ARTICLE

Effects of nutrient depletion on bioethanol production of indigenous Philippine freshwater *Chlorella vulgaris* SP17

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The interest in microalgae-based biofuels is a result of the world's concern on energy crisis and climate change. The use of this third generation feedstock is deemed to be the most sustainable alternative for agricultural crops because it circumvents disputes on food security and competition on arable lands. Bioethanol has the highest demand in the world market among all the types of biofuels since it can be readily blended with traditional petrol fuels without any engine modification. In the study, Philippine indigenous *Chlorella vulgaris* SP17 was used for the production of bioethanol because of its high amount of carbohydrates in the form of starch and cellulose. Several strategies are being employed to trigger accumulation of fermentable sugars including nutrient deficiency such as nitrogen (N) and phosphorus (P) deplete (dep) culture medium. In this study, NP-deplete culture, resulted to a 2.4-fold increase in the total carbohydrate content (60.89%), and a threefold increase in total lipids (40.77%) in comparison to the control. However, the growth rates and total biomass (g/L) were all significantly reduced in the absence of N and P. Since the NP-dep setup obtained the highest carbohydrate content, the biomass from this culture was used as feedstock for bioethanol production. The highest reducing sugars (RS) after saccharification of the biomass was achieved by using 0.5 N HCl (10.02 g/L), and the maximum ethanol (EtOH) yield after the 60-h fermentation was produced by *Aspergillus niger* BIOTECH 3080 (0.307 g-ethanol/g-reducing sugars). Thus, the results of the study show that *C. vulgaris* can be used as feedstock for bioethanol production.

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INTRODUCTION

The world's dependency on fossil fuels has started in the mid-1700s during the start of industrial revolution era. Since then, people have heavily and constantly relied on the consumption of non-renewable energy resources (Fouquet 2011; IEA 2013). As a result, the amount of fossil fuels has considerably declined over the past years; and the growing world's population has further fueled the energy crisis. This has consequently led to a search for a new source of alternative energy that is sustainable, renewable, and environment-friendly (Smil 2010; EIA 2013; Chinnammai 2014). Biofuels such as bioethanol and biodiesel are deemed to be promising substitutes to conventional fuels because of less CO_2 emissions and would thus help alleviate issues concerning global warming and climate change (Demirbas 2009; Escobar et al. 2009; Pimentel et al. 2009; Dutta et al. 2014). Generally, bioethanol is regarded to be the most acceptable liquid biofuel because it is biodegradable, less toxic, and it can be blended with traditional petrol fuels without any

engine modification. It also oxygenates conventional fuels, which promotes complete combustion and thereby lowers polluting emissions (John et al. 2011). The common agricultural feedstocks used for ethanol production are cassava and cane sugar, but this has sparked an international debate on food/feed vs fuel, which has eventually led to the discovery of third-generation biofuels using microalgal biomass as feedstock (Niven 2005; Cardona and Sanches 2007; Ibeto et al. 2011; Hanif et al. 2017).

Microalgae found in natural environments are known to have large quantities of lipids, carbohydrates (predominantly starch) and proteins that can be utilized as raw materials for biofuel production. Polysaccharides (e.g. cellulose, starch and sugar) in particular are the main components of bioethanol production, where the biomass is subjected to hydrolysis/saccharification in order to release all fermentable sugars (John et al. 2011; Chaudhary et al. 2014; Medipally et al. 2015; Zhu 2015; Chng et al. 2017). However, many species of microalgae have relatively low carbohydrate content unless cultivated in specific conditions. Among all the microalgal strains that can be used commercially, *Chlorella vulgaris* is regarded as one of the best candidates for bioethanol production due to its high carbohydrate content amounting to 30-60% of its total dry cell weight. Unfortunately, this is still not sufficient to meet the industrial requirements for a large-scale production (Harun et al. 2010; Kim et al. 2013; Jung et al. 2013; Moncada et al. 2013; Agwa et al. 2017).

Reportedly, controlling the culture conditions could alter the physiology and metabolism of microalgae. For instance, the exposure to certain environmental stressors (e.g. nutrient depletion) will most likely induce the accumulation of carbohydrates and lipids through a redirection of carbon flux and fixation. Macro- and micronutrients are essential to microalgae's growth, but the limitation of these fundamental nutrients prompts a shift in various metabolic pathways (Illman et al. 2000; Dragone et al. 2011; Ho et al. 2012; Kim et al. 2014). For instance, carbon precursors intended for protein and membrane lipid metabolism is redirected towards the biosynthesis of carbohydrates and neutral or storage lipids, but at the expense of growth and biomass (Yamada and Sakaguchi 1982; Singh and Gu, 2010; Markou et al. 2012). Thus, the main objective of the study was to determine the effects of nitrogen and phosphorus deplete conditions on both growth and bioethanol production of an indigenous freshwater *C. vulgaris* isolated from Pampanga, Philippines. The combined effects of N and P depletion on growth and production of biofuel precursors was also closely examined since this is still poorly studied.

MATERIALS AND METHODS

Cultivation of *Chlorella vulgaris*

Previously isolated freshwater *Chlorella vulgaris* strain SP17, from a shrimp culture pond in Sasmuan, Pampanga, Philippines was cultivated in sterile BG-11 culture broth (HiMedia Laboratories Pvt. Ltd., India) containing (g/L): sodium nitrate (1.5 g), dipotassium hydrogen phosphate (0.0314 g), magnesium sulphate (0.036 g), calcium chloride dihydrate (0.0367 g), sodium carbonate (0.020 g), disodium magnesium EDTA (0.001 g), citric acid (0.0056 g) and ferric ammonium citrate (0.006 g). It was incubated at an average temperature of 28°C under constant aeration of filtered air at the flow rate of 1 vv^{-1} m^{-1} (volume air per volume medium per minute), and continuous illumination of 50 μmol photons m^{-2} s^{-1} for 14-d. For nitrogen and phosphorus deplete conditions, the nitrogen and phosphorus were not included in the culture broth. *C. vulgaris* was identified through morphology and confirmed by 18S rDNA sequencing of the ITS1 region.

Measurement of microalgal cell growth rate and cell count

For growth rate, the initial cell density was adjusted to OD_{750} of 1.00, and growth was monitored by measuring optical density at 750 nm using UV/Vis spectrophotometer (UV-2600, Shimadzu, Japan) for 10-d. Meanwhile, 1 mL was collected per day from each sample containing 100 mL culture (starting cell density = 2.5 x 105), and 10 μL was transferred onto the hemocytometer (Neubauer chamber, Marienfeld, Germany) to determine the algal cell count.

