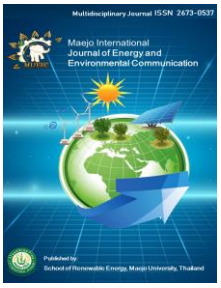
Maejo International Journal of Energy and Environmental Communication

Journal homepage: https://ph02.tci-thaijo.org/index.php/MIJEEC



ARTICLE

Alkali pretreatment and enzymatic saccharification of blue-green alga *Nostochopsis lobatus* for bioethanol production

Pattranan Junluthin[1], Thunvarat Pradechboon[2],*

[1]Biomedical Science, Faculty of Science (Salaya Campus), Mahidol University, Nakhon Pathom, 73170, Thailand
[2]Department of Physics, Faculty of Science, Naresuan University, Phitsanulok, 65000, Thailand

ARTICLEINFO

Article history:
Received 23 January 2022
Received in revised form
19 February 2022
Accepted 25 February 2022

Keywords:
Blue-green alga
Alkali pretreatment
Enzymatic saccharification
Fermentation

ABSTRACT

Using various Sodium hydroxide (NaOH) concentrations (0, 1, 2, and 3%), we studied the alkaline pretreatment of microalgal biomass from the blue-green alga *Nostochopsis lobatus* in this work. In this pretreatment stage, the entrapped polysaccharides within the microalgae cell walls are broken down to form fermentable subunits. The selected pretreatment procedure (2% NaOH) was then followed by enzymatic saccharification for the generation of bioethanol. This pretreatment step aims to release and break down entrapped polysaccharides in the microalgae cell walls into fermentable subunits. The best result of sugars after enzyme hydrolysis was obtained in total and reducing sugars 210.22 and 98.54 g/L, respectively. In addition, Saccharomyces cerevisiae was used to ferment the hydrolysate; the highest ethanol yield was 14 g/L by 48 hours of fermentation. The alkaline pretreatment method was a promising option for pretreating microalgal biomass for bioethanol production.

1. Introduction

The massive extraction of petroleum caused a dramatic surge in gasoline prices worldwide. As a result, a plethora of new challenges arose, including increased inflation and consumer price. This entire situation has focused scientists' attention on the need for alternative energy sources to sustain long-term growth. Concerns about growing urbanization and motorization contribute to the rising global demand for petroleum oil. This is just one way a country's economy can be affected. Concerns about the availability of sustainable fuels have increased due to the decreasing supply of fossil fuels and rising greenhouse gas emissions (Palanisamy et al., 2021a). Using bioenergy or biofuels as an alternative to conventional fuels is undoubtedly a reliable and endless source of supply. Researchers are constantly looking at the possibility of making biofuel from renewable resources, as biofuel reduces one's dependence on fossil fuels. Biofuels can be described as an energy source that is both non-toxic and renewable. They are made from biological resources such as plants and bacteria. There are many forms of biofuels, such as pyrolysis oil and biogas, and bioethanol and biodiesel.

Bioethanol is the renewable fuel with the greatest potential for widespread application in transportation. Fermentation is the biochemical process that converts biomass into bioethanol. This process is carried out on biomass. A wide variety of microorganisms are responsible for this. Bioethanol (C_2H_5OH), or ethyl alcohol, can be used to replace petroleum oil due to its low

* Corresponding author.
E-mail address: thunvaratp60@nu.ac.th (Thunvarat Pradechboon)

toxicity and high efficiency when mixed with gasoline. It is possible to replace petroleum oil without any engine modifications. These benefits are not limited to bioethanol's high octane, which reduces engine knocking; its higher oxygen content, which lowers the emission of pollutants gases into the atmosphere; and the possibility that it can be used directly as pure alcohol in gasoline-powered industries. Before bioethanol production can be significantly increased, it is important to find suitable feedstock. The following are other significant hurdles: Bioethanol production has been divided into three distinct generations according to the feedstock used as well as the processing methods employed by each generation. Bioethanol is a viable alternative to petroleum. Bioethanol can be used to replace fossil fuels. Bioethanol can be used as a fuel for a variety of reasons, both economically and environmentally. Even though starch and sugarcane juice can be used to make bioethanol these ingredients reduce the availability of bioethanol and compete with food production.

