again, *Heterochlorella* sp. MAS3 is a better acid-tolerant strain than *Desmodesmus* sp. MAS1.

The response of the two microalgae to acidic conditions (pH 3.0) was studied employing three growth parameters such as the activity of CA, chlorophyll content and biomass production. The growth of microalgae in autotrophic conditions is primarily dependent on carbon concentration mechanisms (CCMs) through CA activity and passive diffusion uptake of CO_2. The activity of CA depends upon the presence of bicarbonate ions (HCO_3^-) in the culture medium that regulates overall microalgal growth. Since CA activity can serve as an indicator of growth in photoautotrophs, this enzyme was also considered to assess the response of selected microalgal strains to acidic pH. In general, CA activity in the present study significantly declined when both the strains were grown for three weeks at pH 6.7 (Fig. 3a). Thus, the reduction in enzyme activity in *Heterochlorella* sp. MAS1 was 53% while it was 21% in the case of *Desmodesmus* sp. MAS3. A similar trend in decline of CA activity was observed in the cultures grown at pH 3.0. However, particularly after two weeks of growth at pH 3.0, the enzyme activity increased by 31 and 247% in *Heterochlorella* sp. MAS3 and *Desmodesmus* sp. MAS1, respectively, over the activities of CA observed at pH 6.7. This differential activity of CA over incubation period indicates that CCM is species-dependent. Also, the increase in CA activity corresponds to the bicarbonate transport, which is probably active as growth precedes with an increase in pH (Moroney and Ynalvez, 2007). Furthermore, this observed trend of increase in CA activity may be due to the adaptive response to the bicarbonate availability in microalgae.

The present observation also suggests that both the microalgae survived in the first week at pH 3.0 through passive diffusion uptake of CO_2 rather than CA activity. Similarly, El-Ansari and Colman (2015) observed that an acid-tolerant microalga, *Chlorella kessleri*, grew well at pH 4 under continuous illumination (50 µmol m^{-2} s^{-1}) by maintaining near neutral internal pH through passive diffusion of CO_2. The passive diffusion of CO_2 is seemingly more in *Heterochlorella* sp. MAS3 than in *Desmodesmus* sp. MAS1. Thus, *Heterochlorella* sp. MAS3 must have maintained growth at acidic pH similar to that at pH 6.7 through the accumulation of CO_2 in high concentrations at the active site of Rubisco as suggested by El-Ansari and Colman (2015). Interestingly, both the strains showed low CA activity after three weeks probably due to increase in pH by triggering carbonate (CO_3^{2-}) synthesis from HCO_3^-. In all, the above results indicate that the two microalgae grew at pH 6.7 through CCM involving CA activity while both the mechanisms (passive diffusion uptake of CO_2 followed by CA activity) were used at pH 3.0.

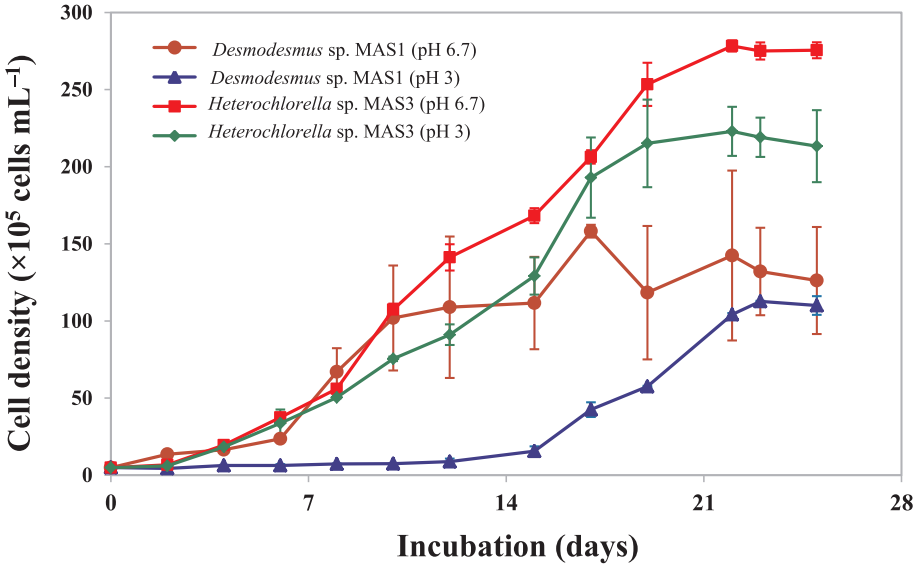


Fig. 2. Growth of *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3 at pH 6.7 and 3.0. Error bars represent standard deviation (n = 3).

