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# Valorization of cassava ethanol waste as carbon and nutrient sources for microalgae cultivation

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**Abstract**

The potential of lipid or carbohydrate-rich biomass production by cultivation of isolated microalgal species i.e., *Coelastrella* sp. KKU-P1 and *Acutodesmus* sp. KKU-P2 under photoautotrophic conditions was investigated. The strain KKU-P1 was able to survive at 45°C for 24 h with a 6% mortality rate caused by heat with tolerance of $CO_2$ in concentrations up to 60%. The stain KKU-P2 survived at 40°C for 24 h with a 12% mortality rate and could grow with $CO_2$ concentrations up to 30%. Total carbohydrate contents of KKU-P1 reached 45% in dry biomass while KKU-P2 could accumulate lipid to over 40% under nitrogen-limited condition. *Acutodesmus* sp. KKU-P2 was further tested with mixotrophic cultivation using cassava stillage as a nutrient and carbon source with no external nutrient supplementation. A stillage concentration of 12 g-COD/L, initial pH 6.0, and light intensity of 7000 lux were the optimal conditions for carbohydrate production yielding biomass with a carbohydrate content and productivity of 28.22% and 278 mg/L.d, respectively. The same conditions but with a lower light intensity of 5000 lux yielded a lipid-rich biomass (28.32% lipid) with a lipid productivity of 137 mg/L.d. The research highlights that isolated microalgae are crucial sources of biomass and can potentially utilize ethanol industrial wastes as carbon and nutrient sources for microalgal carbohydrate and lipid production.

**Keywords:** Green microalgae, Cassava stillage, Mixotrophic, Autotrophic, Thermotolerant

## 1. Introduction

Ethanol is used globally for a wide range of industrial applications. High purity ethanol possesses the same characteristics and properties as fossil fuel and it is widely used as a gasoline additive to increase oxygen content of fuel [1]. Fuel ethanol can be produced from various types of biomass such as cane molasses, corn, cassava, and sugar beet. Cane molasses and cassava are the main feedstocks for fuel ethanol in Thailand due to their low cost and locally available. Ethanol production generates a $CO_2$-rich (>80%) exhaust gas from sugar fermentation and stillage which is an organic-rich stream from distillation. Sugar fermentation for 1 L of ethanol theoretically releases 0.76 kg of $CO_2$ [2]. Additionally, for every ton of ethanol produced, approximately 1-14 tons of stillage are generated [3]. The stillage comprises a material with high chemical oxygen demand (COD), total suspended and soluble solids [4,5] that cannot be directly discharged to the environment without adequate treatment. With the rapidly increasing numbers of ethanol plants in response to fuel demand, it is of great interest to recover these ethanol wastes as the feedstocks of value-added products.

Microalgae have received increasing attention as feedstocks of value-added products because of their capability to accumulate biomolecules (mainly carbohydrates, lipids, proteins, and pigments) and their ability to fix $CO_2$ via photosynthesis. Growth rates and yields of many microalgae are higher than terrestrial crops. Microalgae can grow under photoautotrophic, heterotrophic, and mixotrophic conditions. Photoautotrophic microalgae convert $CO_2$ into organic substances and can utilize $CO_2$ from a feed gas. Heterotrophic microalgae

grow on organic carbon with no light to produce their biomass and bio-products. Mixotrophic microalgae cultivated with inorganic and/or organic carbon and light illumination usually have higher growth rates than autotrophic and heterotrophic microalgae and accumulate high lipid contents in their cells. However, the high cost of organic substrates is an obstacle in this cultivation process. Various researchers reported the use of wastewater as a growth medium for microalgae, suggesting the possibility of producing microalgae biomass in an economically feasible way [6,7,8]. Additionally, ethanol stillage has also been suggested as a promising substrate for cultivation of various microalgal species such as *Micractinium* sp. ME05 [7], *Chlorella* sp. [9, 10] and *Scenedesmus* sp. [10,11].