To avoid potential conflicts between fuel and food, substantial research has been conducted on second-generation bioethanol. However, due to the complex nature of lignocellulose's structural makeup, this method is difficult and economically unviable. The production of lignocellulosic fuel crops can indirectly affect food availability because it takes so much land. People have noticed that algae could be used to replace lignocellulosic bioethanol feedstock (Palanisamy et al., 2022a). Algae grow fast, do not need any land, are not invasive to food-fuel conflicts, and can be grown in many strains that can be adapted to different environments. Algae do not contain any lignin, so their composition is simpler than that of lignocellulose (Palanisamy et al., 2022b). The hydrolysis of polysaccharides to fermentable sugars is a simple process. Algal biomass can also produce cold-active enzymes that can be used in bioethanol production. These enzymes are responsible to hydrolyze starch, cellulose, and algal polysaccharides. This study demonstrates the potential of algae to be used in the production and conversion of bioethanol.

The food, pharmaceutical and fuel industries have been interested in Cyanobacteria as a potential source of bioresources. *Nostochopsis*, a filamentous diazotrophic Cyanobacterium that lives in freshwater lakes and slow-moving rivers in the tropics or subtropics, forms luxuriantly on rock surfaces, in the form of mucilaginous ball-shaped clinging cyanobacterium. Native people in the region use the natural expansion of unialgal populations, which is manifested as mucilaginous ball-shaped balls, to supplement their food intake. Pandey (2003) states that *N. lobatus* provides a rich source of all three macronutrients, protein, carbs, and fatty acid. There are many ecological factors that influence the growth of photosynthesis-based organisms within their natural environments. It is generally believed that the main factor in the development and maintenance of water-blooming organisms is the concentration of phosphorus. Phosphorus is responsible for controlling both the biomass and physiology of cyanobacteria.

Nostococcus Lobatus, blue-green algae, is an example of a naturally occurring ecosystem. It can be found in Northern Thailand. It is a great feedstock for making bioethanol because it has a lot of cellulose fibres and a lot of biomasses. Another benefit is that it grows faster than other perennial and annual lignocellulosic feedstocks. However, the presence of lignocellulose within its biomass is a significant barrier to enzymes that attempt to degrade cellulose. The presence of lignin in the biomass creates a physical barrier that limits the availability of hydrolysing enzymes. This is because lignin adsorbs both enzymes in a non-specific way, which together results in lower saccharification yields. The present study aimed to optimize alkali pre-treatment conditions and the process parameters for hydrolysis pre-treated blue-green alga biomass. This was done while considering both the potential and challenges of delignification. These methods would be valuable additions to the process of converting lignocellulosic biomass into digestible sugars. In the present study, naturally occurring algal blooms were investigated for high fermentable sugars for bioethanol production. Because of its high biochemical content for blooming, the chosen alga could provide a reliable source of biomass for environmentally friendly bioethanol production.

2. Materials and Methods

2.1. Plant materials

Nostococcus Lobatus, blue-green algae, was grown in the roadside ponds were growing at Mae Rim located at 18°57'17.68" N 98° 45' 43.46" E, Chiang Mai Province, Thailand. Bloomed algal biomass was harvested and transferred to Energy Research Center, Maejo Univerity, for further experimental analysis. After being dried, the biomass was cleaned to remove any contaminants, milled to a uniform size of 1 mm, and then stored at room temperature in an airtight container for subsequent pretreatment, enzymatic hydrolysis, compositional analysis, and structural analysis.