Determination of total dry biomass concentration

A total of 20 mL per sample was harvested every day in a period of 10-d. Algal cells were washed twice with deionized water through centrifugation at 8,000 rpm for 5 min to remove any ashes. The cell pellets were then transferred into a pre-weighed Whatman® membrane filter (Sigma-Aldrich, USA) with a 45 μm pore diameter. After the filtration process, membrane filter was oven-dried at 60°C for 12 h or until the weight remained constant. The total biomass content was estimated by using this formula:

$$Biomass\ (g/L) = \frac{\text{Final weight (g)} - Initial\ weight\ (g)}{\text{Volume (L)}}$$

Extraction and spectrophotometric determination of cholorophylls (chl) *a* and *b*

Briefly, 1 mL of *C. vulgaris* strain SP17 was transferred into a 1.5 mL microtube for centrifugation at 13,000 rpm for 15 min. The supernatant was discarded, while the remaining microalgal pellets was resuspended in 1 mL of 90% (v/v) MeOH. Tubes were then placed in a digital BSW1500 heat block (Benchmark Scientific, New Jersey, USA) set at 60°C for 10 min to facilitate complete extraction of pigments. The resulting supernatant containing chlorophylls *a* and *b* was quantified spectrophotometrically by using OD_{665} and OD_{653}. The following equations were used to determine the total chlorophyll content of each sample:

Chlorophyll *a* (μg/mL) = (15.65 (A_{665}) – 7.34 (A_{653})) – A_{750}
Chlorophyll *b* (μg/mL) = (27.05 (A_{653}) – 7.34 (A_{665})) – A_{750}

Extraction and quantification of lipid content by gravimetric analysis

Ten milligram (10 mg) of freeze-dried microalgal biomass was added into a microcentrifuge tube containing 1 mL of 4:5 (v/v) chloroform:methanol with C17:0 internal standard and 0.1% BHT. The mixture was bead beated 8x at 2500 rpm for 60 s with a 2-min interval in between each beating. The solution was then transferred into a clean test tube containing 2.5 mL of MilliQ H_2O with 1M NaCl and 50 mM Tris. After vortexing for 5 s, each tube was centrifuged for 5 min at 3000 rpm. Then the entire chloroform phase (bottom layer) was pipetted into a new tube. The method was repeated twice for maximum lipid extraction, and the chloroform fractions were pooled. To determine the lipid-recovery yield by reference to the weight of the recovered lipids, chloroform was evaporated by using a nitrogen gas stream.

$$Lipid\ content\ (\%) = \frac{\text{Weight of lipid (g)}x\ 100}{\text{Dry cell weight (g)}}$$

Analysis of total carbohydrate content

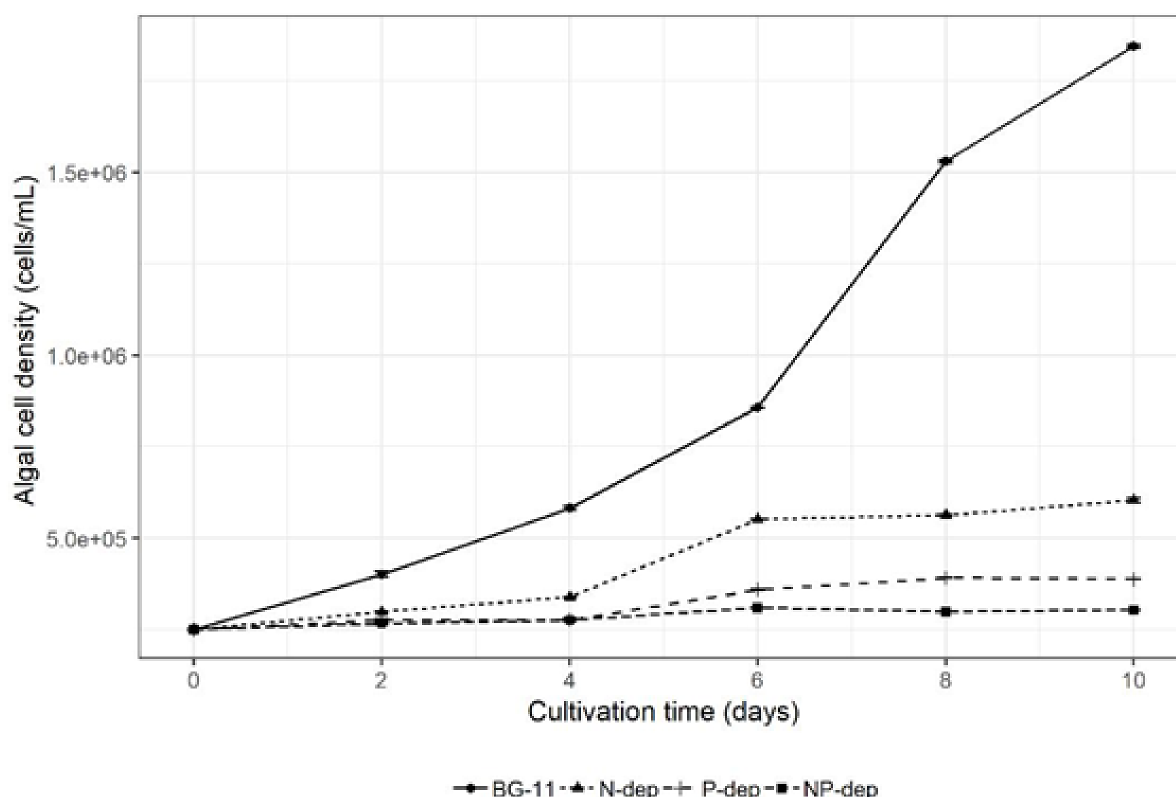

Figure 1: Cell density of *Chlorella vulgaris* strain SP17 cultivated in BG-11 (control), nitrogen and phosphorus deplete culture medium for 10-d. Initial cell concentration is 2.5 x 105 cells/mL-1. The plotted values are the average cell number ± standard deviation (n=3).

The total carbohydrate content of each sample was measured via the phenol-sulfuric acid method (Laurens et al. 2012). Approximately 5 mg of freeze-dried algal biomass was added to a glass tube containing 0.5 mL of 5% (v/v) phenol and 0.5 mL of concentrated sulfuric acid. The mixture was vortexed for 30 s, and the tubes were left to cool at room temperature for 30 min. Then a total of 200 µL of solution was pipetted out and transferred into a cuvette. The absorbance was measured at 490 nm using UV/Vis spectrophotometer (UV-2600, Shimadzu, Japan).

Pretreatment process of microalgal biomass

Cells were harvested in flasks by centrifugation at 8000 rpm for 15 min. The algal pellets were then washed twice with deionized water to remove excess culture broth and ashes. Then the cells were freeze-dried for 48 h. The algal cells were sonicated using Q700 ultrasonicator (QSonica, Connecticut, USA) for 10 min at 28 kHz on 35% amplitude, while diluted in 30 mM sodium acetate buffer adjusted to pH 4.8.

Acidic hydrolysis

The pre-treated microalgal biomass (15g) was mixed with 200 mL of three different types of acids (HCl, H_2SO_4, and HNO_3) in varying concentrations (0.5 N, 1.0 N, 1.5 N and 2.0 N). Hydrolysis process was carried out in 1-L flask and each mixture was autoclaved (Labtron IVA-I11, Scotland, United Kingdom) for 20 min at 120°C. The flasks were then left to cool at room temperature. Afterwards, solid debris and residual algae were removed from the mixture by filtration using Whatman® membrane filter (Sigma-Aldrich, USA) with a 45 µm pore diameter, followed by neutralization with NaOH at 5.0 pH. The hydrolyzed filtrates were then centrifuged at 9000 rpm for 15 min, and the resulting supernatant was sterilized again. The reducing sugar of the hydrolysates was subsequently measured by 3,4-dinitrosalicylic acid (DNS) method following the protocol of Miller (1959), and D-glucose was used as standard.