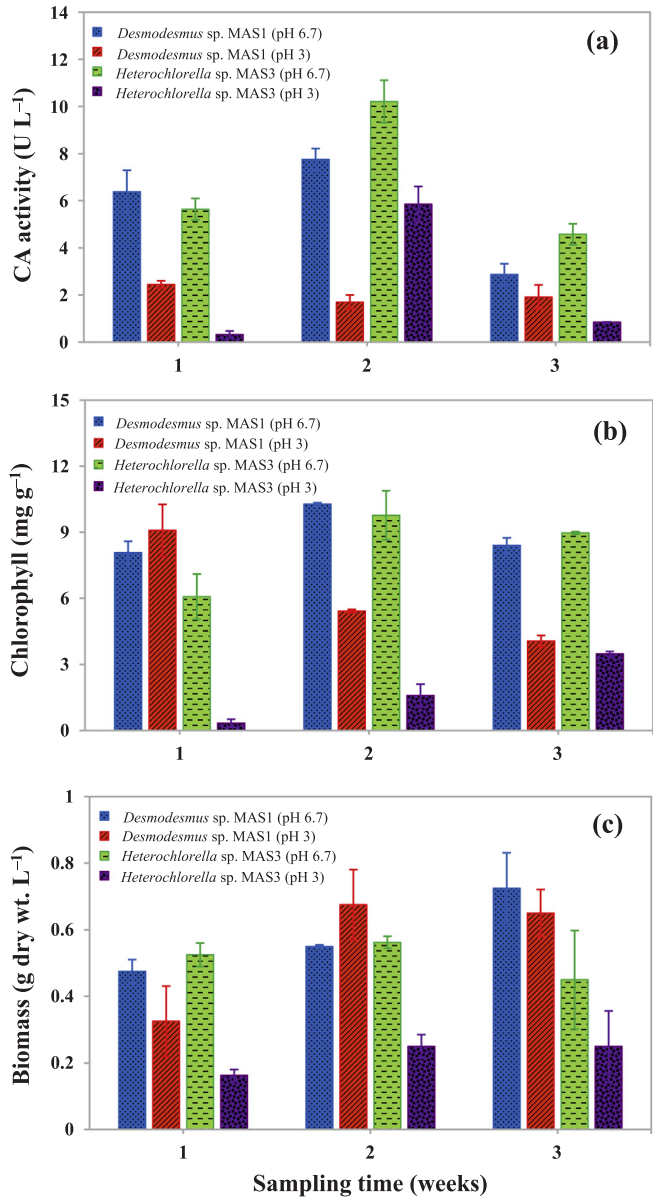


Fig. 3. (a) Carbonic anhydrase (CA) activity, (b) Chlorophyll and (c) Biomass in *Heterochlorella* sp. MAS3 and *Desmodesmus* sp. MAS1 after growth at pH 6.7 and 3.0. Error bars represent standard deviation (n = 3).

The chlorophyll content in *Heterochlorella* sp. MAS3 grown at pH 6.7 for one week was 8.08 mg g^{-1} whereas the corresponding value in *Desmodesmus* sp. MAS1 was 9.08 mg g^{-1} (Fig. 3b). Interestingly, chlorophyll decreased significantly after three weeks of incubation and reached a concentration of 4.05 mg g^{-1}. Such a decrease in chlorophyll content is expected since nitrogen depletion even at pH 6.6 after 20 days may impair the photosynthetic activity thereby decrease the pigments like chlorophyll as in case of a red microalga, *Porphyridium cruentum* (Zhao et al., 2017). Overall, the growth response in terms of chlorophyll content at pH 3.0 was also like that observed with biomass productivity. However, there was a drastic decrease (9-fold) in chlorophyll of *Desmodesmus* sp. MAS1 at pH 3.0 after the first week, indicating impairment of photosystem and the contribution of nitrogen pool mostly for survival rather than chlorophyll metabolism (Jiang et al., 2011). But, the concentration of chlorophyll increased from 0.33 mg g^{-1} (dry wt.) to 3.48 mg g^{-1} in strain MAS1 after three weeks of incubation at pH 3.0. Vaquero et al. (2014) showed that microalgae growing at pH 2.5 with a high inoculum density and light intensity of 400 μmol m^{-2} s^{-1} accumulated higher concentrations of lutein rather than chlorophyll without compromising biomass productivity.

When grown at pH 3.0 for one week, there was no change in biomass of *Heterochlorella* sp. MAS3 while a significant decrease was evident in *Desmodesmus* sp. MAS1 (Fig. 3c). Thus, the reduction in biomass of the strain MAS1 after one week of growth at pH 3.0, when compared with that at pH 6.7, was 50% and was 43% at the end of two weeks. However, after three weeks of incubation at pH 3.0, the decrease of biomass in *Desmodesmus* sp. MAS1 was 40%, whereas the reduction in case of *Heterochlorella* sp. MAS3 was only 28% during this period. Jian et al. (2012) also observed > 1-fold decrease in biomass production in non-adapted *Scenedemus dimorphus* grown at pH 3.0. Similarly, Eibl et al. (2014) reported low biomass production (0.5 g L^{-1}) even in acidophilic microalgae after 35 days. However, the overall yield of biomass in *Heterochlorella* sp. MAS3 decreased in acidic medium continuously for three weeks, while there was a significant increase in biomass of *Desmodesmus* sp. MAS1, clearly suggesting that the acclimation response in the latter strain was higher when compared with the former strain. However, the data also indicate that both the microalgal strains produced higher biomass at pH 3.0 when compared with those acidophilic microalgae grown in the presence of 15–20% CO_2 (Neves et al., 2018), suggesting that microalgae potential for biomass production even in hostile environments such as AMDs.