2.2. Alkali pretreatment

Blue-green algae *Nostococcus Lobatus* biomass was immersed in double-distilled water at 100 °C for five minutes with a 10% biomass loading. Then, the extra water was taken out, and the biomass was used to prepare the alkali. Following that, samples were incubated with different NaOH concentrations (0, 1, 2 and 3%) at room temperature, and duration (24hrs) in an Erlenmeyer flask. To achieve a pH of neutrality, the samples were repeatedly rinsed with water and buffer. For physico-chemical analysis, samples were dried at room temperature, and the pretreated biomass was employed just as it had been for saccharification. Experiments followed all the experimental procedures 20 g dried power with 4 (0, 1, 2 and 3% NaOH solution) treatments. The optimal conditions for a subsequent large-scale fermentation were then chosen by estimating the total sugar and decreasing sugar in all the pretreatment samples.

2.3. Saccharification

In a 150 ml Erlenmeyer flask with the necessary amount of biomass loaded in citrate buffer, pretreatment jute biomass was

enzyme saccharified (pH 4.5 to 5). Following the pretreatment, the pH level was brought down to 5, and then 2% of the commercial enzyme cellulase was added. It was hydrolyzed at 45 °C for 48 hours at 120 rpm in an incubator shaker. The hydrolysate was regularly withdrawn, and the technique with 2, 5-dinitrosalicylic acid was used to determine the reducing sugars (Miller, 1959).

2.4. Microorganisms and culture conditions

The biotechnology program at Maejo University in Chiang Mai, Thailand, has a culture collection from which the fast-fermenting yeast strain Saccharomyces cerevisiae was obtained. In yeast extract peptone dextrose (YEPD) broth (w/v %) [Yeast extract 1, peptone 2, dextrose 2, pH 6.5 0.2] at 30 °C, yeast culture was routinely cultivated at 150 rpm for 48 hours.

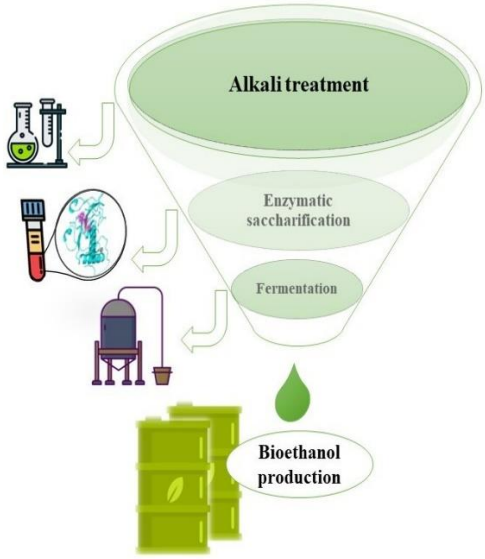


Figure 1 Schematic diagram of the experimental study processes

2.5. Fermentation Sugar analysis and alcohol determination

The schematic diagram of the experimental study processes is presented in Figure 1. Separate from fermentation and hydrolysis, the fermentation process was used in the research (SHF). First, the materials underwent a pre-treatment step to get the sugars out that yeast needs to digest. The pH was then adjusted to 5. Utilizing 2% (v/v) of yeast in 1 L fermenters (Triplicates) and incubating the mixture for three days at 30 °C to 35 °C allowed for the successful completion of the fermentation process. Both the ethanol levels and sugar content were measured. Then, using an ebulliometer, estimate the yield of ethanol (Vu et al., 2018). Utilizing the phenol-sulfuric acid and DNS standard method for total sugar and reducing sugar analyses, respectively (Miller, 1959; Dubois et al., 1956). The current investigation results were given as the mean of three replicates. Additionally, data from three duplicate observations are provided as mean + SD.