Fermentation process

Saccharomyces cerevisiae BIOTECH 2055 and *Aspergillus niger* BIOTECH 3080 were purchased from the Philippine National Collection of Microorganisms (PNCM) at the National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baňos, Laguna, Philippines. *S. cerevisiae* BIOTECH 2055 was cultured using yeast peptone dextrose medium (10.0 g/L yeast extract, 20.0 g/L dextrose and 20.0 g/L peptone), while *A. niger* BIOTECH 3080 was grown in potato dextrose agar medium (4.0 g/L potato extract and 20.0 g/L dextrose) for 48 h inside a shaking incubator set at 30°C and 150 rpm. The initial cell density for both microorganisms is 5 x 10^6 cells/mL.

In a 1-L Erlenmeyer flask, the *C. vulgaris* strain SP17 hydrolysate was added with ethanol fermentation medium consisting of 2 g/L NH_4SO_4, 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.2 g/L $ZnSO_4$, 0.2 g/L $MgSO_4$, and 2 g/L yeast extract. The pH of the reaction mixture was adjusted to 4.5 before sterilization for 25 min at 121°C. After cooling at room temperature, 10% v/v of *S. cerevisiae* BIOTECH 2055 and *A. niger* BIOTECH 3080 were inoculated separately in each flask containing the hydrolysates. It was incubated under static conditions at 32°C for a period of 60 h.

Ethanol yield determination

The ethanol concentration per sample was quantified using the method of Caputi *et al*. (1968) with minimal modifications. Briefly, 1 mL from each sample was added to 1 mL of dichromate solution and 3 mL of perchloric acid. It was then incubated in water bath (WNB 10, Germany) at 80°C for 10 min. The mixture was left to cool for 2 h, and the absorbance per sample was measured at 600 nm. Absolute ethanol was utilized as standard to extrapolate ethanol yield from the standard curve generated.

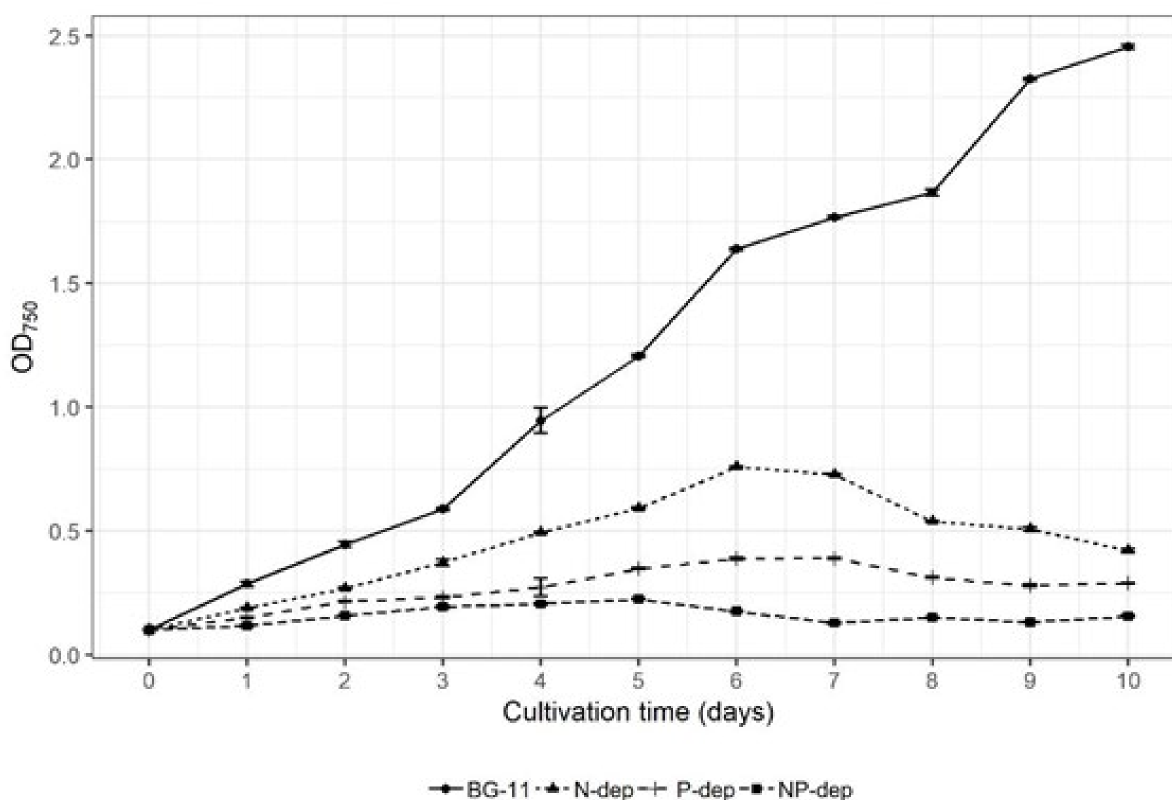

Figure 2: Growth curve of *Chlorella vulgaris* strain SP17 under mixotrophic conditions cultivated in BG-11 (control), N-, P-, and NP-deplete medium. The cell density was normalized to an OD750 of 1.00; and results represent the average and standard deviation of three replicates.

RESULTS

Nitrogen and phosphorus depletion on growth of *C. vulgaris*

Freshwater *C. vulgaris* strain SP17 was previously isolated from a shrimp culture pond located in Sasmuan, Pampanga, Philippines. The effects of nitrogen and phosphorus on growth were observed by removing these macronutrients from BG-11 culture medium. Optical density, cell concentration, biomass and total chlorophyll content were monitored to determine the magnitude of N and P deprivation on the growth of *C. vulgaris* strain SP17. Experiments were carried out in 1-L flasks for 10 days under mixotrophic conditions.

In the absence of N and P (Figure 1), the growth of *Chlorella* was sternly affected. A five-fold decrease in the final cell count at day 10 was observed in both NP-dep ($3.03x10^5$ cells/mL) and P-dep ($3.87x10^5$ cells/mL) setups. These two cultures have also displayed an extended lag phase of 4 days, and entered the stationary phase as early as day 6. The cell concentration did not increase after this period, and has even further declined as shown in Figure 1. On the other hand, the culture without $NaNO_3$ has also maintained a steady cell count after 48 h followed by a logarithmic phase, but growth ceased at day 6. The culture did not continue to flourish because the absence of nitrogen results to deprivation of $NADP^+$ (an example of electron acceptor), which leads to impaired growth. The optical density of each culture was also measured at 750 nm by UV/Vis spectrophotometer (Shimadzu UV-2600, Japan) (Figure 2). All setups were normalized to an OD_{750} of 1.00. The results in Figure 2 exhibited a similar trend to algal cell concentration wherein both NP-dep and P-dep cultures obtained the lowest growth rates. Evidently, the presence of nitrogen and phosphorus is important to *C. vulgaris*'s growth as the maximum cell density of $1.84x10^7$ cells/mL was observed in complete BG-11 (control), higher than all the other setups due to nutrient deprivation in the latter.

