

Final Report

Characterization and Isolation of Duckweed for Biomass and Starch Production for Bioethanol Conversion

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May, 2020

Abstract

Project Code : MRG5580162

Project Title : Characterization and Isolation of Duckweed for Biomass and Starch Production for Bioethanol Conversion

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Project Period : 7/2/2013– 5/30/2020

Abstract:

Lemnaceae or duckweed has been known as an aquatic plant with a great potential for wastewater treatment. It also has a potential as a good resource of proteins and starch, which can be utilized for the production of value-added products such as animal feed and fuel ethanol. In this project, 24 duckweed strains were collected and isolated from the northern part of Thailand. They were morphologically characterized and categorized into 4 groups, which were *Wolffia arrhiza* (2 samples), *Lemna aequinoctialis* (9 samples), *Lemna perpusilla* (9 samples) and *Landoltia punctata* (4 samples). The best growing strains were selected for each species, which were *W. arrhiza* SC004, *L. aequinoctialis* SC022*, L. perpusilla* SC024 and *L. punctata* SC016 with biomass productivity of 16.3 g/m^2, 93.5 g/m^2, 78.6 g/m^2 and 129.2 g/m^2 and starch content of 9.7%, 21.4%, 17.8% and 26.2% respectively. Nutrients starvation, 6-BA and ABA could induce starch accumulation of those strains up to 70.5% (8.2 g/m^2), 88.9% (56.3 g/m^2), 63.9% (37.0 g/m^2) and 66.5% (61.6 g/m^2). The starch and cellulose of those strains were used as the substrate for SSF (simultaneous saccharification and fermentation) with the ethanol yield of 0.12 $\pm$ 0.009 g/g, 0.34 $\pm$ 0.012 g/g, 0.37 $\pm$ 0.018 g/g and 0.28 $\pm$ 0.010 g/g respectively. Pilot scale of polyculture of those strains was performed for 12 months with the average biomass productivity of 23.5 t DM/ha/yr, average starch production of 12 t DM/ha/yr. and ethanol yield of 6,521 l/ha. The duckweed cultivation system in this experiment could be improved to be more efficiency and eco-friendly by using wastewater as the main source of nutrients, which could cut down the fertilizer requirement and reduce methane gas emission from waste water pond.

Keywords : 3-5 words Duckweed, Biomass, Starch, Ethanol

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Introduction to Research

Energy Problem

The world has been facing problems of fossil fuels. Energy consumption around the world has increased 17-fold in the last century, and if the demand is continuingly growing, the fossil depletion is inevitable. Another problem about fossil fuels is the energy price uncertainty which has a profound effect in global economy as observed in many "oil price crisis" incidents around the world in the past century. The energy price uncertainly hinders the developmental progress for developing country and cause energy independence problems. Moreover, increasing use of fossil fuels can have an environmental impact due to the oxidation of CO_2, SO_2 and NOX emission, which are greenhouse gases (GHGs). This phenomenon causes the atmosphere concentrated with GHGs, creating the greenhouse effect. As the consequent, the global warming and the climate change problem arise (Ture et al., 1997; IPCC 2001). Thus, biofuels such as bioethanol and biodiesel have been gaining world attention recently. Biofuels can be used to substitute for petroleum fuels. It can be used alone or blends together with the petroleum (Demirbas 2002; Demirbas 2003). Biofuels give more benefit since it comes from renewable resources. It reduces greenhouse gases emission and stimulates regional development, social structure and agriculture and security supply (Demirbas 2006; Demirbas2008; Unal and Alibas, 2007; Ikilic and Yucesu 2008). Unfortunately, with current technology, most feedstock for biofuels is crop food, and global demand and consumption of agricultural crops for food, feed, and fuel has been increasing at a rapid pace. This demand for plant materials has been expanding for many years. Recent increases in meat consumption in emerging economies together with accelerating use of grain for biofuel production in developed countries have placed new pressures on global grain supplies. Even as the competitive dynamic among food, fossil fuels and biofuels are a singular blend of politics and economics, the rapid expansion of biofuels production from maize, sugarcane, oil seed and from conversion of edible oils has raised serious concerns on preserving the food security of the planet, and also the high price of food products, which creating a popular controversial as "Food vs Fuel". Moreover, the production cost of conventional biofuels keeps rising because the raw material price keeps going up. Therefore, alternative raw materials with low production cost for biofuels need to be explored.

Proposal

To mitigate energy problem with low cost investment may seem to be intricate but it may be possible with the small, tiny plant called “duckweed” or Lemnaceae. “Duckweed” is a common name given to the simplest and smallest flowering plant that grows all over on fresh or polluted water throughout the world. Duckweeds function as food and shelter for aquatic invertebrates and fish (Pandit 1984; Newman 1991), while also acting as reservoirs for nutrients and trace elements (Shilla et al., 2006). Utilization of duckweed for municipal wastewater treatment through recovery of polluting nutrients by duckweed growth is commonly observed. These plants have been used for tertiary treatment of municipal wastewater for about two decades and limited commercialization in the US has occurred. In Thailand, duckweeds are normally used for animal feeding, and there are some species that are consumed by human. Many people still consider duckweed as an aquatic weed instead of a potential valuable plant. Though duckweed is rich of protein and in 1978, William Hillman and David Culley made a compelling case for the development of wild-type duckweed as an aquatic crop for protein production, uses for the massive amounts of duckweed biomass that would be produced by large-scale cropping systems do not exist in Thailand. Duckweed can grow both in sunlight and in shade, and can grow in slightly brackish water. Duckweed grows best in shallow nutrient-rich pools (e.g., with decaying vegetation). Duckweed has the highest photosynthesis and growth rate of any higher plant. In optimal conditions (sufficient nitrates and phosphates), the surface area covered by duckweed can double in less than 2 days. The Indian species, *Wolffia microscopica*, can bud off a new daughter every 30-36 hours. Therefore, for several decades, researchers have been intrigued by the idea that duckweeds could be developed as a major crop. Developing a duckweed cropping system could create cheap biomass production system of industrial scale. However, without a readily available supply of massive amounts of duckweed via cropping, there has not been a drive to find products that could be made from duckweed. However, the energy/climate change challenge and the role of plant biomass as a source of carbon compounds to replace petroleum as an energy and chemical feedstock has inspired the idea of developing duckweed as a crop for biofuels.