Typically, microalgae are able to grow effectively at $CO_2$ concentrations of 1-10%. Higher $CO_2$ concentrations can cause negative effects on microalgae growth [12]. However, $CO_2$ in the exhausted gas is typically higher than this range, such as 10-30% from the flue gas of a coal burner [13], 20–60% from anaerobic digestion processes, and even above 80% from ethanol fermentation [2]. Therefore, it is important to obtain microalgal strains capable of tolerating high $CO_2$ concentrations. Additionally, the ambient temperature and light intensity are key factors requiring consideration in large-scale microalgae cultivation. Tropical and subtropical zones consistently have long duration of daylight throughout the year. Hence, from this perspective, they are suitable locations for microalgae cultivation. However, elevated ambient temperatures increase the water temperature in outdoor photobioreactors to levels that can exceed 38ºC. This can cause the death of microalgal cells or inhibit their activity and temperature control in large bodies of water making outdoor cultivation costly. The use of thermo-tolerant microalgal strains might be a promising solution to these problems since their cultivation can be conducted without the need for temperature control.

This research isolated thermo-tolerant freshwater green microalgae with the capability of tolerating high concentrations of $CO_2$. The effects of temperature on mortality and the effects of $CO_2$ on the growth of the isolated microalgae were investigated under photoautotrophic cultivation. The optimal conditions for biomass and macromolecule production were evaluated under photoautotrophic cultivation using a synthetic medium and under the mixotrophic condition with cassava stillage as a growth medium with no nutrient supplementation.

## 2. Materials and methods

### *2.1 Cassava stillage*

The cassava stillage used in this study was kindly provided by Thai Ethanol Power Public Co., LTD (Khon Kaen, Thailand). Samples were collected in polypropylene carboys and stored at 4 °C for a week to allow sedimentation of solid particles. The supernatant was siphoned from the settled stillage and filtered through muslin cloth to remove fine particles. The filtered supernatant (pH 4.24) comprises 28.30 g/L of total soluble solids, 23.23 g/L of volatile solids, 20.04 g/L of total COD, 17.98 g/L of soluble COD, 1.05 mg/L of total nitrogen, 0.025 mg/L of total phosphorus and 2.53 g/L of total sugars. The supernatant was diluted with distilled water, its pH adjusted using 1 M HCl or 1 M NaOH, and then sterilized at 110ºC for 28 min before use as a growth medium for microalgae cultivation.

### *2.2 Microalgae isolation and characterization*

A green water sample was collected from a freshwater fish farming area in Nakornratchasima Province, Thailand, and used as a source for microalgae isolation. Five mL of green water was inoculated into a one-liter laboratory glass bottle containing 800 mL of 3N Bold Basal Medium (BBM) (sodium nitrate concentration 0.75 g/L) [14]. The bottle was exposed to the natural light (0-110,000 lux) and ambient temperature (22-49ºC) with 30% $CO_2$ enriched air supplied at 0.2 vvm for 90 days with 15 days of subculture. This step was conducted to select high $CO_2$ concentration and heat tolerant microalgae. After 90 days of cultivation, the culture was serially diluted, spread onto 3N BBM agar, and incubated at room temperature (32±4ºC) under 5000 lux illumination from cool white fluorescent lamps. A single colony was picked and streaked onto 3N BBM agar. This process was repeated until bacteria- and fungi-free algal colonies were obtained. The purity of a single algal isolate was investigated under a microscope. Morphology of the isolated microalgae was observed to identify the specific strains. Additionally, the microalgal isolates were identified using a molecular technique. Colony PCR was conducted to amplify the ITS1-5.8S-ITS2 region in the DNA of each isolate. The ITS forward1: ‘‘ACCTAGAGGAAGGAGAAGTCGTAA’’ and ITS reverse1: ‘‘TTCCTCCGCTTATTGATATGC’’ [15] were applied. The obtained PCR products were sent to Macrogen, Korea for DNA sequencing.

### *2.3 Photo autotrophic cultivation test*

Unless otherwise stated, three biological replicates of photoautotrophic cultivations were conducted in one liter laboratory glass bottles containing 800 mL 3N BBM with 10% $CO_2$ enriched air supply at 0.2 vvm at room

temperature (32±5ºC). The experiments were conducted in a dark, temperature-controlled room. Cool white fluorescent lamps were used for continuous illumination at an average light intensity of 5000 lux. Culture samples were taken at regular time intervals for analysis of biomass concentration and composition (carbohydrate, lipid, and protein). The isolated microalgae were grown under the stated conditions for 4 days to obtain seed inocula for further experiments.

The seed inoculum of each isolate was grown under different temperatures of 30, 35, 40, 42, 45, and 48ºC for 24 h in a temperature-controlled incubator to assess the thermotolerant capability of isolated microalgae. The availability of the microalgae was then determined using to a colorimetric assay of viability for algae (CAVA) technique and the mortality of the microalgae was calculated [16].