Figure 3 shows the time course biomass profile of *C. vulgaris* strain SP17 grown mixotrophically in N-dep, P-dep, and NP-dep culture medium . Results revealed that both nitrogen and phosphorus deficiency limited biomass production, specifically apparent in P-dep and NP-dep cultures that obtained a very low biomass productivity of 0.0323 g/L^{-1} and 0.0234 g/L^{-1}. Meanwhile, N-depleted culture medium has the second highest total biomass of 0.501 g/L at day 10. Nonetheless, the data indicates that both nitrogen and phosphorus are essential to cell growth and biomass production since these macronutrients are considered to be one of the building blocks of all structural and functional proteins in algal cells (Chu et al. 2013; Kalla and Khan 2016; Agwa et al. 2017).

The total chlorophyll content of microalgae is another representation of algal biomass, and a measurement of its photosynthetic activity (Chisti 2008). Therefore, the effects of N and P starvation on the ratio of chlorophyll was determined by extracting both Chl *a* + *b* using MeOH. In Figure 4, chlorophyll production peaked on the 6th day for N-dep, P-dep, and NP-dep with a total Chl content of 3.033 µg/mL, 1.632 µg/mL, and 0.897 µg/mL. Under the fluorescence microscope (data not shown), the cells also appeared to be bleached compared to algal cells grown in nutrient replete medium. Moreover, a stable increase in the production of chlorophyll was observed in *Chlorella* cultivated in complete BG-11, and obtained the highest Chl yield of 11.06 µg/mL at day 10. The Chl content of the control is 12-fold higher compared to NP-dep culture, which had the lowest chlorophyll yield. Many other studies have reported a decrease in photosynthetic rates of the green alga *C. vulgaris* and other microalgae species under N and P limitation (Hecky and Kilham 1988; Litchman et al. 2003; Eixler et al. 2006; Barghbani and Rezaer 2012; Chu et al. 2013). Irrefutably, the availability of N and P in the culture medium is critical in chlorophyll production for photosynthesis and thus in cell division of any photosynthetic microorganism. In the absence of nitrogen, algal

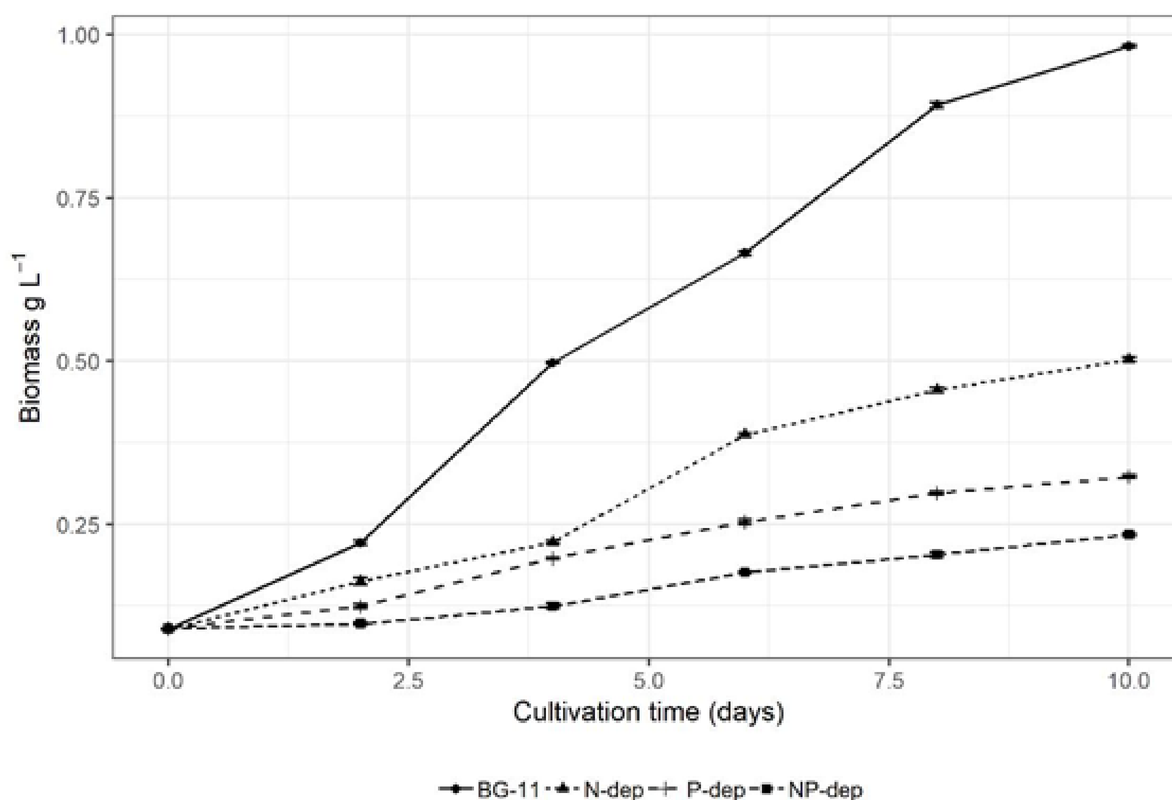

Figure 3: Dry cell biomass (gdw/L) of *Chlorella vulgaris* strain SP17 cultivated in BG-11 (control), nitrogen and phosphorus deplete medium. The biomass production was monitored every two days. Error bars are SEM (n=3).

cells also tend to metabolize chlorophylls, hence there is an increase in carotenoid to chlorophyll ratio (Benaventes-Valdes et al. 2014).

Lipid and carbohydrate composition of *C. vulgaris* under nitrogen and phosphorus deficiency

The intracellular lipids and carbohydrates were quantified after 10-d of cultivation in N-, P- and NP- deplete medium. Lipids from freeze-dried biomass of *Chlorella* were extracted following the method of Breuer *et al.* (2013), while phenol-sulfuric acid method was utilized in carbohydrate extraction. Figure 5 shows that the lipid yield of *Chlorella* grown in complete BG-11 medium was significantly lower than NP-dep (40.77%; $p < 0.001$), P-dep (34.65%; $p < 0.001$), and N-dep (28.53%; $p < 0.001$) cultures. The combined effect of N and P deficiencies has clearly resulted to an accumulation of lipids, an almost three-fold increase in comparison to the control (14.60%). In nitrogen limiting conditions, other studies have reported that algae can even accumulate up to 70% of intracellular lipids in the absence of $NaNO_3$ (Litchman et al. 2003; Beardall et al. 2005; Chu et al. 2013; Griffiths et al. 2014; Ikaran et al. 2015; Kalla and Khan 2016). The same results were observed under P deficiency. Carbohydrates was also enhanced under nutrient deplete conditions. NP-dep had the highest carbohydrate content of 60.89%, a 2.4-fold increase from the control.