3. Results and discussion

Cyanobacteria is a prokaryotic microalga. Some of them form trichomes or extracellular polysaccharides, which can then be used to create macroscopic colonies. Certain types of cyanobacteria are known to be eaten worldwide for many years. For example, the blue-green algae *N. Lobatus* is considered edible in Thailand. *Nostoc Lobatus*, a terrestrial cyanobacterium, is difficult to wash until it is suitable for consumption. *N. lobatus*' branched filaments form rare intercalary heterocysts within the main trichome (Pandey & Pandey, 2008). The short lateral branches (1 to 4-celled) typically produce a terminal heterocyst if the alga has been grown in a medium that is devoid of combined Nitrogen. Intercalary heterocysts do not form in the presence of ammonium, but the lateral heterocysts grow to a balloon-like shape and undergo division in situ. They then release 3-5 so-called germlings. The cycle continues until the germlings stop growing and continue to produce heterocysts (Babić et al., 2016). Heterocysts form at the terminal ends of long lateral branches in the late stages of growth in ammonium media. They are not formed in cultures that are nitrogen-free.

Moreover, the possible role of lateral homocysts when controlling the growth and development of long laterals (Skinner & Entwisle, 2001). Consequently, a large amount of biomass could be generated during the algal bloom. The major lignocellulosic substances found in algal biomass are cellulose, pectin, hemicellulose, and lignin. The conversion of cellulose and/or hemicellulose into their respective sugars is the basis for producing various value-added chemicals, including fuel ethanol.

Pre-treatment is necessary to maximize the yield of reducing glucose and minimize degradation products from lignocellulosic material (Manmai et al., 2019; Palanisamy et al., 2022a). Because

they are relatively inexpensive, less energy-intensive and more effective towards many lignocellulosic feedstocks such as agricultural residues or forages than other pre-treatment methods, alkaline pre-treatment methods have been in demand (Manmai et al., 2020).

Alkaline pre-treatments can lead to delignification and disruption of structural links, decrystallization, cellulose depolymerization, and delignification. Most previous studies used NaOH at temperatures greater than 100 °C. Alkaline reagents like lime and ammonia are effective at lower temperatures, i.e., Temperatures below 50 °C, and for a longer time. Concentrated or dilute acid can be used to hydrolyse cellulose and hemicellulose. Hemicellulose is more easily solubilizable than cellulose polymer (Nguyen et al., 2020; Palanisamy et al., 2022b). Acid hydrolysis has the advantage that it is faster than enzyme hydrolysis (Mussatto et al., 2012). However, glucose could be further degraded into toxic compounds.

In this study, sugar concentrations of *N. lobatus* biomass after pretreatment and hydrolysis are presented in Table 1. Concentrated acid hydrolysis can produce more monosaccharides than dilute acid hydrolysis but produces fewer degradation products. Concentrated acid (>30%) is used at a lower temperature (100°C) to reduce the formation of toxic substances. For fermentation, acid hydrolysate must be processed. The following steps are required: neutralization, concentration, detoxification if necessary and supplementing different nutrients to ferment the sugar mixture (Sunarti et al., 2017). Different alkaline pretreatment methods (NaOH + urea, ammonia) were used in the current investigation for delignification at ambient temperature (30 °C).

Table 1 Sugar concentrations of *N. lobatus* biomass after pretreatment and hydrolysis

Dried Biomass (g)	NaOH Concentration (%)	Total Sugar (g/L)	Reducing Sugar (g/L)
After pretreatment			
20	0	108.24±1.88	48.58±1.08
	1	141.74±0.92	55.67±1.11
	2	162.15±1.55	62.92±1.03
	3	150.38±1.45	57.88±1.56
After hydrolysis			
20	2% NaOH + 2% enzyme	210.22±1.11	98.54±0.89

Note: Data are presented as means ± SD of three replicates.

Optimized variables were biomass loading, alkali strength, residence time, and time for pre-treatment (Asachi & Karimi, 2013; Palanisamy et al., 2021a). The optimization also included optimizing acid concentration, time, and reaction time to obtain maximum fermentable sugars (Galindo-Leva et al., 2016). The naturally occurring yeast can ferment most of the sugars in lignocellulosic biomass, which has been used in the present study to ferment the acid hydrolysate (Ramaraj et al., 2021; Palanisamy et al., 2021b). However, *S. cerevisiae* is most widely used for ethanol production from hexose sugars because of its high ethanol productivity and tolerance to ethanol and inhibitors, unable to ferment the pentose sugars (xylose), which are the second most abundant sugar present in the lignocellulosic biomass.