Beside a potential biomass source, duckweed is also a potential starch source. Duckweed starch contents ranging from 3 to 75% have been reported (Landolt and Kandeler 1987, Reid and Bieleski 1970). Duckweed starch content can be manipulated by adjusting growth

conditions, e. g., pH, nutrient starvation, which affect frond proliferation. Therefore, this project investigated the possibility of duckweed cropping system for ethanol production. Duckweed populations were collected around the northern part of Thailand, and their diversity was studied. They were morphologically characterized in order to make a collection of duckweed in the northern part of Thailand. Using local or regional duckweed would avoid the use of an invasive exotic species. Later, those duckweeds was investigated for ability to produce biomass and starch under the laboratory conditions. Duckweed population that performs well in either category was selected. The selected duckweed strains would be tested for the appropriate parameters (nutrients and plant hormones) for duckweed cropping system as sources for biomass and starch. Pilot test for duckweed cropping was performed throughout the year at Research Institute of Agricultural Research, Lampang. This part made this project unique because it was a year-round pilot test, the seasonal effect on the system could be tested and the collected data would be more appropriate for impact assessment. Next, the possibility to utilize duckweed as an energy crop for bioethanol production was explored. Ethanol production from duckweed cellulose, starch and total biomass with standard protocols was studied. Finally, the impact of duckweed cropping system for bioethanol production was evaluated to for sustainability assessment. Advantage of this idea were; to avoid the potential collision between demands for energy with increasing demands for crop commodities such as grains and legumes, to use duckweed cropping system to directly to capture CO_2 to mitigate global warming effect and to develop incentive program for farmers to employ duckweed cropping system in their communities for low cost biofuel production.

Literature review

What is Duckweed?

The duckweed is the common name given to the simplest and smallest flowering plant that grows all over on fresh or polluted water throughout the world. Duckweed function as food and shelter for aquatic invertebrates and fish (Pandit 1984; Newman 1991), while also acting as reservoirs for nutrients and trace elements (Shilla et al., 2006). Duckweed is able to grow in many parts of the world except for very cold regions. With a longer growing period than most plants, duckweed produces a continual biomass supply for 9–12 months every year depending on the agricultural zone. For research study, duckweeds have great application in genetic or biochemical research, similar to the use that that drosophila (fruit flies) and bread molds. For general use, duckweed is normally used for consuming, animal feeding and fertilizer making. The duckweed or Lemnaceae is a monocotyledonous family of aquatic plants with four genera and a total of 37 species: *Spirodela*, *Lemna*, *Wolffia* and *Wolfiella*. All members of the duckweed are small, free-floating, fresh-water plants whose geographical ranges span the entire globe (Crawford et al., 2006; Landolt, 1976). The duckweeds normally populate in freshwater ponds and pools without flowing water. One species, *Spirodela polyrrhiza*, is the most widely distributed. The duckweeds are the most morphologically reduced species of higher plants.

Spirodela and *Lemna* plants have disc-shaped fronds of varying sizes, shapes and thickness depending on species, a hair-like root or roots. Sometimes they have one or two flowers. *Lemna* and *Spirodela* species vary from less than one to several millimeters in diameter, with roots elongating to no more than several centimeters in length. The morphologies of *Wolffia* species are further reduced, with plants consisting of tiny fronds resembling geometric solids, no roots and rarely single flowers. *Wolfiella* species are the most varied in morphology. The fronds of *Wolffia* species are less than 2 mm in diameter. All Lemnaceae species proliferate primarily through vegetative budding of new fronds from parent fronds. Newly budded fronds remain attached to the parent frond to varying degrees. *Lemna* and *Spirodela* species are forming frond clusters of varying number and *Wolffia* species remaining single. Although parent fronds are limited in the number of progeny fronds that are produced before the parent frond dies, duckweed cultures achieve near exponential growth rates. Doubling times vary by species and

environmental conditions and are as short as 20 to 24 hours and many species have doubling times of 2 to 3 days. The growth of duckweed is dependent on the ability of the roots to recover nutrients from the water (Marin and Oron, 2007; Myriam et al., 2009). Intensive laboratory culture of duckweed has achieved high rates of biomass accumulation per unit time at culture densities of 1–2 kg/m^2 (Landolt and Kandeler, 1987). Greenhouse production levels of 1 kg fresh weight/m^2/wk have been achieved. With wastewater treatment, there has been reported that a growth rate of 0.2 kg dry weight/m^2/wk. had been achieved (Cheng et al., 2002). To achieve these growth rates, only low concentrations of nutrients are required. Oron and co-workers achieved optimal growth rates at 20 ppm nitrogen utilizing municipal wastewater (Oron 1994). Duckweed proliferation creates a floating photosynthetic surface that both maximizes capture of sunlight per unit area and shades out competing algal growth. It consumes carbon dioxide from the atmosphere by photosynthesis, which is beneficial for reducing the greenhouse effect. Duckweed grows 28 times faster than corn and accumulates biomass at a greater rate than most other plants including field crops. The rate of biomass accumulation is 2.3 g of dry weight produced per original unit (g) of dry weight per week for corn (Culley et al., 1981) and up to 64 g/g/week for the duckweed species *Lemna paucicostata* (Krishna and Polprasert, 2008). Oron reported that the annual biomass yield for the duckweed Lemna gibba was about 55 tons of dry weight per hectare using domestic wastewater (Oron 1994). Without continual harvesting, proliferation creates dense mats of multiple frond layers that float at the surface of the water. To optimize production continuous harvesting of duckweed biomass is necessary. The floating particulate growth habit of duckweed facilitates harvesting and a variety of methods have been devised to corral and harvest duckweed biomass. Duckweed's small size gives the plant a large surface area to volume ratio. Duckweed also lacks a waxy cuticle, present on land plants to prevent water loss. Both of these characteristics mean that duckweed can be dried quickly with low energy inputs. The chemical composition of duckweeds as reported in the literature varies considerably due to the age of the duckweed and type and amount of nutrients supporting the growth of the duck weed. In spite of variation, it is acceptable that duckweed contains high level of protein and carbohydrate (Table 1; Landolt et al., 1987).

Table 1: Estimation of organic composition of duckweed (Landolt et al., 1987).