Nutrient deficiency stimulates the synthesis of carbohydrates and lipids at the expense of proteins since N and P starvation instigates a shift from protein to lipid synthesis (Sheehan et al. 1998; Simionato et al. 2013; Guccione et al. 2014; Agirman and Cetin 2015). Under NP-dep and P-dep, the protein content (data not shown) has diminished substantially.

Evaluation of different acid types and concentrations on the hydrolysis of algal biomass

C. vulgaris strain SP17 cultivated in NP-dep medium had the highest carbohydrate content among all the four setups. Therefore, the biomass from this culture was used as feedstock for bioethanol production. The harvested biomass was hydrolyzed using three different types of acids in varying concentrations to evaluate which acid would be best for saccharification process. Acidic hydrolysis was preferred in the study to degrade carbohydrates in the form of cellulose and starch into reducing sugars for fermentation. Results in Figure 6 indicate that among the three acid types, 0.5 N hydrochloric acid produced the highest reducing sugars of 10.02 g/L, followed by 2.0 N nitric acid (7.11 g/L; $p < 0.05$) and 1.5 N sulfuric acid (3.71 g/L; $p < 0.01$), respectively. The same results were observed in the study of Park et al. (2016) wherein the hydrothermal acid hydrolysis of *C. vulgaris'* biomass by HCl showed the highest RS amounting to 10.71 g/L. The optimal concentration of the acid was determined to ensure high sugar recovery. As shown in Figure 6, lower concentrations of hydrochloric acid led to an increase of RS. The opposite was observed on both sulfuric and nitric acids, whereas reducing sugars yields peaked when 2.0 N acid concentration was used.

Determination of reducing sugars (RS) and ethanol production (EtOH) during fermentation of hydrolysates assisted by *S. cerevisiae* and *A. niger*

After saccharification, released sugars consisting mainly of glucose and galactose are subjected to ethanol production. The fermentation broths are inoculated separately with *S. cerevisiae* BIOTECH 2055 and *A. niger* BIOTECH 3080 with an initial cell density of 5 x 10^6 cells/mL, in order to determine which microorganism would have the highest consumption rate of RS and ethanol conversion.

The initial concentration of RS was 10.02 g/L. Figure 7 shows that all reducing sugars were consumed for the two setups after the fermentation period indicating that all polysaccharides from *C. vulgaris* strain SP17 are fermentable. The broth supplemented with *A. niger* BIOTECH 3080 had the highest consumption rate of reducing sugars with a final yield of only 0.0186 g/L after

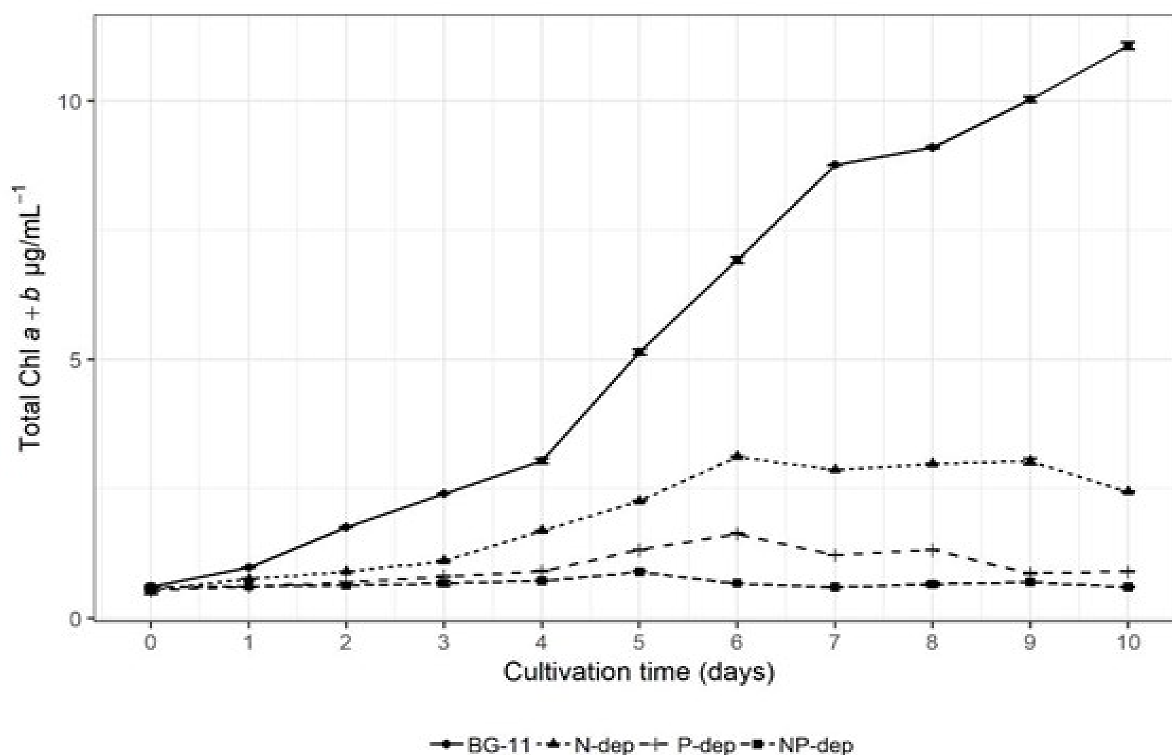

Figure 4: Total chlorophylls a+b ratio per µg DCW of *Chlorella vulgaris* strain SP17 grown in BG-11 (control) and under nitrogen and phosphorus deprivation for a period of 10-d. Error bars represent the standard deviation of triplicate cultures.

60 h, while *Saccharomyces cerevisiae* BIOTECH 2055 has poorly fermented the RS (0.559 g/L) as shown in Figure 7A. On the other hand, data on ethanol production (Figure 7B) strongly support the previous results that *A. niger* was more suitable for fermentation due to its complete and rapid conversion of RS to ethanol than *S. cerevisiae*. The maximum yield of ethanol was 0.307 g-ethanol/g-reducing sugars at the end of the fermentation period, while *Saccharomyces* obtained 0.248 g/L. Difference is considered statistically significant at $p < .05$.

DISCUSSION

Energy security, climate change, and food supply are intersected issues brought about by our continued dependence on fossil fuels, which are finite in supply (Lal 2010; Karp and Richter 2011). These non-renewable energy reserves continue to diminish over the years as a result of swelling demand especially in countries with emerging economies. To address these pressing problems, energy from plant biomass or biofuels are explored. Renewable carbon from microalgae seems like an attractive alternative to agricultural crops because of its (1) high surface biomass productivity, (2) ability to accumulate huge amounts of lipid and carbohydrates, (3) sequester CO_2 emissions, (4) capable of all-year round production, (5) grow at high rates, (6) capacity to grow in wastewater and harsh conditions, (7) no requirement for fertilizers, and (8) high efficiency conversion of solar energy into chemical energy (Pulz and Gross 2004; Wijjfels and Barbosa 2010; Brenna and Owende 2010; Li-Beisson and Peltier 2013). At present, much of the on-going research studies on biofuels make use of green algae such as *Chlorella* species, *Chlamydomonas reinhardtii*, and *Botryococcus braunii* due to their inherent high lipid/starch content and rapid growth rate (Metting 1996; Dragone et al. 2010; Demirbas 2011; Jung et al. 2013; de Farias Silva and Bertucco 2016). However, in order for the algae biofuel industry to move beyond research to commercial production, there is a need to further increase the amount of intracellular biofuel precursors to an extent that it would be able to pass industrial standards. A common way of inducing lipid and starch production in microalgae is exposure to environmental stressors such as nutrient depletion and adjustment in light intensity, salinity, temperature, and pH (Spolaore et al. 2006; Harun et al. 2010). These strategies, however, are observed to slow down growth and limit biomass production. Therefore, it is imperative to balance out the tradeoffs between lipid/starch and biomass productivities (Ewin and Msangi 2009; Adams et al. 2013).