The effects of $CO_2$ concentration were investigated by cultivating the isolated microalgae with 0.2 vvm air enriched with $CO_2$ at various concentrations of 10%, 20%, 30%, 50%, 60%, and 80%. The cultivation was conducted in a temperature-controlled room (30±2ºC) at a 4500 lux illumination. Each isolated microalga was grown with optimal $CO_2$ concentration while sodium nitrate concentrations in the BBM were varied from 0 to 1.0 g/L, which is equivalent to 0-164.7 mg-N/L, to determine the effects of nitrogen concentration.

*2.4 Mixotrophic cultivation of the isolated Acutodesmus sp. KKU-P2 microalga*

Two strains of microalgae, *Acutodesmus* sp. KKU-P2 and *Coelastrella* sp. KKU-P1, were successfully isolated. Photoautotrophic cultivation tests indicated that the KKU-P2 strain possesses higher growth and lipid accumulation than KKU-P1. Additionally, a preliminary experiment was performed to evaluate the growth potential of both microalgal strains using cassava stillage (4 g-COD/L) as carbon and nutrient sources. Better growth performance was observed with the strain KKU-P2 (data not shown). Based on these results, the isolated microalga *Acutodesmus* sp. KKU-P2 was selected for the mixotrophic cultivation test. This microalga was grown photoautotrophically to obtain a seed inoculum as described above. Except when otherwise stated, the seed cells were grown in 500 mL flasks containing 200 mL of sterile diluted cassava stillage (initial pH 7.0±0.05) with no nutrient supplementation. The flasks were incubated at 35ºC and shaken at 150 rpm with continuous light illumination at 3000 lux. The effect of stillage concentrations of 2, 4, 6, 8, 10, 12, and 14 g-COD/L on microalga growth and macromolecule (protein, lipid, carbohydrate) accumulation was first examined. The obtained optimal stillage concentration was further used to determine the effect of nitrogen source supplementation, initial pH, and light intensity. The effect of nitrogen supplementation was evaluated by supplying urea at different concentrations of 1, 2, 3, and 4 g/L to the diluted cassava stillage. The effect of light intensity was examined at various average light intensities of 3000, 5000, 7000, and 9000 lux measured at the top of the flasks. Culture samples were taken at regular time intervals during cultivation for analysis of biomass concentration and composition. All experiments were performed using three biological replicates.

*2.5 Analytical methods*

The pH of the stillage and culture was determined using a pH meter. Total solids (TS) and volatile solids (VS) concentrations of the stillage were determined using standards method of the American Public Health Association (APHA) [17]. Total COD (tCOD) and soluble COD (sCOD) of the stillage were determined using a closed reflux method [17]. The total nitrogen concentration was determined using Hach TN reagent set HR TNT (method 10072) and total phosphorus concentration was assayed using Hach phosphorus (total) TNT reagent set, HR, Molybdovanadate kit (method 10127).

Culture samples were centrifuge at 10,000 rpm for 5 min. The cell pellets obtained after centrifugation were washed using distilled water and recentrifuged. These washed pellets were used for the determination of cell dry weight after drying at 80ºC for 24 h. Additionally, the protein and carbohydrate contents were analyzed according to Li et al. [18], and lipid analysis was performed according to Mishra et al. [19].

## 3. Results and discussion

*3.1 Microalgae identification and photoautotrophic cultivation test*

Two strains of thermo-tolerant freshwater green microalgae, KKU-P1 and KKU-P2 (Figure 1), were isolated and identified using morphological and molecular analyses. The blast results indicated that KKU-P1 and KKU-P2 strains are closely related to the microalgae genus *Coelastrella* (98.42% similarity) and *Acutodesmus* sp. (99.51% similarity), thus they were named *Coelastrella* sp. KKU-P1 and *Acutodesmus* sp. KKU-P2, respectively. The sequences of their ITS1-5.8S-ITS2 region were deposited in GenBank under the accession numbers of MW581273 and MW555785, respectively.