3.2. Biochemical and stress response in microalgae to acidic pH

The impact of acidic pH on biochemicals, viz., total proteins, carbohydrates and lipids in microalgae are very crucial in understanding the changes in metabolism. *Heterochlorella* sp. MAS3 accumulated high concentrations (nearly 3-fold) of carbohydrates while *Desmodesmus* sp. MAS1 recorded a significant decrease (23%) (Fig. 4a) when grown at pH 6.7 for three weeks. Such a differential response among the microalgal strains even at neutral pH could be due to sharp changes in nutrient status of the medium. For instance, Rizza et al. (2017) observed low levels of carbohydrates in *Scenedesmus* sp. under limited conditions of nitrogen. Although carbohydrate accumulation in *Heterochlorella* sp. MAS3 decreased by 50% at pH 3.0 after one week; there was > 5 and > 7-fold increase at the end of two and three weeks of incubation, respectively. On the other hand, the accumulation of carbohydrates increased by 48% in *Desmodesmus* sp. MAS1 after two weeks when grown at pH 3.0, but significantly decreased (2-fold) after three weeks. Khalil et al. (2010) also reported such a decrease in *Dunaliella bardawil* and *Chlorella ellipsoidea* since endogenous carbohydrates are used for survival at pH 4.0. Contrary to the response of carbohydrates, the extent of protein accumulation in the selected microalgae was entirely different. Thus, even at pH 6.7, the protein increases in *Desmodesmus* sp. MAS1 after a week was 3.5-fold when compared with *Heterochlorella* sp. MAS3 (Fig. 4b). Interestingly, there was a significant decrease over time in strain MAS1 and increase in strain MAS3. The observed decline in *Desmodesmus* sp. MAS1 could be ascribed to nitrogen limitation since Yilancioglu et al. (2014) perceived that certain microalgae shift to accumulate higher lipid and less protein especially under nitrogen starvation at a near neutral pH. It has been well established in the literature that the response to limitation and consequent depletion of nitrogen results in a decrease in photosynthetic pigment as well as protein content and an increase in lipid content of microalgae (Vo et al., 2018). Overall, protein accumulation significantly increased after three weeks in both the strains when grown at pH 3.0. Thus, the increase in protein content after three weeks in *Heterochlorella* sp. MAS3 was 34% as against to 58% enhancement in *Desmodesmus* sp. MAS1, indicating that protein-coding genes may have been upregulated in acidic pH. The total lipids increased significantly in *Desmodesmus* sp. MAS1 as compared to *Heterochlorella* sp. MAS3 after one week at pH 6.7 (Fig. 4c). Incubation of the strain MAS1 for two weeks at pH 3.0 significantly increased (119%) the protein content, but the increase declined to only 15% at the end of three weeks. Some

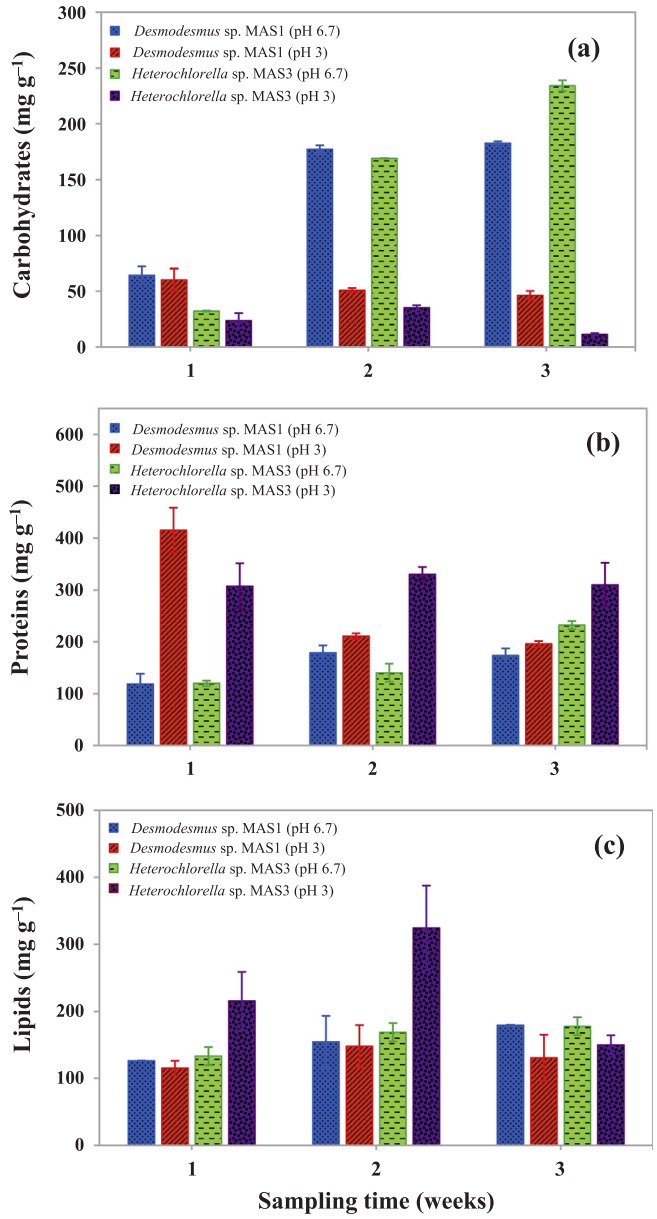


Fig. 4. (a) Carbohydrates, (b) Proteins, and (c) Lipids in *Heterochlorella* sp. MAS3 and *Desmodesmus* sp. MAS1 after growth at pH 6.7 and 3.0. Error bars represent standard deviation (n = 3).

reports indicated that nitrogen deprivation caused lipid induction even in acidic algae (Hirooka et al., 2014; 2017). Eibl et al. (2014) observed a three-week lag phase and appearance of green to yellow color in microalgae isolated from mine environment (pH 3.0) and correlated this change to the synthesis of lipids. There was a two-fold increase in lipid content of *Desmodemsus* sp. MAS1 at pH 3.0 while the increase was only one-fold in *Heterochlorella* sp. MAS3 after two weeks of incubation. The above results indicate that *Desmodesmus* sp. MAS1 yields proteins and total lipids better than *Heterochlorella* sp. MAS3 under acidic conditions.