Factors affecting microalgal growth are categorized into two groups: (1) chemical (e.g. nutrients) and (2) physical (e.g. temperature, pH). In the study, *C. vulgaris* strain SP17 were grown in nitrogen and phosphorus deplete culture medium to examine the impact on growth and production of bioethanol precursors. Results in Figures 1 to 4 show that all the parameters used to measure growth were severely affected by N and P starvation, in accordance to many other published reports (Litchman et al. 2003; Beardall et al. 2005; Ji and Sherrell 2008; Chu et al. 2013; Griffiths et al. 2014; Ikaran et al. 2015; Kalla and Khan 2016). Among all the setups, P-dep and NP-dep cultures were the most affected. As compared with other studies, the NP-dep culture (Fig. 4) had a four-fold decrease in biomass (0.234g/L) in comparison to the control (0.982 g/L). Macronutrients such as nitrogen and phosphorus are essential in the metabolism of functional bioactive molecules and in regulating normal cell growth. Nigam et al. (2011) mentioned that the amount of nitrogen source in the medium is directly proportional to biomass production since N is a key element for protein, nucleic acid, and chlorophyll synthesis. This impaired growth caused by N-deficiency was also attributed to the loss of electron acceptors (e.g. $NADP^+$) during photosynthesis. While the limited chlorophyll production shown in Figure 4 is a consequence of *Chlorella*'s inability to synthesize amino acids such as glycine, glutamate and succinate, the chief precursors of 5-aminolevulinic acid. This endogenous protein is involved in porphyrin synthesis pathway under abiotic stress (Li et al. 2012, Kim et al. 2013; Kamalanathan et al. 2015).

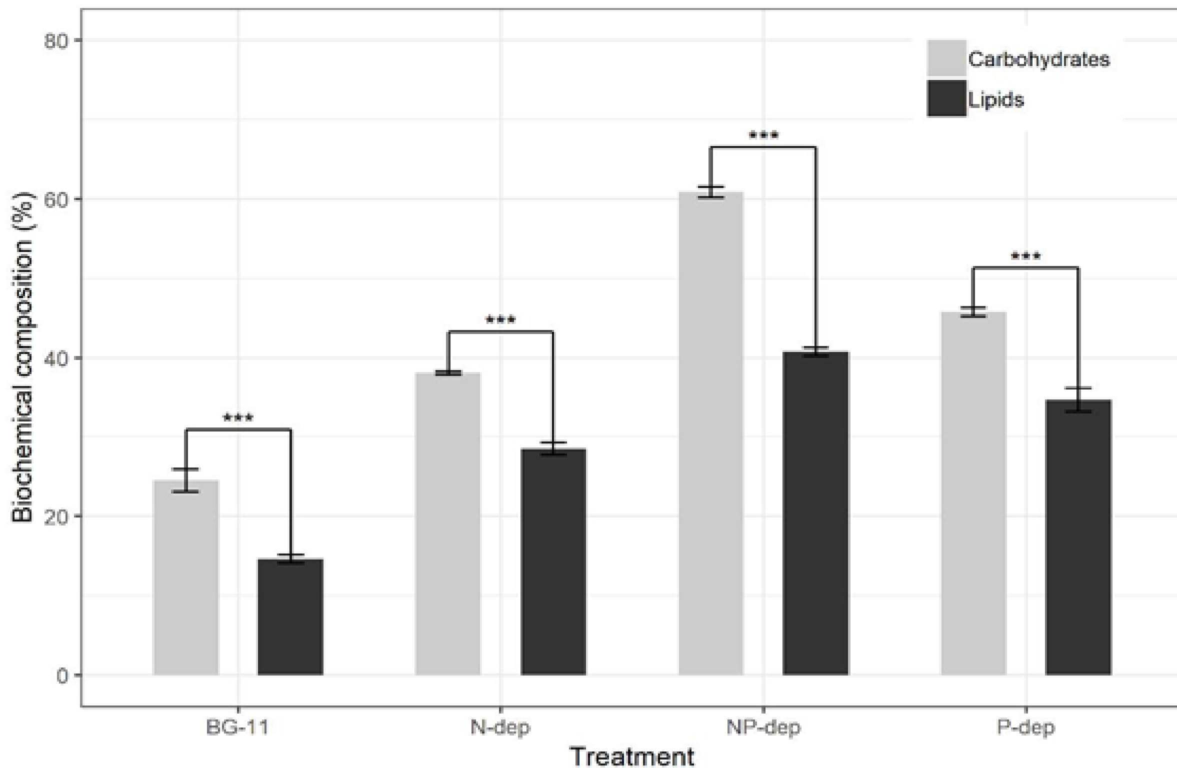

Figure 5: Biochemical profile of *Chlorella vulgaris* strain SP17 grown in BG-11 and under nitrogen and phosphorus limitation after 10-d cultivation period. Each value represents the mean of three replicates ± standard deviation (n=3). Significant differences: *P < 0.05>, **P< 0.01 >, ***P < 0.001 >.