Organic composition in the Lemnaceae	% of dry weight
Protein	6.8 — 45.0
Lipid	1.8 — 9.2
Crude fiber	5.7 — 16.2
Carbohydrate	14.1 — 43.6
Ash	12.0 — 27.6

Duckweed growth can be optimized to produce high levels of protein or high levels of starch. Protein content of a number of duckweed species grown under varying conditions has been reported to range from 15 to 45% dry weight (Chang et al., 1977; Porath et al., 1979; Appenroth et al., 1982). These values place the protein content of dry duckweed biomass between alfalfa meal (20%) and soybean meal (41.7%) (Hillman, 1961). Starch contents ranging from 3 to 75% have been reported (Landolt and Kanderler, 1987; Reid and Bieleski, 1970). A duckweed starch content of 75% is comparable to corn, whose starch content ranges from 65 to 75% (Lin and Tanaka, 2006). The possibility to manipulate growth to produce either high-protein or high-starch duckweed provides two opportunities to use duckweed biomass. The high-starch content suggests that duckweed could be used as an industrial feedstock for ethanol production for fuel. The high-protein content suggests that duckweed could be used as the protein component for animal feed. However, to commercialize either of these potential products requires a cropping system that can consistently produce stable duckweed biomass in massive quantities.

Duckweed and Bioethanol

In recent years there has been a growing interest in renewable energy production worldwide because of the limited reserve of crude oil and natural gas and environmental concerns of using fossil fuels. Ethanol production from dedicated crops or agricultural residues is one form of renewable energy that addresses the critical need for sustainable transportation fuels. Utilization of solar energy in the form of carbohydrates stored to biomass is one of the most effective ways to address the current energy concern. The energy produced during photosynthesis is stored in the form of starch in many plants. Starch is a good feedstock for the bioethanol industry, because of its relatively simple conversion process (Cheng, 2010). Ethanol is considered one of best alternative energy sources and sugarcane and cassava is currently the

dominant feedstock for the production of fuel ethanol in Thailand. Bioethanol can be used as liquid fuel and gasoline mixed agent to increase oxygen content and reduce emissions, so many countries and regions are in the implementation of ethanol fuel program (Sánchez and Cardona, 2008). There is already a well-developed market for ethanol in Thailand. Currently, sugar cane and cassava are the primary raw material for fuel ethanol production in Thailand. Sugarcane and cassava conversion for energy purposes is very easy and efficient. However, it raises much concern because sugarcane and cassava are also food/feed sources (Sun and Cheng, 2002). It may not be practical to substantially increase ethanol production from those raw materials because of the competition against food and feed production. Moreover, intensive sugarcane and cassava cultivation have also caused environmental problems including nutrient pollution and soil erosion (Pimentel, 2003). Therefore, it is required to explore novel starch or sugar sources to supplement sugarcane and cassava starch to achieve a sustainable development of ethanol industry. Thus, there is a great interest in exploring alternative feedstock for ethanol production.

Duckweed is a potential starch source. Duckweed starch contents ranging from 3 to 75% have been reported (Landolt and Kandeler 1987; Reid and Bieleski 1970). Duckweed starch content can be manipulated by adjusting growth conditions, e. g., pH, nutrient starvation, which affect frond proliferation. Satake and Shimura (1983) reported that *Spirodela polyrrhiza* takes more CO_2 from water at low pH than at high pH, because the dissolved CO_2 content in the water is much higher. Other factors which trigger starch accumulation, are P deficiency (Reid and Bieleski 1970), K + -deficiency (White 1939), supply of branched-chain amino acids (Van Mazijk 1975) and N deficiency (Scholz 1962). Cheng and Stomp (2009) reported that a high starch content of 45.8% could be reached in their laboratory experiment through simple transfer of fresh duckweed fronds from a nutrient-rich solution to tap water for 5 days. At nutrient starvation, the protein synthesis supported by nitrogen assimilation is substantially reduced while the continual photosynthesis causes an increase in the relative proportion of starch in duckweed plants. Xu et al. (2011) reported that enzymatic hydrolysis of the duckweed biomass with amylase could produce sugar yield almost 50% of dry duckweed. The fermentation of the hydrolysate could produce 25% ethanol from the dry duckweed. These results indicate that duckweed biomass can produce starch in appreciable quantities that can be readily fermented into ethanol.

In addition, duckweed biomass has several characteristics that make duckweed biomass to-ethanol process advantages and that could lower overall costs when compared to corn or

cassava. Cheng and Stomp (2009) reported that duckweed could produce starch in a rate of approximately 28 tons per hectare per year compared to corn starch production of about 5.0 tons per hectare per year. Duckweed biomass would require little or no mechanical grinding because of the small size of the plants and because it is a green, hydrated biomass. The lack of a milling step to prepare biomass for fermentation translates into a substantial savings in energy, one of the major costs in the corn-to-ethanol process. Duckweed has a protein content ranging between 15 and 45% dry weight compared to 9% protein content for corn. This suggests that supplementation of the yeast fermentation mash with an N source may not be necessary when using duckweed biomass. High protein content may also make “distilled grain”, a by-product of the ethanol fermentation, from duckweed biomass a livestock feed supplement superior to that derived from corn. Although producing high-starch duckweed as a supplement to corn starch for fuel ethanol production seems to be a promising technology and some lab-scale experiments have shown encouraging results, an investigation based on a larger scale operation is required to better evaluate its technical viability.

Objective

1. To classify and make a collection of duckweed populations, which suitable for biomass and starch production, from the northern part of Thailand

2. To develop a duckweed cropping system for high biomass yield and high starch content

3. To investigate the potential of ethanol production from duckweed's cellulose, duckweed's starch and duckweed's total biomass

4. To analyze the impact of ethanol production from duckweed and to compare to ethanol production from conventional energy plants

Research methodology

1. Plant material collection

1.1 In this study, various duckweed samples were collected from different regions of the northern part of Thailand. The locations were recorded with a GPS.

1.2 After collection, the plants were surface-sterilized in a 10% to 50% bleach (sodium hypochlorite) solution and they were propagated in Hoagland's E-Medium under laboratory condition according to Zue et al. (2001) with 10g/l sucrose at pH 5.6. The fronds were maintained in room temperature under wide spectrum fluorescent light and sub-cultured once a month in fresh medium.

2. Duckweed characterization

2.1 Systematic of duckweed was classified according to the method of Les et al. (1997), which use the morphological and anatomical data. About 20 plants of various growth stages was used to represent each population and was investigated for morphological and anatomical characteristics.

2.2 Classification analysis of morphological and anatomical data was generated based on the collected data.

3. Duckweed for biomass production

3.1 Healthy duckweed of each population was sterilized and sub-cultured into Hoagland's E-Medium under laboratory condition in 1L flask. There was triplicate for each duckweed population. The following data was collected daily to measure the growth rate of each duckweed population by counting fronds. The number of fronds was recorded daily. Although it is a time-consuming process, it had lesser effect to overall duckweed growth. Each day, approximately 10 individuals were collected from each flask (30 individuals per population) and they were observed under a stereo-microscope for 9 days. Every visible new frond of each individual was counted. The obtained data was statistically analyzed for variance using ANOVA (analysis of variance) and to compare the difference between the averages with Duncan’s new multiple range test (DMRT). The data would later be used to calculate the growth rate. Duckweed strains that have a great potential for biomass production were identified.