Since it is also essential to understand the stress response to extreme environmental fluctuations (Yilancioglu et al., 2014), advocates for the use of flow cytometry analysis in the present study. Thus, ROS, membrane permeability and neutral lipids were used as the criteria for stress response in microalgae. The ROS (Fig. 5a) profile showed that the stress was apparent only in *Desmodesmus* sp. MAS1. After the first week, there was a 2-fold increase in ROS at pH 3.0 but dropped at the end of the second week, and the microalga was relieved entirely from stress after three weeks. Eibl et al. (2014) also observed identical pattern of increased stress after one week in an acidophilic microalga, *Scenedesmus* sp. Lig 290, isolated from a waterbody with pH 4.5. On the contrary, there was no stress in *Heterochlorella* sp. MAS3 even after two weeks, indicating its high potential in adapting quickly to the changed pH. The data presented in Fig. 5b indicate changes in the membrane permeability of the non-acidophilic microalgae when grown in acidic conditions. *Desmodesmus* sp. MAS1 exhibited a significant (> 2-fold) decrease in fluorescence signal at pH 3.0 when compared to its growth at neutral pH, indicating the low permeability of the dye into the cells in acidic conditions. However, the fluorescence signal gradually increased in *Heterochlorella* sp. MAS3 upon growth at pH 3.0. These differences in membrane permeability observed because of structural and physiological changes in both the strains grown at pH 3.0 corroborate with the results on ROS. Similarly, neutral lipid profile (Fig. 5c) indicates a tremendous increase in fluorescence intensity in *Desmodesmus* sp. MAS1 is grown at pH 3.0. Thus, the observed 3.3-fold increase in neutral lipids in the first week corroborates with the enhancement of membrane permeability and ROS production noticed during this period. This increase in lipid was also observed even after two weeks, which may be due to the limitation of nutrients, especially nitrogen. Yao et al. (2016) also found an increasing trend in the lipid content of *Dunaliella salina* under osmotic shock through Nile red fluorescence. Thus, the present results on neutral lipids in *Desmodesmus* sp. MAS1 suggest that the microalga during acclimation to extreme acidic conditions changed its metabolic pathway to protein and lipid, especially higher neutral lipid faction, synthesis resulting in decreased carbohydrate reserves. In contrast, *Heterochlorella* sp. MAS3 did not show significant changes in lipid profile, a response similar in case of ROS production.

3.3. Biodiesel production in microalgae at acclimated acidic pH

Since FTIR-based microalgal FAME (biodiesel) analysis is considered as a rapid method compared to the conventional techniques (Mathimani et al., 2015), and this approach employed in the study to monitor the biofuel production from algal biomass through *in-situ* transesterification. FTIR spectral analysis of FAME exhibit strong peak areas as suggested for the presence of ester molecules and the characterization of biodiesel from microalgae (details can be found in the Supplementary Data). The appearance of peaks in the region of 1730–1743 cm^{-1} is due to C═O stretching vibrations from the mixture of carboxylic acid esters, indicating the conversion of triglycerides (Viêgas et al., 2015). Again, the peak region at 1106–1114 cm^{-1} is due to asymmetric vibrational stretching of esters group arising from methyl C—O, confirming that the biodiesel is predominantly a monoalkyl ester (Sitthithanaboon et al., 2015). Furthermore, the bending vibrational frequency of C—O as evident in the region 1232–1236 cm^{-1} confirms the presence of ester molecules as has been established for a fatty acid ester derived from hydrothermal liquefaction of *Dunaliella teriolecta* biomass by Zou et al. (2009). The occurrence of rich aliphatic hydrocarbons in the present samples, as evident at 2835 cm^{-1} and 2943–2945 cm^{-1} through $-CH_2/-CH_3$ symmetric and asymmetric stretching vibrations, respectively, indicates the good quality of biodiesel (Mathimani et al., 2015). Interestingly, another peak has also been observed at 1650–1652 cm^{-1} that corresponds to the C═C stretching vibration arising from *cis*-olefins, suggesting a degree of unsaturation that is found in vegetable oils (Timilsena et al., 2017). The 3D-IR image analysis for the region 1700–1800 cm^{-1} after deconvolution of the peaks as shown in Supplementary Data confirms the proper transesterification in both the microalgal strains. It also proves that the conversion of triglyceride to ester is high in biomass samples collected at pH 3.0 when compared to those grown at pH 6.7.

The yield of FAME was 11 and 13% from *Desmodesmus* sp. MAS1 when grown at pH 6.7 and 3.0, respectively, and the corresponding values for FAME yield in *Heterochlorella* sp. MAS3 were 7 and 15% (Fig. 6). While Laurens et al. (2012) reported 9–10% yield of FAME in