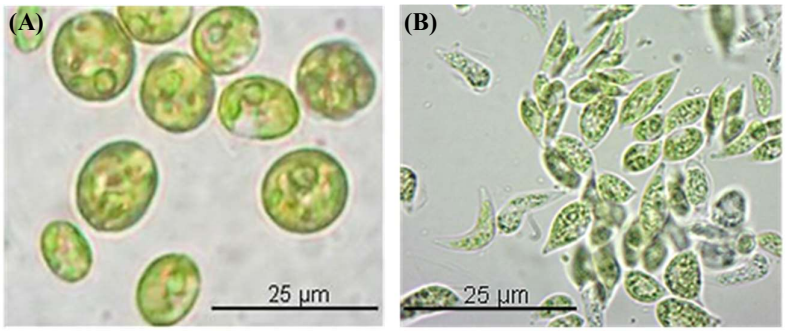


**Figure 1** Morphology of (A) *Coelastrella* sp. KKU-P1 and (B) *Acutodesmus* sp. KKU-P2 under light microscope.

*3.2 Photoautotrophic cultivation test*

The strain KKU-P1 was able to survive at 45 °C for 24 h with a 6.10% mortality rate caused by heat. Mortality increased to 23.70% when the temperature was increased to 48°C. The strain KKU-P2 survived at 40°C for 24 h with a 12% mortality rate. A dramatic increase in mortality (38.31 to 84.12%) was observed at higher temperatures of 42 to 48°C (Figure 2A). $CO_2$ concentrations affected biomass production by both strains. Relatively high biomass concentrations for KKU-P1, in the range of 1.76 to 2.13 g/L, were observed with the $CO_2$ concentrations ranging between 10-50%. Increased $CO_2$ concentrations to 80% markedly decreased the biomass concentration to 0.42 g/L. The strain KKU-P2 yielded high biomass concentrations of 1.95-2.16 g/L at $CO_2$ concentrations of 10%-30%. More than 6 times lower biomass concentrations were observed for KKU-P2 with $CO_2$ concentrations of 50% (Figure 2B).

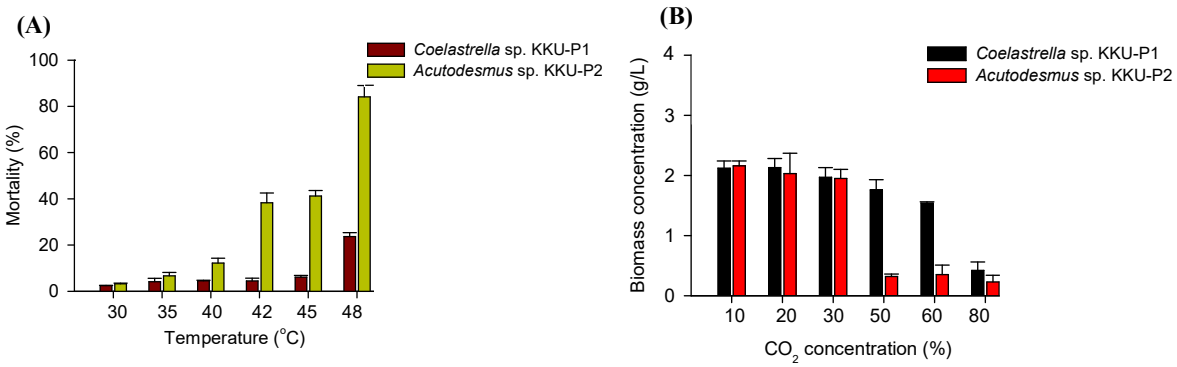


**Figure 2** (A) Mortality of isolated microalgae after incubation at various temperatures for 24 h and (B) biomass production of isolated microalgae grown in 3N BBM at 30ºC with various $CO_2$ concentrations.

Nitrogen concentrations affected the biomass production and biomass contents of the isolated microalgae (Figure 3). Biomass concentrations and protein contents increased with nitrogen concentration. The maximum biomass concentration reached 2.64 (KKU-P1) and 2.91 (KKU-P2) g/L at a nitrogen concentration of 164.7 mg/L. Under nitrogen-limited conditions (0.00-41.2 mg-N/L), the KKU-P1 strain showed a potential of carbohydrate accumulation while low contents of lipid were observed. In contrast, significant lipid accumulation was observed in the strain KKU-P2 with less carbohydrate accumulation. Total carbohydrate contents of KKU-P1 reached 45% in dry biomass while KKU-P2 possessed the potential of total lipid accumulation over 40% under nitrogen-limited conditions (Figure 3).