3.2 Studying for optimal condition for duckweed growth under laboratory condition was performed from previous step. The control factors were temperature, pH of the water and trace elements. The variable factors were amount of usable nitrogen and phosphorus and air pump application. The selected population was grown in minimum medium supplied with different concentration of nitrogen (0, 10, 20, 30, 40 and 50 mg/L) or phosphorus (0, 1, 2, 3, 4 and 5 mg/L) with or without air supply. There was triplicate for each treatment. The growth rate of each treatment was measured as method described above. These data were statistically analyzed for variance using ANOVA (analysis of variance) and to compare the difference between the averages with Duncan's new multiple range test (DMRT).

4. Pilot test for duckweed biomass production

4.1 The pilot test of the outdoor duckweed cropping system was operated using 1.5m diameter cement tank. The appropriate parameters obtained from previous step was employed. There were ten replicates for each treatment. Because duckweed fronds can double their mass in 2 days (Hillman 1961), duckweed in the tank was harvested three times a week to remove newly grown biomass and keep a two-layer-frond coverage for 300 days period to cover all season during the year.

4.2 The duckweed yields were recorded. The data was statistically analyzed for variance using ANOVA (analysis of variance) and to compare the difference between the averages with Duncan's new multiple range test (DMRT). The potential of biomass production from duckweed cropping was determined.

5. Duckweed and Starch Accumulation

Duckweed with high starch content is desirable because it can be used as a starch source for ethanol production. Research showed that duckweed can be a potential starch source for ethanol production (Cheng and Stomp, 2009). Duckweed starch contents ranging from 3-75% dry weight have been reported (Landolt and Kandeler, 1987; Reid and Bieleski, 1970).

5.1 The ability to accumulate starch in duckweed was pre-screened by staining the plants with KI (potassium iodide) reagent. Plant with presence of starch would show dark-blue color.

5.2 Populations that showed potential was cultivated in Hoagland's E-Medium under laboratory condition in 1L flask. There was triplicate for each duckweed population. When

duckweed reached stationary phase/equilibrium phase (determined from the above experiment), duckweed was collected and fresh duckweed samples was dried in an oven at 70 ^{o}C for overnight. The starch content of dried duckweed biomass was analyzed by enzymatic hydrolysis of the duckweed biomass with a-amylase (Sigma A3404), pullulanase (Sigma P2986), and amyloglucosidase (Sigma 10115). Glucose in hydrolysate will be measured using 3, 5-dinitrosalicylic acid reagent method adapted from Millar (1959) and Ghose (1987). All treatments in this study were conducted in triplicate. Analysis of variance (ANOVA) was used to determine the effects of various factors on treatments. The data was statistically analyzed for variance using ANOVA (analysis of variance) and to compare the difference between the averages with Duncan's new multiple range test (DMRT). Duckweed strains that have a great potential for starch production will be identified.

5.3 Enhancing starch accumulation by nutrients starvation was evaluated on duckweed populations selected from the method described above. Starch accumulation in duckweed plants can be triggered at manipulated growing conditions like nutrient starvation (Cui et al., 2010). Cheng and Stomp (2009) reported that a high starch content of 45.8% could be reached in their laboratory experiment through simple transfer of fresh duckweed fronds from a nutrient rich solution to tap water for 5 days. Therefore, the selected duckweed was tested for nutrient starvation by transferring healthy fresh duckweed and transferring into "clean water" for 5 days and the starch contented was measured as described above. More than 20% of starch content is considered to be acceptable.

6. Pilot test for duckweed starch production

6.1 The pilot test of the outdoor duckweed cropping system was operated using 1.5 m diameter cement tank. The appropriate parameters obtained from previous step was employed. There were ten replicates for each treatment. Duckweed in the tank was harvested three times a week to remove newly grown biomass and kept a two-layer-frond coverage for 300 days period to cover all season during the year.

6.2 The duckweed yields and starch content was recorded. The data was statistically analyzed for variance using ANOVA (analysis of variance) and to compare the difference between the averages with Duncan's new multiple range test (DMRT). The potential of starch production from duckweed was determined.

7. Effect of plant hormone on duckweed biomass and starch production

Plant hormones such as auxin, cytokinin, ABA and gibberellins (GA) are known for their effects on plant growth and development. Gibberellins, cytokinin and auxin are also implicated with protein and starch accumulation in plants (Zie et al., 2003; Cao and Shannon, 1007). Therefore, effect of plant hormones on duckweed biomass and starch production was also investigated.

7.1 Duckweed selected from previous steps which suitable for biomass production and starch production was tested. They were cultivated in Hoagland's E-Medium under laboratory condition in 1L flask supplied with plant hormones. The variable factors were type and concentration of plant hormones. IAA (auxin), kinetin (6-BA), ABA and GA (GA_3) with various concentrations (0μM, 0.1μM, 0.01μM, 0.001μM, 0.0001μM, 0.00001μM and 0.000001μM) were applied. There was triplicate for each treatment.

7.2 The growth rate and starch content of duckweed in each treatment were measured as method described above. These data were statistically analyzed for variance using ANOVA (analysis of variance) and to compare the difference between the averages with Duncan's new multiple range test (DMRT). The appropriate plant hormones application was analyzed.

8. Duckweed and Ethanol Production

8.1 Approximately 1 kg of fresh duckweed biomass was harvested from the previous step using a strainer, and then scattered on a concrete board and sun dried for three days. The dried biomass was collected in plastic bags for enzymatic hydrolysis and yeast fermentation. To improve hydrolysis efficiency, the dried duckweed was ground first.