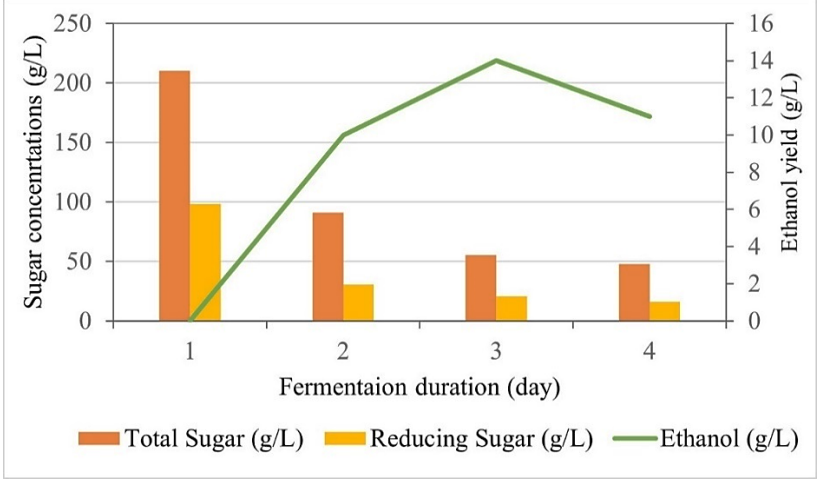


Figure 2 Schematic diagram of the experimental study processes

This study's fermentation results are shown in Figure 2. An early investigation into the generation of bioethanol by *S. cerevisiae* in a 1L bioreactor was carried out utilizing optimal parameters derived from a shake flask. The shake flask contained

75% of the substrate concentration together with yeast extract at a regulated pH. For bioethanol production, it was increased from 0 h until 48 h of fermentation time and decreased to 11 g/L at 72 h of fermentation time. The profile showed that the growth of *S. cerevisiae* continued to increase after the glucose was depleted.

Table 2 Ethanol production from different feedstocks

Feedstock	Pretreatment	Ethanol	Reference
Prosopsis julifora	Acid	7.13	Gupta et al. 2009
Water hyacinth			
Coconut meal waste	Alkaline	8.5 g/L	Sangkharak et al. 2020
Wheat bran hemicellulose	Liquid hot water + alkaline	9.5 g/L	Menon et al. 2010
Spent coffee grounds	Acid	9–10 g/L	Mussatto et al. 2012
Coffee silverskin	Liquid hot water	9–10 g/L	Galindo-Leva et al. 2016
NMMO-treated wheat straw	Liquid hot water	10.6 g/L	Asachi & Karimi, 2013
Laminaria japonica	Thermal	2.9 g/L	Sunarti et al. 2017
N. lobatus	Alkaline	14 g/L	This study

From 0 to 48 hours, the concentration of bioethanol rose in the opposite direction of the sugar content. According to studies that were conducted in the past, the maximum concentration of ethanol in the fermentation process was typically attained after 24 or 48 hours and then stayed constant after that. The most favorable circumstances for fermentation will result in the highest possible yield of ethanol. Because of their significant impact on the overall efficiency of the fermentation process, these parameters need to be optimized. It has been found that the fermentation of enzymatic needy sates results in higher fermentation efficiency compared to the fermentation of alkaline hydrolyzed lignocellulosic mate. Ethanol production from different feedstocks results as depicted in Table 2. The amount of ethanol that *N. lobatus* produced was significant, coming in at 14 g/L compared to other organisms' outputs (Asachi & Karimi, 2013; Galindo‐Leva et al., 2016; Gupta et al., 2009; Sangkharak et al., 2020; Menon et al., 2010; Mussatto et al., 2012; Sunarti et al. 2017). As a result of this, one might inevitably arrive at the conclusion that *N. lobatus* can be utilized in the production of bioethanol.