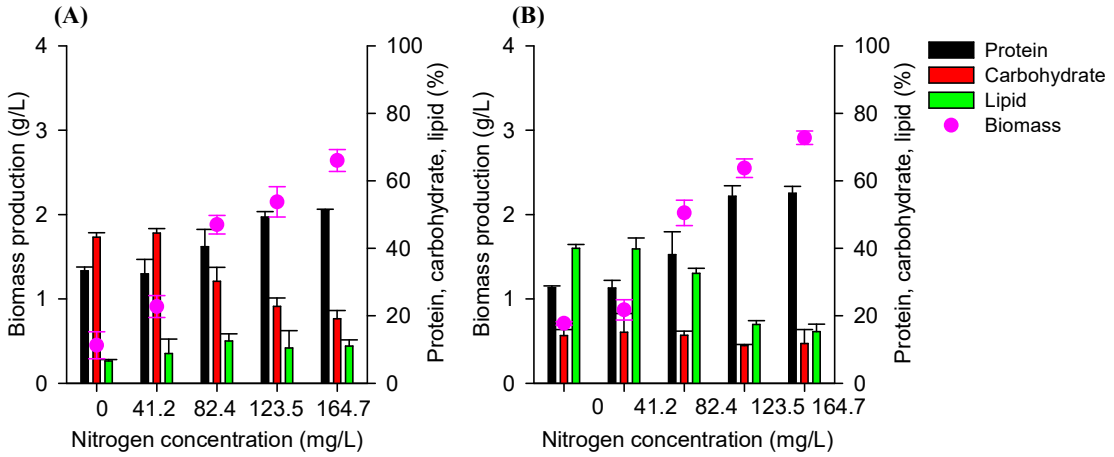


**Figure 3** Effect of nitrogen concentration on biomass production and biomass compositions (protein, carbohydrate, and lipid contents) of (A) *Coelastrella* sp. KKU-P1 and (B) *Acutodesmus* sp. KKU-P2.

The present results indicated successful isolation of green microalgae with the capability of tolerating high temperatures and high concentrations of $CO_2$. The isolated microalgae strains are promising biomass sources in tropical and subtropical areas with the potential for used mitigating $CO_2$ from emitted gases of various systems such as ethanol fermentation as well as biomass or fuel burning processes. Various genera of microalgae have been isolated and shown to have heat and/or $CO_2$ tolerance, as previously reviewed by Raeesossadati et al. [20]. For example, *Scenedesmus* sp. was able to grow at $CO_2$ concentrations ranging from air level to 70% in which the highest biomass concentration of 3.92 g/L was achieved at a $CO_2$ concentration of 10%. About a 30% decreased biomass production was observed at 70% $CO_2$. High $CO_2$ concentrations (30-70%) were favorable for lipid accumulation of *Scenedesmus* sp. [21]. *Coelastrella* sp. F50 could withstand a temperature of 50ºC for 8 h, however, a decrease in growth rate was observed compared that at 45ºC [22]. The results of biomass production and biomass content obtained from this study are comparable to those reported in published research studies. Various strains of thermotolerant *Desmodesmus* sp. produced approximately 2.5 g/L of biomass when they were cultivated in BBM. Nitrogen depletion promoted both lipid and carbohydrate accumulation (greater than 35% of dry weight) by strains F44 and F51, while strains F2 and F32 tended to accumulate lipid (approximately 47%) and carbohydrate (approximately 50%), respectively, under a nitrogen depleted condition [23].

*3.3 Mixotrophic cultivation of Acutodesmus sp. KKU-P2*

Growth of *Acutodesmus* sp. KKU-P2 with various stillage concentrations (based on COD) is depicted in Figure 4A. The microalga was able to grow in 2 to 12 g-COD/L stillage without a lag phase. At a stillage concentration of 2 and 8 g-COD/L, rapid growth was only observed until 48 h of cultivation and decreasing growth were seen thereafter. Microalga cultivated in 10 and 12 g-COD/L stillage tended to grow continuously from days 0 to 6 and reached a stationary phase after 8 days. An increase in stillage concentration from 2 to 12 g-COD/L supported microalgal growth and biomass production. After 10 days of cultivation, a maximum biomass concentration of 3.36 g/L was obtained with 12 g-COD/L stillage while the lowest biomass concentration of 1.17 g/L was obtained with 2 g-COD/L stillage. A 96 h lag period of growth was observed at a high stillage concentration of 14 g-COD/L, indicating a substrate inhibition effect on the KKU-P2 strain. However, the biomass concentration increased rapidly after entering the exponential phase, reaching 3.16 g/L at the end of cultivation. A slight decrease in biomass production was observed compared to those obtained with 12 g-COD/L stillage. This might have resulted from decreased light penetration with higher stillage concentrations. The cassava stillage turned to dark in color when its pH was adjusted to values higher than 5.5 to facilitate microalgal growth. The dark color of cassava stillage could hinder photosynthesis by microalga and decrease its growth and biomass production. The inhibition of biomass production at high stillage concentrations as affected by substrate inhibition and reduction of light penetration has been reported elsewhere [11,7,24,25].