8.2 Alkaline/oxidative pretreatment (A/O pretreatment) (Mishima et al., 2008) was applied to the dried biomass as described briefly below. The samples were reacted in 1% (w/v) NaOH at room temperature for 12 h with subsequent addition of 31% H_2O_2 (w/v) to the final concentration of 1% (w/v). The resultant suspension was left to react for another 12 h. The pretreated samples were collected and washed with tap water until the pH value of the drained water was neutral. Then the samples were dried at 60 ^{o}C and powdered. For ethanol production from duckweed, ethanol production in SHF (simultaneous saccharification and fermentation) was performed. The SSF reaction mixtures consisted of 8.0 g of the pretreated biomass of duckweed with filter-sterilized cellulase (20 FPU/g substrate^{-1}) or the amylase mixture (containing 47.2 mg/g g

substrate^{-1} of α-amylase (A9857; Sigma-Aldrich Corp., USA) and 0.625 mg/g g substrate^{-1} amyloglucosidase (A1602; Sigma-Aldrich Corp., USA)) in 0.1 M sodium phosphate, and with the basal medium to constitute a working volume of 80 ml. The yeast (Saccharomyces cerevisiae (ATCC 24859)) preculture was inoculated. Fermentation was conducted for 60 h at 30 ^{o}C and 120 rpm on a rotary shaker. All experiments were conducted at least twice.

9. Impact Assessment of Duckweed Production

To analyze the impact of duckweed cropping system, LCA (Life Cycle Assessment) method will be employed. The LCA method is one of the most important information tools of environmentally oriented product policy. Within the meaning of ISO 14040 LCA method can be defined as compilation and evaluation of the inputs, outputs and the potential environmental impacts of a product system throughout its life cycle.

The function unit of assessment was area 1 ha that producing duckweed every day at the average biomass obtained from the pilot tested. This assessment was performed as “Cradle-to-Gate”, which the ethanol production, the product usage stage and waste treatment are omitted because those activities were not performed in pilot scale in this experiment. All material and energy flows within the product system were recorded. For input/output that can’t be measured, the data from commercial LCA inventory and previous study were used. For environmental impact, the SimaPro 7.12 software was used to assess only the greenhouse effect (or potential global warming). The result was also compared to the conventional energy plants.

Results

1. Plant material collection

The 52 duckweed samples were collected from the different regions of the northern part of Thailand. After surface-sterilization, 2-3 healthy duckweeds with similar morphology were picked in each sample to propagate in Hoagland's E-Medium under laboratory condition, resulting in 68 samples were selected. After 1 month, only 24 samples were alive, and they were used for further studies (Table 2)

2. Duckweed characterization

Systematic of duckweed was classified according to the method of Les et al. (1997), which use the morphological and anatomical data. Those samples were classified into 4 group of species.

1) *Wolffia arrhiza* : Plant bodies are small (about 0.4 – 1.3 mm long and 0.2 - .0 mm wide). It had globoid to ovoid-ellipsoid or cylindrical shape. It did not have root system. The distinctive morphology of this species were the no veins, dark green upper surface, flattened surface, no pigment (brown pigment) and more than 15 stomates. The sample that belong to this species were SC001 and SC004.

2) *Lemna aequinoctialis* : Plant bodies were asymmetrical and about 2.0 – 3.5 mm long. The 3 veins were easily observed per plant body. It had single root with length of 1.0 – 3.0 cm. Root sheath with 2 obvious, wing - like appendage at base and one apical papules on the dorsal side of the leaves were key features of this species. There were 9 samples that belong to this species, which were SC002, SC007, SC008, SC012, SC013, SC015, SC019, SC021 and SC024.

3) *Lemna perpusilla* : This species was very similar to *Lemna aequinoctialis*. They shared the common distinct structures which were 3 veins and winged root sheath near the basal attachment node. The difference between *Lemna perpusilla* and *Lemna aequinoctialis* was the presence of several papules on the dorsal side of the leaves whereas *Lemna aequinoctialis* has only one. The sample that belong to this species were SC005, SC011, SC012, SC013, SC015, SC019, SC021 and SC024.

4) *Landoltia punctata* : The plant body was big compared to other species with the length of 3-5 mm. The plant body was flattened, oblong-obovate in outline and asymmetrical. The color was dark green with reddish-purple ventral surface. The dead ones usually showed brown

pigment cells (punctae) in epidermis. The root was long (about 3-5 cm). There were 5 samples that belong to this species, which were SC003, SC006, SC010, SC016 and SC020.

Table 2: Information of duckweed samples collected from the northern part of Thailand (scale bar = 1 mm)

Code	Location	Pictures	Species
SC001	Baan Klang sub-district, San Patong district, Chiangmai 18°34'49.4"N 98°52'34.8"E		*Wolffia arrhiza*
SC002	Baan Klang sub-district, San Patong District, Chiangmai 18°34'49.4"N 98°52'34.8"E		*Lemna aequinoctialis*
SC003	Thapha sub-district, Ko Kha district, Lampang 18°11'34.7"N 99°23'30.7"E		*Landoltia punctata*
SC004	Hauykaew sub-district, Phu Kam Yao district, Payao 19°21'43.1"N 99°59'50.6"E		*Wolffia arrhiza*
SC005	Weing sub-district, Maung district, Chiangrai 19°55'04.0"N 99°50'26.8"E		*Lemna perpusilla*
SC006	Wiang Phang Kham sub-district, Mae Sai district, Chiangrai 20°24'14.0"N 99°53'06.8"E		*Landoltia punctata*
SC007	Pa Sak sub-district Mueang district, Lamphun 18°32'45.7"N 99°03'33.5"E		*Lemna aequinoctialis*

Table 1 (continued)

SC008	Ban Klang sub-district, Mueang district, Lamphun 18°35'02.8"N 99°02'31.7"E		*Lemna aequinoctialis*
SC009	Makhuea Chae sub-district, Mueang district, Lamphun 18°35'38.8"N 99°02'32.8"E		*Lemna aequinoctialis*
SC010	Klang Wiang sub-district, Wiang Sa district, Nan 18°34'59.5"N 100°44'28.3"E		*Landoltia punctata*
SC011	Sathan sub-district, Pua district, Nan 19°12'01.7"N 100°55'55.1"E		*Lemna perpusilla*
SC012	Pai sub-district, Pai district, Maehongson 19°20'57.0"N 98°26'06.5"E		*Lemna perpusilla*
SC013	Sop Pong sub-district, Pang Mapha district, Maehongson 19°32'31.0"N 98°12'41.2"E		*Lemna perpusilla*
SC014	Mae Kham Mi sub-district, Mueang district, Phrae 18°14'29.6"N 100°12'51.8"E		*Lemna aequinoctialis*
SC015	Nam Cham sub-district, Mueang district, Phrae 18°11'31.1"N 100°13'58.4"E		*Lemna perpusilla*

Table 1 (continued)