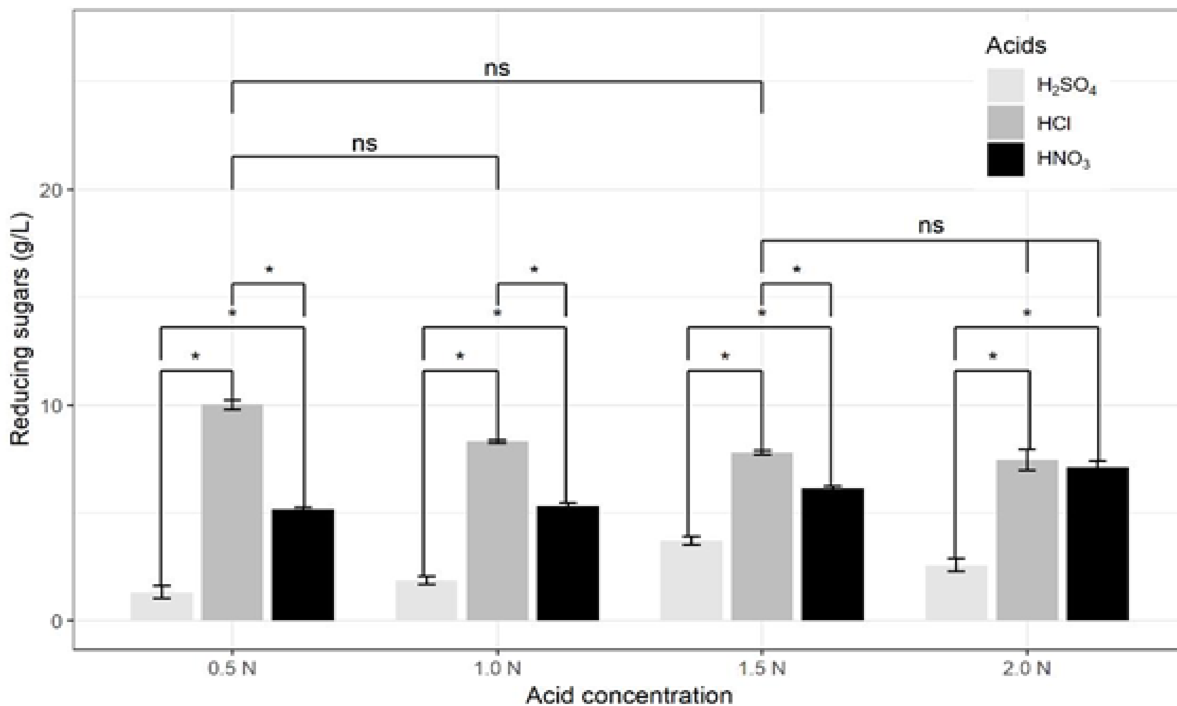

Figure 6: The effect of different acid types and concentrations on the reducing sugars of biomass harvested from *C. vulgaris* strain SP17 cultivated in NP-dep medium. Each value represents the mean of three replicates ± standard deviation (n=3). Significant differences: *P <0.05>, **P < 0.01 >, ***P < 0.001 >.

On the other hand, phosphorus is critical for the formation of phosphorylated sugars (e.g. ester phosphates), DNA, RNA, and phospholipids (Eixler et al. 2006; Roopnarain et al. 2014). Absence of P would normally cause a decline in the production and transformation of metabolic energy in the form of ATP and NADP, leading to the termination of cell division (Litchman et al. 2003; Chu et al. 2013; Kalla and Khan 2016). This elucidates the extremely low cell densities and total biomass in NP-dep and P-dep cultures (Figures 1 and 3). Generally, chlorophylls do not contain P, so the decrease in the ratio of chlorophylls *a* + *b* (Figure 4) cannot be credited to the breakdown of chlorophyll for phosphorus sequestration. Roopnarain et al. (2014) stated that P-starved algal cells' inability to produce ATP and NADPH, which are mainly responsible for chlorophyll synthesis is a

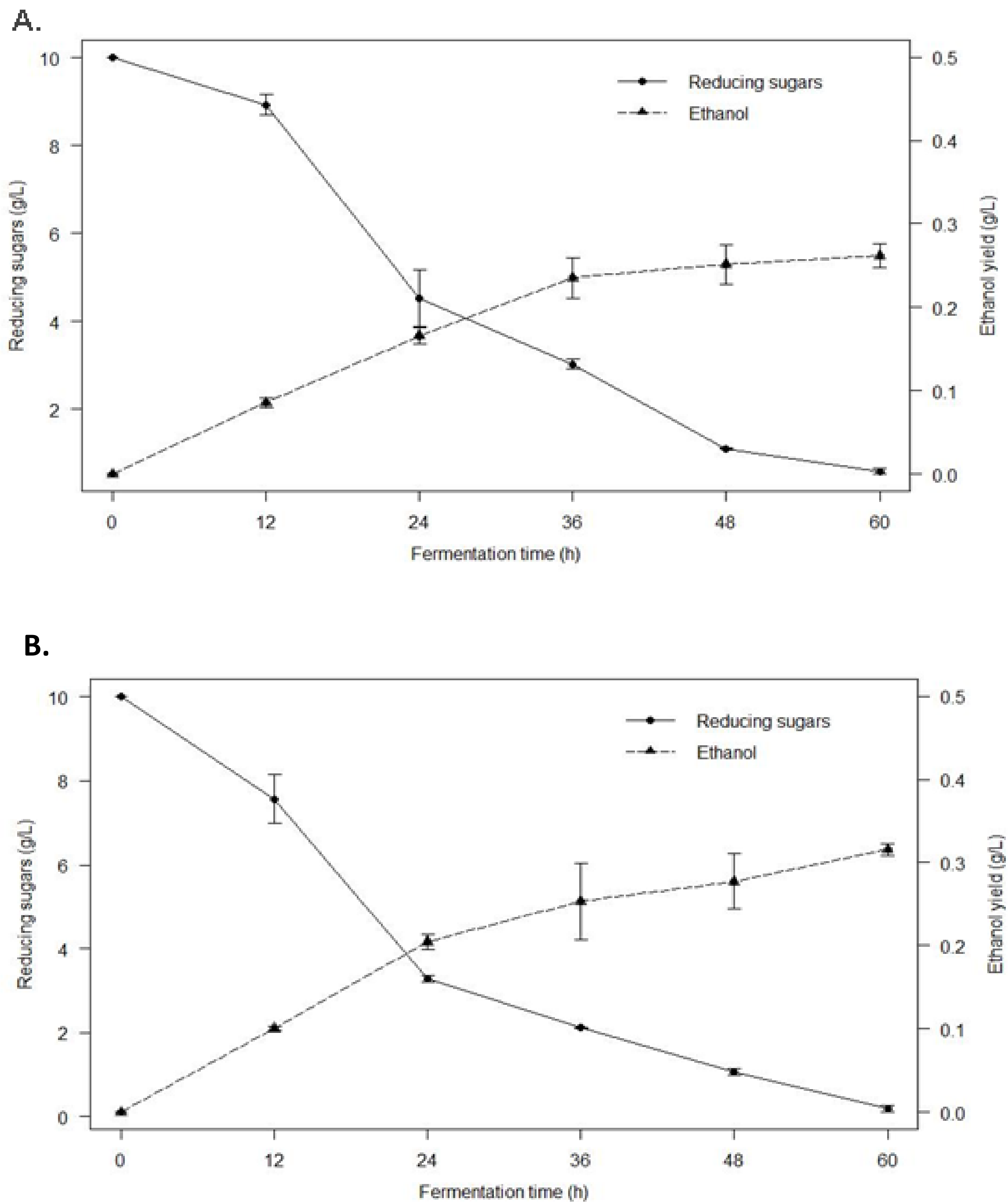

Figure 7: Reducing sugar and ethanol yield during fermentation *of C. vulgaris*' strain SP17 hydrolysates by (A*) S. cerevisiae* BIOTECH 2055 and (B) *A. niger* BIOTECH 3080 for 60-h under static conditions. Error bars represent the standard deviation of triplicate cultures (n=3).

biological response of microalgae when P becomes limiting; while previously synthesized chlorophylls are subjected to degradation since they naturally have a short half-life. The carotenoid concentration of the cultures was not measured, but a lot of studies have revealed that during N-/P- limited conditions, microalgae tend to have a higher ratio of carotenoid to chlorophyll, suspecting that this is a protective mechanism of algal cells against photo-oxidative stress (Berges et al. 1996; Masojidek et al. 2000; Cade-Menun and Paytan, 2010; Pirastru et al. 2012).