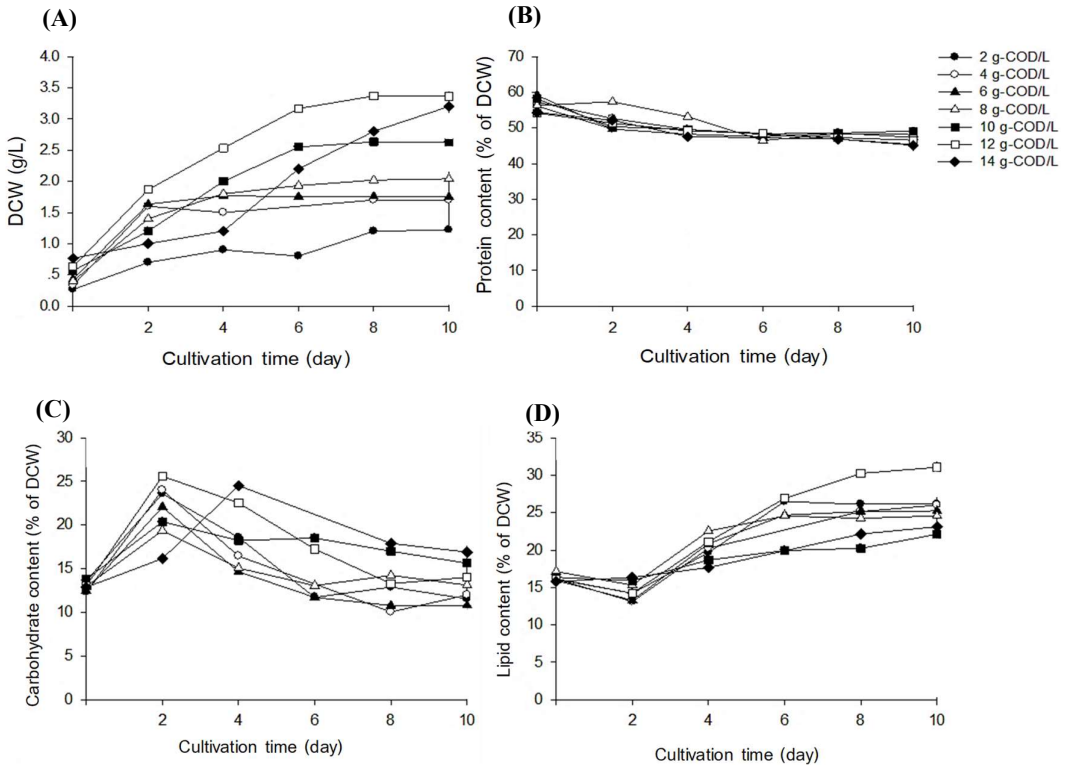


**Figure 4** Effect of stillage concentrations (in g-COD/L) on biomass production expressed as dry cell weight (CDW) (A) protein contents (B), carbohydrate (C), and lipid (D) of *Acutodesmus* sp. KKU-P2

Contents of the biomass obtained from microalga cultivation with the various stillage concentrations are depicted in Figures 4B-C. Initially, the microalga biomass was comprised of 58.19% protein, 13.14% carbohydrate, and 16.02% lipid. Protein contents for all of the stillage concentrations tested tended to decrease over time during 6 days of cultivation and remained unchanged thereafter (Figure 4B). This coincides with the growth profiles of the microalga (Figure 4A). The carbohydrate contents increased for 2 days for all treatments except for the treatment with 14 g-COD/L stillage, in which the carbohydrate content increased until 4 days of cultivation (Figure 4C). The decrease in starch content thereafter implies degradation of storage starch during microalga growth. A slight decrease in lipid content was observed in 2 days of cultivation and tended to increase afterward (Figure 4D). These results indicated that the KKU-P2 strain initially accumulated carbohydrates along with its growth and accumulated lipid as growth slowed. Microalgae starch and lipid accumulation is species-specific. Generally, microalgae synthesize macromolecules such as cellular proteins, cell wall carbohydrates, and membrane lipids that support cell growth and division. Under unfavorable conditions for growth, some microalgae switched their carbon metabolic pathway toward energy-rich storage compounds such as starch and/or lipid. Starch synthesis shares a precursor (glyceraldehyde-3-phosphate, G3P) with lipid synthesis and cellular starch degradation releases metabolites for fatty acid production [26]. The transitory nature of starch concentrations with lipid accumulation in response to stress conditions, especially nutrient starvation, was found in several strains of green microalgae such as *Chlorella zofingiensis* [27], *Haematococcus pluvialis* [28], and *Pseudochlorococcum* sp. [29].