SC016	Ban Sang sub-district, Mueang District, Phayao 19°09'27.2"N 99°51'40.2"E		*Landoltia punctata*
SC017	Nong Han sub-district, San Sai district, Chiang Mai 18°52'52.0"N 99°00'51.8"E		*Lemna aequinoctialis*
SC018	Pa Sak sub-district, Mueang district, Lamphun 18°30'50.0"N 99°01'20.2"E		*Lemna aequinoctialis*
SC019	Tha Thung Luang sub-district, Mae Tha district, Lamphun 18°26'08.9"N 99°02'54.4"E		*Lemna perpusilla*
SC020	Nong Chom sub-district, San Sai district, Chiang Mai 18°49'45.4"N 99°00'43.7"E		*Landoltia punctata*
SC021	Nong Han sub-district, San Sai district, Chiang Mai 18°55'09.4"N 98°59'44.4"E		*Lemna perpusilla*
SC022	Nong Han sub-district, San Sai district, Chiang Mai 18°55'09.4"N 98°59'44.4"E		*Lemna aequinoctialis*
SC023	Pichai sub-district, Mueang district, Lampang 18°18'59.2"N 99°32'34.2"E		*Lemna aequinoctialis*

Table 1 (continued)

SC024	Wiang Nuea sub-district, Mueang district, Lampang 18°18'03.5"N 99°30'31.4"E		*Lemna perpusilla*

3) Duckweed for Biomass Production

3.1) Selection of Duckweed Strains for Biomass Production

Healthy duckweed of each population was sterilized and sub-cultured into Hoagland's E-medium under laboratory condition for growth investigation. Counting fronds was performed daily to measure the growth rate of each duckweed population. Although it was a time-consuming process, it has lesser effect to overall duckweed growth.

For *Wolffia arrhizal* group, consisting of SC001 and SC004, the results (Figure 1) showed that sample SC004 produced fronds better than sample SC001. At the end of experiment, average of 498 fronds was counted per sample of SC004, while the another was 114 fronds. Therefore, sample SC004 was selected as the representative of *Wolffia arrhiza* group, and would be called *Wolffia arrhiza* SC004

For *Lemna aequinoctialis,* duckweed sample SC002, SC007, SC008, SC009, SC014, SC017, SC018, SC022 and SC023, were evaluated for growth performance. These samples were classified into 3 groups, which were a good growing group, an average growing group and a slow growing group. Duckweed sample SC022, SC017, SC007 could increase frond numbers very fast compared the other groups. At the end of experiment, they could produce 189, 165 and 161 new fronds respectively. Duckweed sample SC018, SC002, and SC014 were classified as average growth group with the new fronds of 137, 105 and 105 respectively. Duckweed sample SC008, SC009 and SC023 were in slow growth group. The new fronds production rate was slow compared to the others. At the end of the experiment, duckweed sample SC008 and SC009 produced 59 and 48 new fronds correspondingly, while duckweed sample SC023 was dead after 7 days cultivation. At the end of the experiment, duckweed sample SC022 was selected

as the representative of *Lemna aequinoctialis* group, and would be called *Lemna aequinoctialis* SC022 in the future experiments.

For *Lemna perpusilla*, duckweed sample SC003, SC005, SC011, SC012, SC013, SC015, SC019, SC021 and SC024, were examined for growth performance. Three groups were divided, which were a good growing group, an average growing group and a slow growing group. Duckweed sample SC024, SC021 and SC015 were in a good growing group. After 9 days, they could make average of 203, 178 and 153 new fronds respectively. Duckweed sample SC013 was only sample in an average growing group, and at the end of experiment, average of 96 new fronds were made for this sample. Duckweed sample SC005, SC011, SC012 and SC019 were in a slow growing group. The growth of duckweed sample SC019 stopped at the 6^{th} day of experiment, and it was dead at the end of experiment. Duckweed sample SC005, SC011 and SC012 made fewer new fronds compared to the others with average of 48, 40 and 38 new fronds respectively. Therefore, duckweed SC024 was selected as the representative of *Lemna perpusilla* group, and would be called *Lemna perpusilla* SC024 in the future experiments.

For *Landoltia punctata*, there were 5 duckweed samples in this group, which were SC006, SC010, SC016 and SC020. All sample showed very good growth performance. Duckweed sample SC006, SC010, SC016 and SC020 made average of 61, 51, 68 and 63 new fronds respectively. At the end of the experiment, duckweed sample SC016 was selected for future works because of the highest new fronds production. This sample would be the representative of *Landoltia punctata* group, and would be called *Landoltia punctata* SC016 in future experiments.

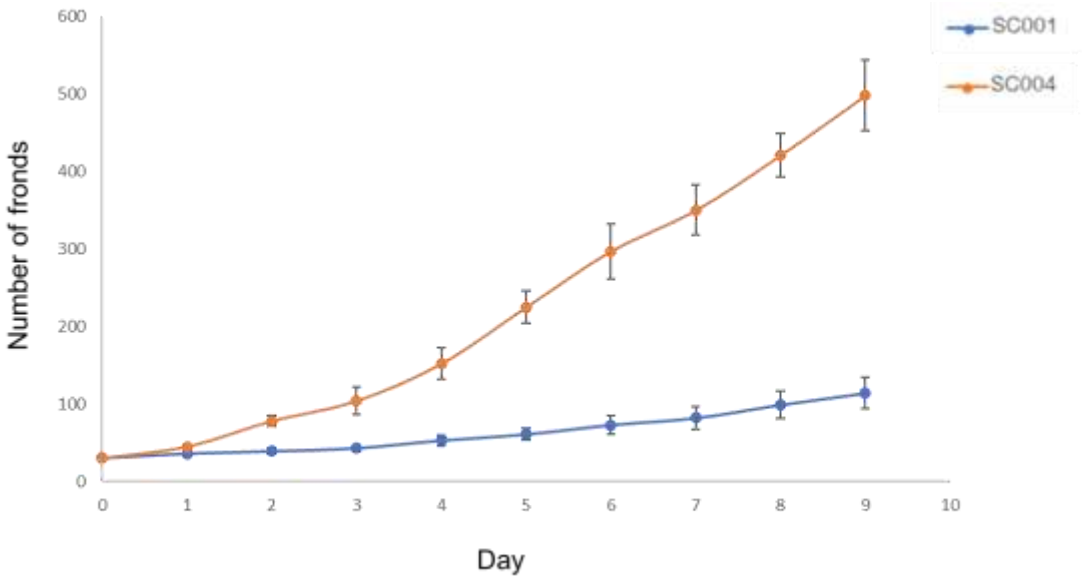

Figure 1: Changes in the number of *Wolffia arrhiza* fronds. Bars are ± SD (n = 3).