4. Conclusion

The production of bioethanol was studied in this study utilizing batch fermentation with *S. cerevisiae* yeast and a substrate of *N. lobatus* biomass. It has been demonstrated that the substrate consisting of *N. lobatus* biomass contains the required quantity of total fermentable sugar. In order to achieve the highest possible level of bioethanol production from *S. cerevisiae* in a shake flask system, the substrate was effectively adjusted and prepared. In a bioreactor using defined conditions of the *N. lobatus* biomass substrate, it was discovered that *S. cerevisiae* had a high capability of making ethanol (14 g/L). On the basis of the research that has been conducted, the potential for the biomass of *N. lobatus* to be used as a low-cost and non-food fermentation feedstock can be researched further for the purpose of increasing the concentration of bioethanol and various other biotechnological products.

Reference

Asachi, R., & Karimi, K. (2013). Enhanced ethanol and chitosan production from wheat straw by Mucor indicus with minimal nutrient consumption. Process Biochemistry, 48(10), 1524-1531.

Babić, O., Kovač, D., Rašeta, M., Šibul, F., Svirčev, Z., & Simeunović, J. (2016). Evaluation of antioxidant activity and phenolic profile of filamentous terrestrial cyanobacterial strains isolated from forest ecosystem. Journal of Applied Phycology, 28(4), 2333-2342.

Chuanchai, A., & Ramaraj, R. (2018). Sustainability assessment of biogas production from buffalo grass and dung: biogas purification and bio-fertilizer. 3 Biotech, 8(3), 1-11.

Galindo-Leva, L. Á., Hughes, S. R., López-Núñez, J. C., Jarodsky, J. M., Erickson, A., Lindquist, M. R., Cox, E.J., Bischoff, K.M., Hoecker, E.C., Liu, S., & Jones, M. A. (2016). Growth, ethanol production, and inulinase activity on various inulin substrates by mutant *Kluyveromyces marxianus* strains NRRL Y-50798 and NRRL Y-50799. Journal of Industrial Microbiology and Biotechnology, 43(7), 927-939.

Gupta, R., Sharma, K. K., & Kuhad, R. C. (2009). Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and Pichia stipitis-NCIM 3498. Bioresource Technology, 100(3), 1214-1220.

Dubois M, Gilles KA, Hamilton JK, Rebers PT, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28(3), 350-356.

Junluthin, P., Pimpimol, T., & Whangchai, N. (2021). Efficient conversion of night-blooming giant water lily into bioethanol and biogas. Maejo International Journal of Energy and Environmental Communication, 3(2), 38-44.

Khammee, P., Ramaraj, R., Whangchai, N., Bhuyar, P., & Unpaprom, Y. (2021). The immobilization of yeast for fermentation of macroalgae *Rhizoclonium* sp. for efficient conversion into bioethanol. Biomass Conversion and Biorefinery, 11(3), 827-835.

Manmai, N., Bautista, K., Unpaprom, Y., & Ramaraj, R. (2019). Optimization of combined pre-treatments on sugarcane leaves for bioethanol production. Maejo International Journal of Energy and Environmental Communication, 1(1), 30-39.

Manmai, N., Unpaprom, Y., Ponnusamy, V. K., & Ramaraj, R. (2020). Bioethanol production from the comparison between optimization of sorghum stalk and sugarcane leaf for sugar production by chemical pretreatment and enzymatic degradation. Fuel, 278, 118262.

Menon, V., Prakash, G., Prabhune, A., & Rao, M. (2010). Biocatalytic approach for the utilization of hemicellulose for ethanol production from agricultural residue using thermostable xylanase and thermotolerant yeast. Bioresource Technology, 101(14), 5366-5373.

Miller, G. L. (1972). Use of DNS reagent for determination of reducing sugars. Analytical Chemistry 31, 426-428.