In contrast, the total carbohydrates and lipid content of *C. vulgaris* strain SP17 under N and P deficiencies were significantly enhanced (Figure 5) in comparison to cells cultivated in nutrient replete medium. Several studies have shown increased lipid and starch content in microalgae after nutrient starvation (Litchman et al. 2003; Beardall et al. 2005; Ji and Sherrell 2008; Chu et al. 2013; Griffiths et al. 2014; Ikaran et al. 2015; Kalla and Khan 2016). The N-deprived *C. vulgaris* cells synthesize high amounts of neutral lipids predominantly triacylglycerols (TAGs) due to the decrease of NADPH consumption. Now the excessive amounts of NADPH in cells result to a surge of acetyl CoA molecules that could not enter the

citric acid cycle because the latter blocks the enzyme citrase synthase from entering the TCA due to its high concentration. This results in the conversion of acetyl CoA into mallonyl CoA by accase (acetyl CoA carboxylase), which is the principal carbon donor for fatty acid synthesis. Similarly, phosphorus limitation has also led to increased lipid and carbohydrate levels in *Chlorella* species, *Chaetoceros* sp., *Isochrysis galbana*, *Chlamydomonas reinhardtii*, and *Tetraselmis* sp (Gopalakrishnan et al. 2015; Goncalves et al. 2016; Kalla and Khan 2016). According to Belotti et al. (2013), N and P limitation usually redirects the inorganic carbon fixation from DNA and protein synthesis to accumulation of carbon reserves for the production of lipids and carbohydrates especially during the stationary phase. Although nitrogen is considered to be the most important nutrient to channel carbon flux to lipid biosynthesis, P-dep (34.65%) seems to induce more intracellular lipids compared to N-dep (28.53%), but the highest lipid yield was induced by the combination of N and P depletion (40.77%), a threefold increase compared to the control. Moreover, Figure 5 also shows that both P-dep and NP-dep obtained the highest total carbohydrate contents of 45.76% and 60.89%, respectively. Reitan et al. (1994) and Liang et al. (2013) reported that although carbohydrate content is directly linked to phosphorus concentration, further studies have to be made in order to elucidate what exactly regulates the increase in starch/lipid under phosphorus starvation. Protein content was also reduced in the cultures without N and P (data not shown). Vitova et al. (2015) and Fernandes et al. (2013) suggested that under nutrient deficiency, there is a redirection of metabolic carbon flow from protein towards TAG/neutral lipid synthesis.

Results on the changes of biochemical composition revealed the increase of both lipids and carbohydrate contents, which are both essential biofuel precursors. In the study, NP-dep induced the highest carbohydrate content in *C. vulgaris*. Hence, the biomass from this particular setup was harvested to be used as feedstock for bioethanol production. Carbohydrates in the form of starch and cellulose are generally stored in the chloroplast and cell wall. To produce ethanol, a pre-treatment method called saccharification is required in order to disrupt the algal cells and release all complex polysaccharides. Acidic or enzymatic hydrolysis can either be applied (Harun et al. 2010; Park et al. 2016; Agwa et al. 2017). In the study, acidic hydrolysis was employed since it is more economically feasible and has a faster reaction time. The most suitable acid type and concentration were also determined.

Three different acids (HCl, HNO_3, and H_2SO_4) and varying concentrations (0.5 N, 1.0 N, 1.5 N, and 2.0 N) were used to evaluate which acid is the most suitable to degrade carbohydrates into reducing sugars. According to Esteghlalian et al. (1997) and Sun and Cheng (2002), diluted H_2SO_4 is generally used for pre-treatment of highly lignocellulosic algal biomass because of high reaction rates. However, results showed in Figure 6 that 0.5 N HCl was the most effective leading to a total RS yield of 10.02 g/L. The data also show that increasing hydrochloric acid concentration would lead to a sharp decline in the amount of reducing sugars, probably because *Chlorella* has been excessively decomposed. Contrastingly, both sulfuric and nitric acids were more effective at high concentrations. Similar results were reported by Zhou et al. (2011); Markou et al. (2013); Park et al. (2016); and Agwa et al. (2017). It is also important to note that hydrolysis using acids must be optimized first because utilization of an inappropriate acid type and concentration could result in partial saccharification, and worse is the decomposition of hydrolyzed glucose (Chng et al. 2017).

The fermentation of hydrolysates containing the released sugars from the algal biomass is assisted by microorganisms capable of converting sugars to ethanol. *S. cerevisiae* BIOTECH 2055 and *A. niger* BIOTECH 3080 were used in the study to produce ethanol from the feedstock. The yeast *S. cerevisiae* is widely used in different ethanol industries due to numerous advantages. However, results on Figure 7A shows an incomplete conversion of reducing sugars to bioethanol when *Saccharomyces* was applied. This is possibly because they are not capable of utilizing complex sugars (e.g. pentoses and hexoses) present in the hydrolysates. The low bioethanol yield might also be due to the inhibition of *Saccharomyces*' growth and metabolism because of high salt concentration formed in the fermentation broth. To resolve this problem, salt-tolerant yeasts can be developed or an addition process called desalination can be employed to remove excess salts (e.g. NaCl, $NaNO_3$, and Na_2SO_4) in the fermentation system. Other possible inhibitors are organic acids and furan derivatives (Jimoh et al., 2009; Chandel et al. 2011; Kim et al. 2013; Markou et al. 2013; Agwa et al. 2017). Meanwhile, the fungus *A. niger* BIOTECH 3080 has a higher conversion rate of RS to ethanol since it has a wider range of consumable sugars. In addition, *Aspergillus* can produce carbohydrate hydrolyses and other useful enzymes (e.g. amylases and cellobiases) that will catalyze degradation reactions eventually resulting to an increase in the amount of reducing sugars in the fermentation broth (Akinyele et al. 2007; Botella et al. 2007; Mamma et al. 2008). This explains the higher ethanol yield in Figure 7B (0.307 g EtOH/g reducing sugars).

CONCLUSION

Collectively, the results of the study show that indigenous freshwater *Chlorella vulgaris* can be used as feedstock for bioethanol production. Removal of both nitrogen and phosphorus in the medium has significantly induced an increase on both total lipids and carbohydrates, but growth was severely affected. Hence, enhancing the accumulation of these biofuel precursors without biomass loss under nutrient deficiency should be further explored. The NP-dep setup obtained the highest carbohydrate content among all the cultures. The biomass was then harvested and used for bioethanol production. To ensure complete conversion of biomass to reducing sugars to ethanol, the appropriate acid type and concentration for saccharification of algal biomass should be determined. In the present study, 0.5 N HCl had the highest sugar recovery of 10.02 g/L. Lastly, *A. niger* BIOTECH 3080 produced the maximum yield of ethanol (0.307 g EtOH/g sugars) in the fermentation system compared to *S. cerevisiae* BIOTECH 2055.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Pintor KL and Vital PG conceptualized the study, analyzed and interpreted the data, and prepared the draft of and finalized the manuscript. Pintor KL performed the experiments. Rivera WL was involved in the design of the study and edited the manuscript.

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