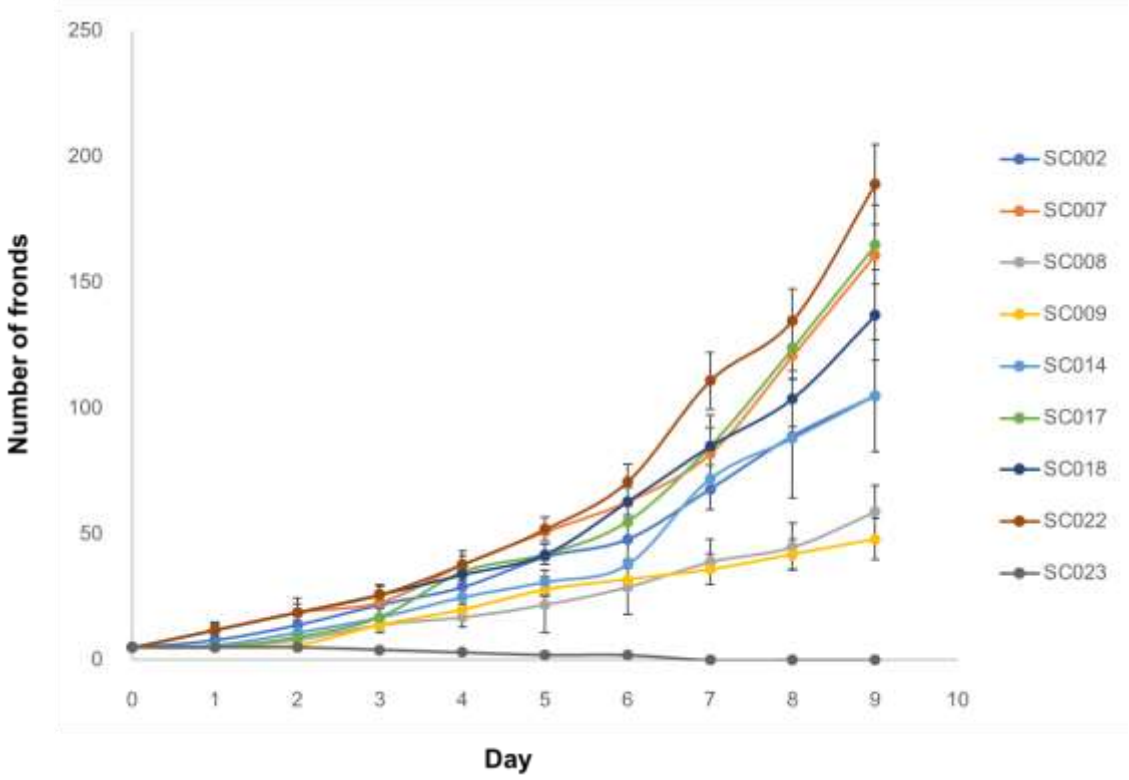

Figure 2: Changes in the number of *Lemna aequinoctialis* fronds. Bars are n ± SD (n = 3).

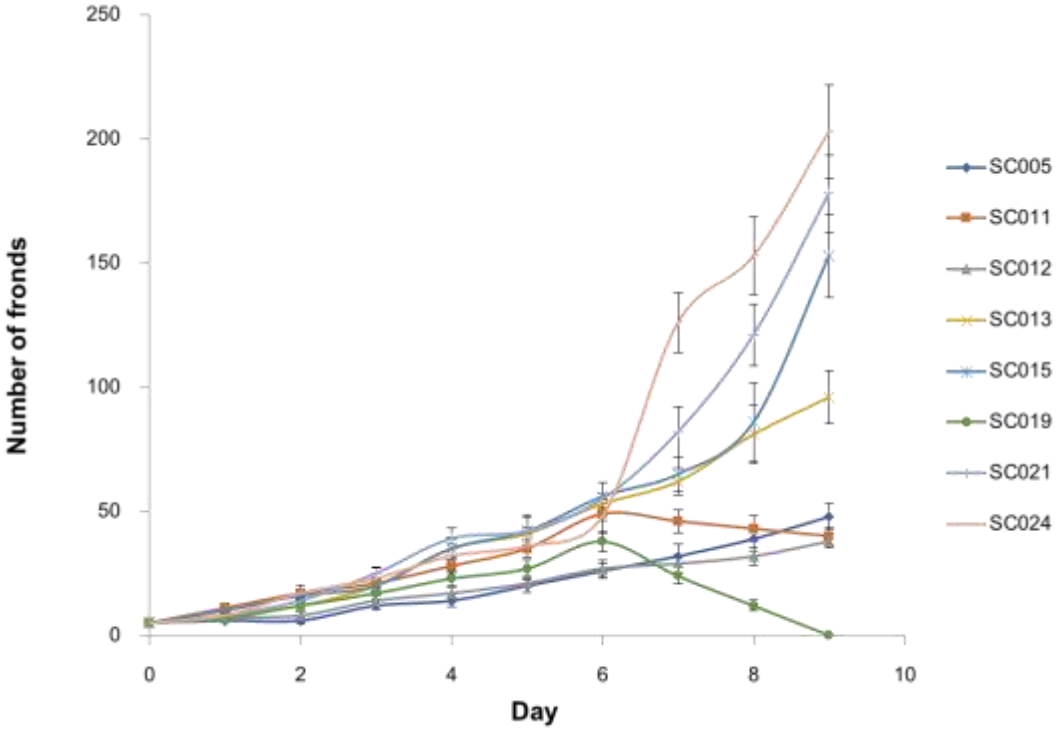

Figure 3: Changes in the number of *Lemna perpusilla* fronds. Bars are ± SD (n = 3).