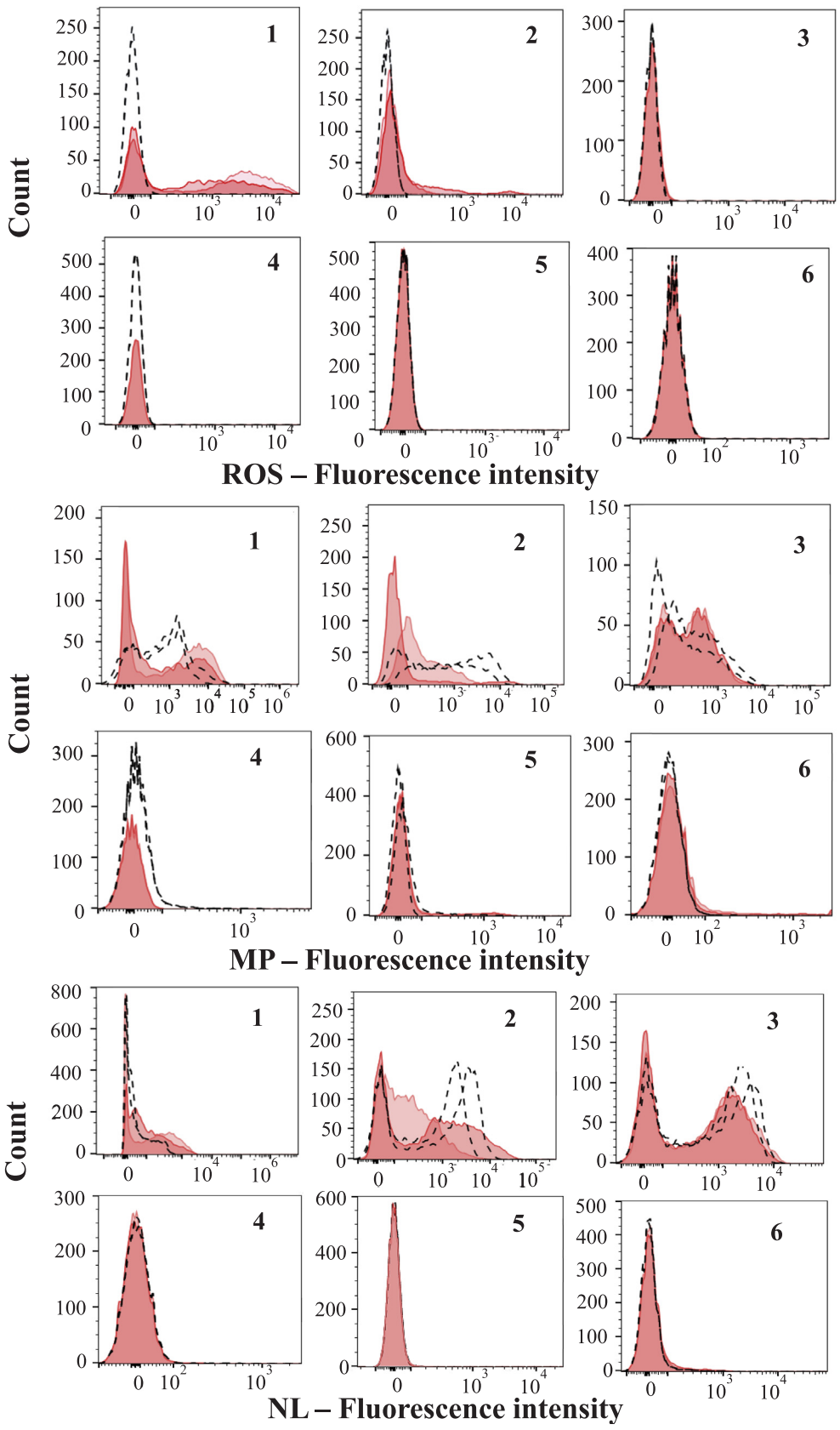


Fig. 5. Three-week response of (a) Reactive oxygen species (ROS), (b) Membrane permeability (MP), and (c) Neutral lipids (NL) in *Desmodesmus* sp. MAS1 (1–3); and *Heterochlorella* sp. MAS3 (4–6) after growth at pH 6.7 (- -) and pH 3.0 ( ).

Chlorella vulgaris and *Nannochloropsis* sp. under normal growth conditions, Tang et al. (2016) observed a FAME yield of 10% (on a dry weight basis) in *Nannochloropsis* sp. The results indicate that the FAME yield in both the microalgal strains when acclimated to acidic conditions is higher than those reported in the literature. Recently, Souza et al. (2017) reported a 15% oil yield in *Chlamydomonas acidophila* LAFIC-004 grown under acidic conditions. In general, the lipid to FAME yield was low in both the microalgae acclimated to acidic pH. Similarly,

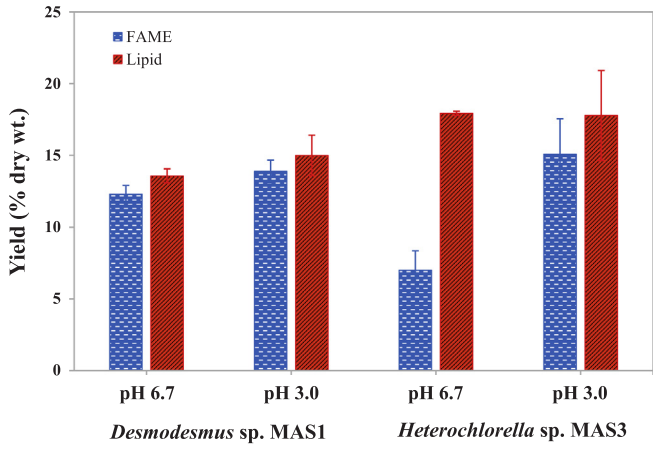


Fig. 6. Yield (% dry wt.) of FAME and lipids in *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3 when grown at pH 6.7 and 3.0. Error bars represent standard deviation (n = 3).