Mussatto, S. I., Machado, E. M., Carneiro, L. M., & Teixeira, J. A. (2012). Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. Applied Energy, 92, 763-768.

Nguyen, T., Unpaprom, Y., & Ramaraj, R. (2020). Enhanced fermentable sugar production from low grade and damaged longan fruits using cellulase with algal enzymes for bioethanol production. Emergent Life Sciences Research, 6(2), 26-31.

Palanisamy, K. M., Paramasivam, P., Maniam, G. P., Rahim, M. H. A., Govindan, N., & Chisti, Y. (2021a). Production of lipids by *Chaetoceros affinis* in media based on palm oil mill effluent. Journal of Biotechnology, 327, 86-96.

Palanisamy, K. M., Maniam, G. P., Sulaiman, A. Z., Ab. Rahim, M. H., Govindan, N., & Chisti, Y. (2022a). Palm Oil Mill Effluent for Lipid Production by the Diatom *Thalassiosira pseudonana*. Fermentation, *8*(1), 23.

Palanisamy, K. M., Rahim, M. H. A., Govindan, N., Ramaraj, R., Kuppusamy, P., & Maniam, G. P. (2022b). Effect of blue light intensity and photoperiods on the growth of diatom *Thalassiosira pseudonana.* Bioresource Technology Reports, *19*, 101152.

Palanisamy, K. M., Paramasivam, P., Jayakumar, S., Maniam, G. P., Rahim, M. H. A., & Govindan, N. (2021b, February). Economical cultivation system of microalgae Spirulina platensis for lipid production. In *IOP Conference Series: Earth and Environmental Science* (Vol. 641, No. 1, p. 012022). IOP Publishing.

Pandey, U., & Pandey, J. (2008). Enhanced production of biomass, pigments and antioxidant capacity of a nutritionally important cyanobacterium *Nostochopsis lobatus*. Bioresource technology, 99(10), 4520-4523.

Pimpimol, T., Tongmee, B., Lomlai, P., Prasongpol, P., Whangchai, N., Unpaprom, Y., & Ramaraj, R. (2020). Spirogyra cultured in fishpond wastewater for biomass generation. Maejo International Journal of Energy and Environmental Communication, 2(3), 58-65.

Ramaraj, R., & Unpaprom, Y. (2019). Enzymatic hydrolysis of small-flowered nutsedge (*Cyperus difformis*) with alkaline pretreatment for bioethanol production. Maejo International Journal of Science and Technology, 13(2), 110-120.

Ramaraj, R., Bhuyar, P., Intarod, K., Sameechaem, N., & Unpaprom, Y. (2021). Stimulation of natural enzymes for germination of mimosa weed seeds to enhanced bioethanol production. 3 Biotech, 11(6), 1-9.

Sangkharak, K., Chookhun, K., Numreung, J., & Prasertsan, P. (2020). Utilization of coconut meal, a waste product of milk processing, as a novel substrate for biodiesel and bioethanol production. Biomass Conversion and Biorefinery, 10(3), 651-662.

Skinner, S., & Entwisle, T. J. (2001). Non-marine algae of Australia: 1. Survey of colonial gelatinous blue-green macroalgae (Cyanobacteria). Telopea, 9(3), 573-599.

Sunarti, T. C., Yanti, S. D., & Ruriani, E. (2017, May). Two-steps microwave-assisted treatment on acid hydrolysis of sago pith for bioethanol production. In IOP Conference Series: Earth and Environmental Science, 65(1), 012052.

Vu, P. T., Unpaprom, Y., & Ramaraj, R. (2017). Evaluation of bioethanol production from rice field weed biomass. Emergent Life Sciences Research, 3, 42–49.

Vu, P. T., Unpaprom, Y., & Ramaraj, R. (2018). Impact and significance of alkaline-oxidant pretreatment on the enzymatic digestibility of *Sphenoclea zeylanica* for bioethanol production. Bioresource Technology, 247, 125-130.