The increased lipid and carbohydrate contents of the microalga cells implies that the cells were grown under stressed environmental conditions. Stress factors such as nutrient starvation and unsuitable ranges of pH, temperatures, and reduced light intensity applied during microalgae cultivation can drive microalgae to accumulate carbohydrates and lipid [30]. The effects of nitrogen (urea) supplementation, initial pH, and light intensity were further investigated at a stillage concentration of 12 g-COD/L. Figure 5 summarizes their effects on maximum biomass concentrations and biomass compositions. A slight increase in biomass concentration from 3.36 to a value in the range of 3.62 to 3.75 g/L was observed when the stillage was supplemented by 1 to 4 g/L of urea (Figure 5A). However, markedly decreased carbohydrate and lipid contents was noticed with increased protein content. These results imply that nitrogen content of the stillage is appropriate for carbohydrate and lipid accumulation by *Acutodesmus* sp. KKU-P2. Supplementing nitrogen to the stillage may alter the microalga metabolic pathways for protein synthesis hence decreasing carbohydrate and lipid

production. Initial pH markedly affected biomass production but not biomass composition (Figure 5B). Maximal biomass concentrations of 3.36-4.12 g/L were achieved after 10 days of microalga cultivation with an initial pH of 5.0-7.0. Lower maximum biomass concentrations, 2.56 and 1.52 g/L, were obtained at days 4 and 2, respectively, with initial pH values of 8.0 and 9.0, respectively. The decrease in biomass concentration after reaching their maximum values may be attributed to cell autolysis at high pH, noting that higher final pH values of 10.24 and 10.64 were observed in the treatments with initial pH values of 8.0 and 9.0, respectively.