Ruiz-Dominguez et al. (2015) observed that lipid to FAME yield was low even in acidophilic microalgae. The FAME yield in *Heterochlorella* sp. MAS3 at pH 6.7 was low probably because of non-optimization of lipid to oil conversion (Ehimen et al., 2010). However, *Heterochlorella* sp. MAS3 gave a higher yield of lipids and FAME than *Desmodesmus* sp. MAS1 at pH 3.0. Likewise, Eibl et al. (2014) observed that acidophilic microalgae are growing at optimum pH of 4.0 accumulated higher lipids relative to those grown at pH 7.0. Pick and Avidan (2017) showed that microalgae under nitrogen limitation assimilated neutral lipids made from C allocation. The present observation of a significant increase in lipids and FAME indicates the stress mediated by both acidic pH and nutrient depletion, with the former occurring initially and the latter in the third week of microalgal growth. Such a sequential stress response was observed earlier in acidophilic microalgae wherein they accumulated more lipids under nitrogen limited conditions at a pH range of 3–5 (Hirooka et al., 2014). The efficiency in conversion of lipid to biodiesel was relatively higher in *Desmodesmus* sp. MAS1 than in *Heterochlorella* sp. MAS3, indicating the accumulation of tranesterifiable lipids (triacylglycerols, TAGs) due to the stress induced by acidic conditions. Based on the results, *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3 is designated as acid tolerant strain.

3.4. Practical applications and prospects of the present findings

Very recent report from NASA (Global Climate Change, 2018) indicates that anthropogenic activities resulted in a relative 93% increase in atmospheric CO_2 during the last 12 yrs. Also, the European Union dedicated to cut CO_2 and other greenhouse gas emissions based on the UN Framework using microalgae (CORDIS, 2013). Sequestration of CO_2 by microalgae is often limited due to mass transfer in raceway ponds and requires the specific design of photobioreactors to produce biomass for revenue generation (Abinandan et al., 2018b). Furthermore, CO_2 reacts with water to form carbonic acid resulting in very acidic pH necessitating the addition of hydroxides to maintain the required bicarbonate for microalgal growth (Van Den Hende et al., 2012). These factors cause more capital and energy investments, thus always underestimating the promising values of the microalgal technology for biomass production. It is imperative to explore and identify the microalgae as a primary indicators/tools to sequester at least the CO_2 emission from industrial activities such as flue gas that contributes about 10–20% CO_2 (Sakarika and Kornaros, 2016). However, the flue gas reaction with water creates a very acidic environment suggesting that the acidophilus microalgae are a possible alternative for CO_2 sequestration (Neves et al., 2018).

The stress for acidophilic microalgae in acidic conditions is much less compared to non-acidophiles and it reflects in less biofuel production by the former group. Hence, non-acidophilic microalgae should be bio-prospected for biomass and biodiesel production under extreme acidic environments. Several studies that exploited microalgae to produce biofuel relied mostly on nutrient stress and varying other growth conditions such as phototrophy, mixotrophy and heterotrophy (Cheirsilp and Torpee, 2012; Huang et al., 2017; Ma et al., 2017; Kose Engin et al., 2018). However, while acclimating non-acidophilic microalgae for lipid production, their response to varying nutrients needs to be thoroughly understood. In fact, this approach would be especially beneficial in many developing countries such as Vietnam, Laos, Cambodia and Myanmar for alleviating the levels of excess nutrients in wastewaters and probable effluents from the mine sites (Reichl et al., 2018). For instance, wastewaters from shrimp farming in Vietnam generate high amounts of nitrogen ($159\,kg^{-1}\,ha^{-1}\,crop^{-1}$) and phosphates ($19.6\,kg^{-1}\,ha^{-1}\,crop^{-1}$) that can be utilized for microalgal biofuel production (Vo et al., 2018). Also, reports suggest that acid-tolerant microalgae produce carotenoids and lutein in response to stress implying their applications for producing commercial nutraceuticals (Vaquero et al., 2014). Interestingly, acidophilic microalgae found in such extreme environments as AMDs develop tolerance to survive rather than remediating the contaminants (Abinandan et al., 2018a). Hence, consortia of acidophilic microalgae and acid-tolerant microalgae obtained from non-acidophilic environments can be the best candidates for remediation of AMDs. Future research must include a wide array of non-acidophilic microalgae to establish their greater potential in production of biomass and biofuel under extreme acidic conditions.

4. Conclusions

Present investigation demonstrated that selective non-acidophilic microalgae can grow well under acidic conditions through passive diffusion of CO_2. This acclimation could be ascribed to downregulation of carbohydrate-pathway and upregulation of protein and lipid-pathways as evidenced in *Desmodesmus* sp. MAS1 and to a lesser extent in *Heterochlorella* sp. MAS3. The FAME yield during acclimation was relatively high in both the strains compared to neutral conditions, indicating the biofuel potential. This study provides a proof-of-the-concept that non-acidophilic microalgae can acclimated to acidic conditions for the potential use in the sustainable production of biomass and biodiesel under extremely acidic conditions as exist in AMDs.

Acknowledgment

SA acknowledges the RTP Scholarships (APA and IPRS) provided by the University of Newcastle (UoN). Also, we thank Analytical and Biomolecular Research Facility (ABRF), UoN for access to Flow-cytometry.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.09.140.

References

Abinandan, S., Subashchandrabose, S.R., Venkateswarlu, K., Megharaj, M., 2018a. Microalgae–bacteria biofilms: a sustainable synergistic approach in remediation of acid mine drainage. Appl. Microbiol. Biotechnol. 102, 1131–1144.

Abinandan, S., Subashchandrabose, S.R., Venkateswarlu, K., Megharaj, M., 2018b. Nutrient removal and biomass production: advances in microalgal biotechnology for wastewater treatment. Crit. Rev. Biotechnol. 38, 1244–1260.

Chae, Y., Kim, D., An, Y.J., 2016. Effect of fluoride on the cell viability, cell organelle potential, and photosynthetic capacity of freshwater and soil algae. Environ. Pollut. 219, 359–367.

Cheirsilp, B., Torpee, S., 2012. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration

and fed-batch cultivation. Bioresour. Technol. 110, 510–516.
Chen, Y., Vaidyanathan, S., 2013. Simultaneous assay of pigments, carbohydrates, proteins and lipids in microalgae. Anal. Chim. Acta 776, 31–40.
CORDIS, 2013. https://cordis.europa.eu/news/rcn/35593_en.html (Accessed 19.09. 2018).
Dempster, A., Sommerfeld, R., 1998. Effects of environmental conditions on growth and lipid accumulation in *Nitzschia communis* (Bacillariophyceae). J. Phycol. 34, 712–721.
Eibl, J.K., Corcoran, J.D., Senhorinho, G.N., Zhang, K., Hosseini, N.S., Marsden, J., Laamanen, C.A., Scott, J.A., Ross, G.M., 2014. Bioprospecting for acidophilic lipid-rich green microalgae isolated from abandoned mine site water bodies. AMB Exp 4, 7.
Ehimen, E.A., Sun, Z.F., Carrington, C.G., 2010. Variables affecting the in situ transesterification of microalgae lipids. Fuel 89, 677–684.
El-Ansari, O., Colman, B., 2015. Inorganic carbon acquisition in the acid-tolerant alga *Chlorella kessleri*. Physiol. Plantar. 153, 175–182.
Ganeshkumar, V., Subashchandrabose, S.R., Dharmarajan, R., Venkateswarlu, K., Naidu, R., Megharaj, M., 2018. Use of mixed wastewaters from piggery and winery for nutrient removal and lipid production by *Chlorella* sp. MM3. Bioresour. Technol. 256, 254–258.
Global Climate Change, NASA, 2018. https://climate.nasa.gov/vital-signs/carbon-dioxide (Accessed 19.09.2018).
Hirooka, S., Higuchi, S., Uzuka, A., Nozaki, H., Miyagishima, S.Y., 2014. Acidophilic green alga *Pseudochlorella* sp. YKT1 accumulates high amount of lipid droplets under a nitrogen-depleted condition at a low-pH. PLoS One 9, 107702.
Hirooka, S., Hirose, Y., Kanesaki, Y., Higuchi, S., Fujiwara, T., Onuma, R., Era, A., Ohbayashi, R., Uzuka, A., Nozaki, H., Yoshikawa, H., Miyagishima, S.Y., 2017. Acidophilic green algal genome provides insights into adaptation to an acidic environment. Proc. Natl. Acad. Sci. USA 114, E8304–E8313.
Huang, A., Sun, L., Wu, S., Liu, C., Zhao, P., Xie, X., Wang, G., 2017. Utilization of glucose and acetate by *Chlorella* and the effect of multiple factors on cell composition. J. Appl. Phycol. 29, 23–33.
Jiang, Y., Peng, X., Zhang, W., Liu, T., 2012. Enhancement of acid resistance of *Scenedesmus dimorphus* by acid adaptation. J. Appl. Phycol. 24, 1637–1641.
Jiang, L., Luo, S., Fan, X., Yang, Z., Guo, R., 2011. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO_2. Appl. Energy 88, 3336–3341.
Khalil, Z.I., Asker, M.M., El-Sayed, S., Kobbia, I.A., 2010. Effect of pH on growth and biochemical responses of *Dunaliella bardawil* and *Chlorella ellipsoidea*. World J. Microbiol. Biotechnol. 26, 1225–1231.
Kose Engin, I., Cekmecelioglu, D., Yücel, A.M., Oktem, H.A., 2018. Enhancement of heterotrophic biomass production by *Micractinium* sp. ME05. Waste Biomass Valori. 9, 811–820.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Laurens, L.M.L., Nagle, N., Davis, R., Sweeney, N., Van Wychen, S., Lowell, A., Pienkos, P.T., 2015. Acid-catalyzed algal biomass pretreatment for integrated lipid and carbohydrate-based biofuels production. Green Chem. 17, 1145–1158.
Laurens, L.M.L., Quinn, M., Van Wychen, S., Templeton, D.W., Wolfrum, E.J., 2012. Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. Anal. Bioanal. Chem. 403 (1), 167–178.
Ma, M., Yuan, D., He, Y., Park, M., Gong, Y., Hu, Q., 2017. Effective control of *Poterioochromonas malhamensis* in pilot-scale culture of *Chlorella sorokiniana* GT-1 by maintaining CO_2-mediated low culture pH. Algal Res. 26, 436–444.
Mathimani, T., Uma, L., Prabaharan, D., 2015. Homogeneous acid catalysed transesterification of marine microalga *Chlorella* sp. BDUG 91771 lipid – An efficient biodiesel yield and its characterization. Renew. Energy 81, 523–533.
Moroney, J.V., Ynalvez, R.A., 2007. Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*. Eukaryot. Cell 6, 1251–1259.
Neves, F.D.F., Hoinaski, L., Rörig, L.R., Derner, R.B., de Melo Lisboa, H., 2018. Carbon biofixation and lipid composition of an acidophilic microalga cultivated on treated wastewater supplied with different CO_2 levels. Env. Technol. https://doi.org/10.1080/09593330.2018.1471103.
Nalewajko, C., Colman, B., Olaveson, M., 1997. Effects of pH on growth, photosynthesis, respiration, and copper tolerance of three *Scenedesmus* strains. Environ. Exp. Bot. 37 (2–3), 153–160.
Ores, J.D.C., Amarante, M.C.A.D., Kalil, S.J., 2016. Co-production of carbonic anhydrase and phycobiliproteins by Spirulina sp. and Synechococcus nidulans. Bioresour. Technol. 219, 219–227.
Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y., 2011. Heterotrophic cultures of microalgae: Metabolism and potential products. Water Res. 45, 11–36.
Pick, U., Avidan, O., 2017. Triacylglycerol is produced from starch and polar lipids in the green alga *Dunaliella tertiolecta*. J. Exp. Bot. 68, 4939–4950.
Reichl, C., Schatz, M., Zsak, G., 2018. World mining data. Min. Prod. Int. Organiz. Commit. World Min. Congres. 32, 1–261.
Ramadass, K., Megharaj, M., Venkateswarlu, K., Naidu, R., 2017. Toxicity of diesel water accommodated fraction toward microalgae, *Pseudokirchneriella subcapitata* and *Chlorella* sp. MM3. Ecotoxicol. Environ. Saf. 142, 538–543.
Ruiz-Domínguez, M.C., Vaquero, I., Obregón, V., de la Morena, B., Vílchez, C., Vega, J.M., 2015. Lipid accumulation and antioxidant activity in the eukaryotic acidophilic microalga *Coccomyxa* sp. (strain onubensis) under nutrient starvation. J. Appl. Phycol. 27 (3), 1099–1108.
Sassenhagen, I., Wilken, S., Godhe, A., Rengefors, K., 2015. Phenotypic plasticity and differentiation in an invasive freshwater microalga. Harmful Algae 41, 38–45.
Sakarika, M., Kornaros, M., 2016. Effect of pH on growth and lipid accumulation kinetics of the microalga *Chlorella vulgaris* grown heterotrophically under sulfur limitation. Bioresour. Technol. 219, 694–701.
Sitthithanaboon, W., Reddy, H.K., Muppaneni, T., Ponnusamy, S., Punsuvon, V., Holguim, F., Dungan, B., Deng, S., 2015. Single-step conversion of wet *Nannochloropsis gaditana* to biodiesel under subcritical methanol conditions. Fuel 147, 253–259.
Souza, L.D.S., Simioni, C., Bouzon, Z.L., Schneider, R.D.C.D.S., Gressler, P., Miotto, M.C., Rossi, M.J., Rörig, L.R., 2017. Morphological and ultrastructural characterization of the acidophilic and lipid-producer strain *Chlamydomonas acidophila* LAFIC-004 (Chlorophyta) under different culture conditions. Protoplasma 254, 1385–1398.
Subashchandrabose, S.R., Logeshwaran, P., Venkateswarlu, K., Naidu, R., Megharaj, M., 2017a. Pyrene degradation by *Chlorella* sp. MM3 in liquid medium and soil slurry: Possible role of dihydrolipoamide acetyltransferase in pyrene biodegradation. Algal Res. 23, 223–232.
Subashchandrabose, S.R., Wang, L., Venkateswarlu, K., Naidu, R., Megharaj, M., 2017b. Interactive effects of PAHs and heavy metal mixtures on oxidative stress in *Chlorella* sp. MM3 as determined by artificial neural network and genetic algorithm. Algal Res. 21, 203–212.
Tang, Y., Zhang, Y., Rosenberg, J., Betenbaugh, M., Wang, F., 2016. Optimization of one-step in situ transesterification method for accurate quantification of EPA in *Nannochloropsis gaditana*. Appl. Sci. 6, 343.
Timilsena, Y.P., Vongsvivut, J., Adhikari, R., Adhikari, B., 2017. Physicochemical and thermal characteristics of Australian chia seed oil. Food Chem. 228, 394–402.
Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Flue gas compounds and microalgae: (bio-)chemical interactions leading to biotechnological opportunities. Biotechnol. Adv. 30, 1405–1424.
Vaquero, I., Mogedas, B., Ruiz-Domínguez, M.C., Vega, J.M., Vílchez, C., 2014. Light-mediated lutein enrichment of an acid environment microalga. Algal Res. 6, 70–77.
Viêgas, C.V., Hachemi, I., Freitas, S.P., Mäki-Arvela, P., Aho, A., Hemming, J., Smeds, A., Heinmaa, I., Fontes, F.B., da Silva Pereira, D.C., Kumar, N., Aranda, D.A.G., Murzin, D.Y., 2015. A route to produce renewable diesel from algae: Synthesis and characterization of biodiesel via in situ transesterification of *Chlorella* alga and its catalytic deoxygenation to renewable diesel. Fuel 155, 144–154.
Vo, H.N.P., Bui, X.T., Nguyen, T.T., Nguyen, D.D., Dao, T.S., Cao, N.D.T., Vo, T.K.Q., 2018. Effects of nutrient ratios and carbon dioxide bio-sequestration on biomass growth of *Chlorella* sp. in bubble column photobioreactor. J. Environ. Manage. 219, 1–8.
Yao, S., Lu, J., Sárossy, Z., Baggesen, C., Brandt, A., An, Y., 2016. Neutral lipid production in *Dunaliella salina* during osmotic stress and adaptation. J. Appl. Phycol. 28, 2167–2175.
Yilancioglu, K., Cokol, M., Pastirmaci, I., Erman, B., Cetiner, S., 2014. Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PLoS One 9, e91957.
Zhao, L.S., Li, K., Wang, Q.M., Song, X.Y., Su, H.N., Xie, B.B., Zhang, X.Y., Huang, F., Chen, X.L., Zhou, B.C., Zhang, Y.Z., 2017. Nitrogen starvation impacts the photosynthetic performance of *Porphyridium cruentum* as revealed by chlorophyll a fluorescence. Sci. Rep. 7, 8542.
Zou, S., Wu, Y., Yang, M., Li, C., Tong, J., 2009. Thermochemical catalytic liquefaction of the marine microalgae *Dunaliella tertiolecta* and characterization of bio-oils. Energy Fuels 23, 3753–3758.