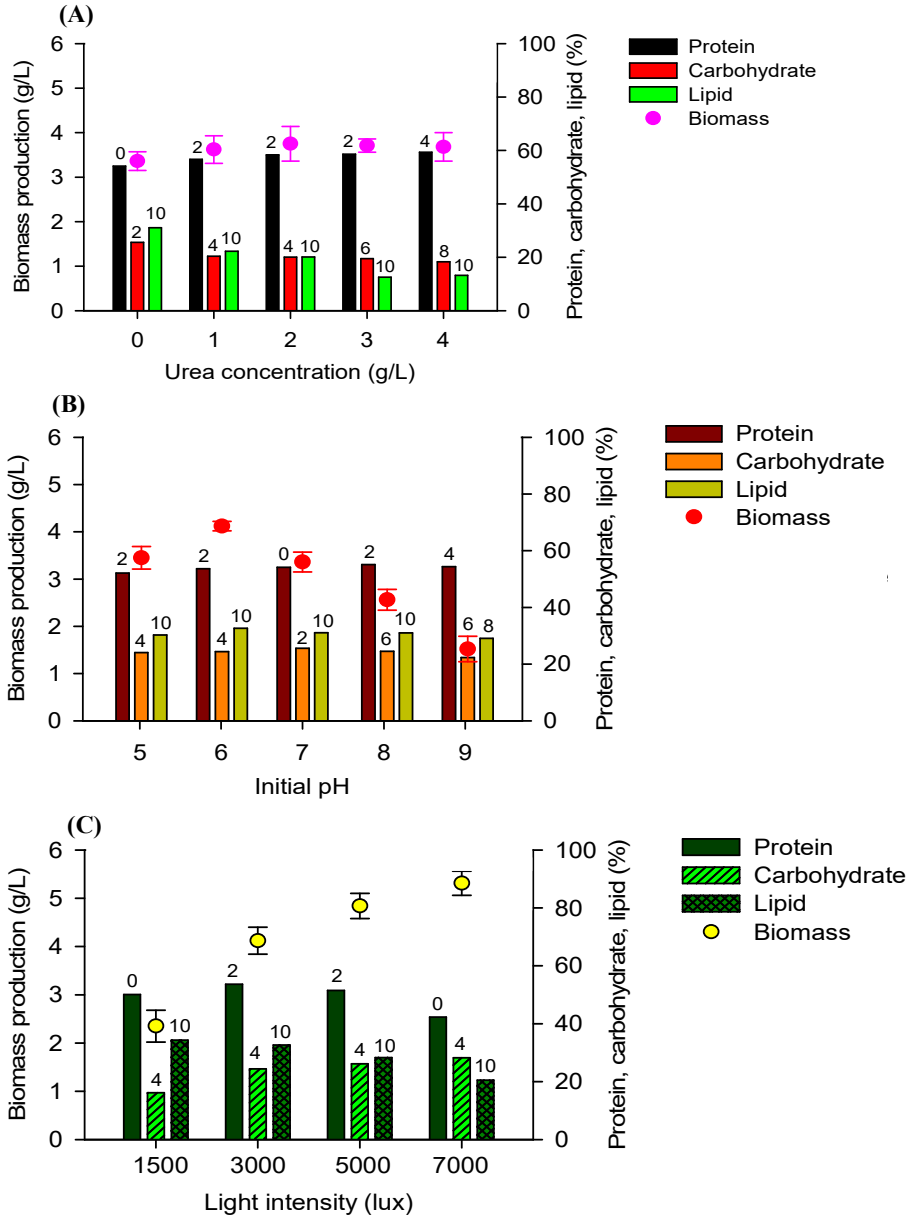


**Figure 5** Maximum biomass production and biomass composition obtained from mixotrophic cultivation of *Acutodesmus* sp. KKU-P2 in 12 g-COD/L stillage with (A) nitrogen (urea) supplementation, (B) initial pH , and (C) light intensity. The number on each vertical bar indicates the cultivation time (days) to obtain the reported value.

Light intensity significantly affected biomass production and composition (Figure 5C). The maximum biomass concentration, achieved at day 10, increased from 2.35 to 5.31 g/L, with increased light intensity from 1500 to 7000 lux, suggesting no light inhibition occurred in this study. Maximum carbohydrate contents were obtained at day 4 of cultivation for all treatments in which the highest value of 28.22% was achieved at the greatest light intensity, 7000 lux. Low light intensity positively affects the lipid content of the microalga cells.

The highest lipid content, 34.33%, was attained at a light intensity of 1500 lux. The carbohydrate and lipid productivities were calculated to justify the optimal light intensity for *Acutodesmus* sp. KKU-P2 cultivation. Maximum carbohydrate productivity, 278 mg/L.d, was obtained with microalga cultivation under 7000 lux light illumination for four days, while the highest lipid productivity, 137 mg/L.d, was achieved at ten days of cultivation under a 5000 lux light illumination. Light illumination is important for photosynthesis. It directly affects microalgae growth, $CO_2$ fixation, and biomass composition. An increase in light intensity positively affects microalgae growth. However, higher light intensities than the optimal values decrease the growth rate as microalga cells are affected by light inhibition [30]. A certain level of light intensity is required for maximum lipid or carbohydrate accumulation and the effect of light intensity is species-specific [31]. During photosynthesis, Adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) are synthesized as an energy source and reducing agent for converting $CO_2$ to G3P, which can be further used in lipid and starch synthesis pathways. Additionally, variation of light intensity can affect the activation of a key enzyme for starch synthesis, phosphoglucomutase, which therefore directly influences the starch accumulation by microalgae [32].

## 4. Conclusion

Two strains of microalgae, *Acutodesmus* sp. KKU-P2 and *Coelastrella* sp. KKU-P1, with the ability to tolerate high temperatures and high concentrations of $CO_2$ were successfully isolated and identified. The growth and biochemical (lipids, carbohydrates and proteins) accumulation of both microalgal strains were investigated under photoautotrophic cultivation using $CO_2$ as a carbon source. The cultivation of *Acutodesmus* sp. KKU-P2 under a mixotrophic condition using cassava stillage as a carbon and nutrient source was also evaluated. Under suitable environmental conditions for cultivation, *Acutodesmus* sp. KKU-P2 could accumulate high contents of lipid while *Coelastrella* sp. KKU-P1 could accumulate high contents of carbohydrate, indicating their potential to be used as feedstock for biodiesel and bioethanol production, respectively. The present study demonstrates the potential use of inexpensive and readily available wastes from the ethanol industry (distillery stillage) as carbon and nutrient feedstock for microalgae cultivation that could reduce the need for expensive organic carbon sources, fresh water, and supplemental nutrients.

## 5. Acknowledgments

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