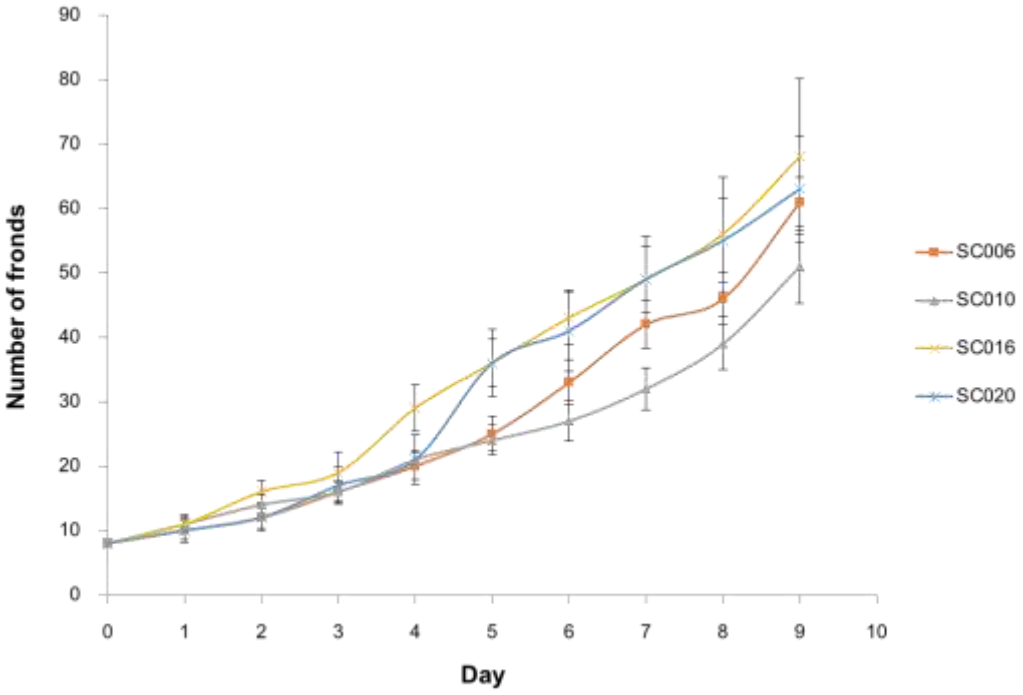

Figure 4: Changes in the number of *Landoltia punctata* fronds. Bars are mean ± SD (n = 3).

3.2 The optimal condition for duckweed growth

1) Nitrogen

The selected duckweed of each specie was grown in different concentration of nitrogen (0, 10, 20, 30, 40 and 50 mg/L). All tested duckweed samples showed similar response to nitrogen concentration. Higher concentration could increase biomass production of those duckweed samples. The optimal concentration for all duckweed samples was 30 mg/L. Higher nitrogen concentration than 30 mg/L could not increase biomass production compared to 30 mg/L treatment. Moreover, when the cultivations were supplied with air pump, the biomass was increased in all treatments tested. From this experiment, the maximum of biomass production of *Wolffia arrhiza* SC004, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 and *Landoltia punctata* SC016 were 16.3 g/m^2, 93.5 g/m^2, 75.1 g/m^2 and 114.6 g/m^2 respectively when cultivated with 30 mg/L of nitrogen with air pump.

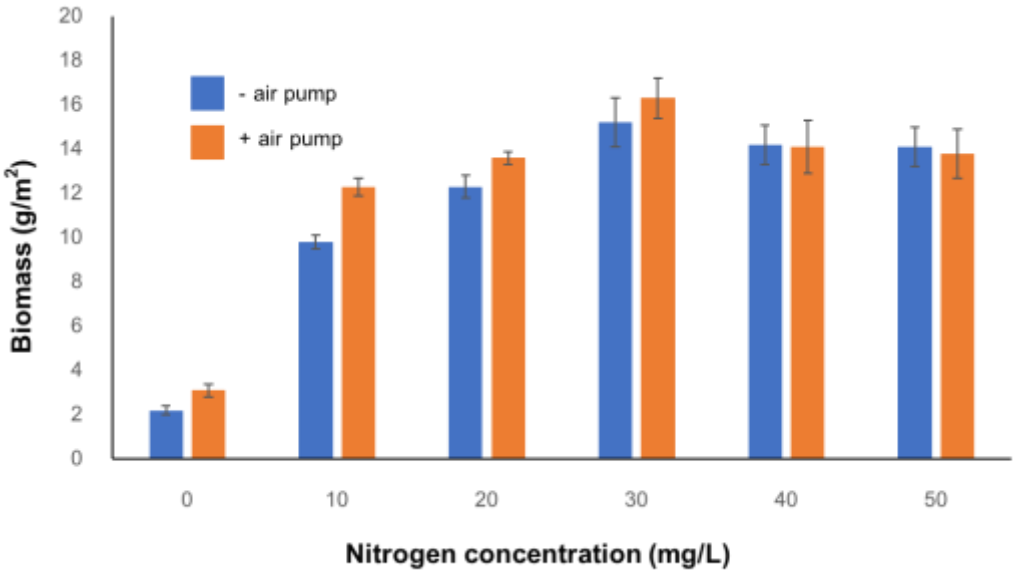

Figure 5: Biomass production of *Wolffia arrhiza* SC002 cultivated in different concentration of nitrogen with and without air pump supplied. Bars are ± SD (n = 3).

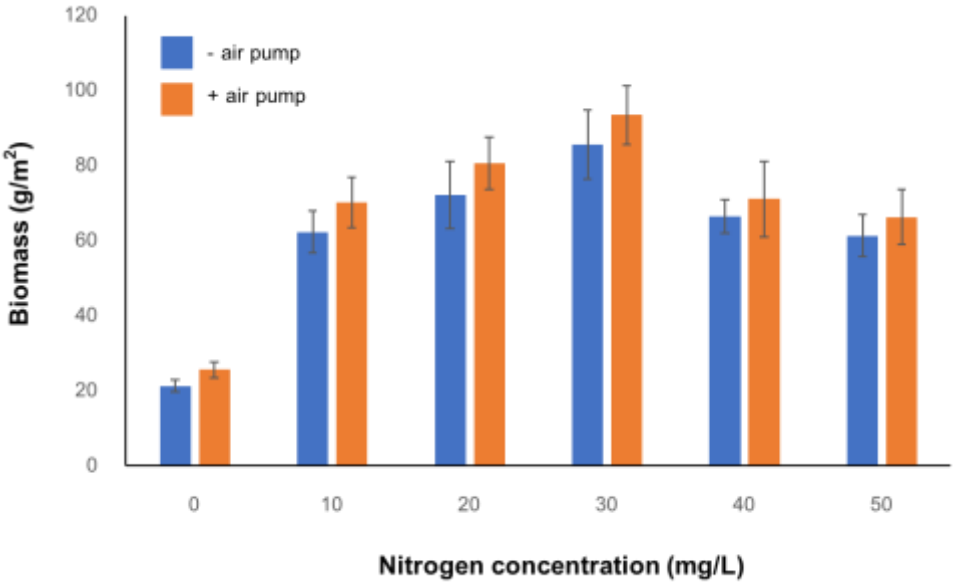

Figure 6: Biomass production of *Lemna aequinoctialis* SC022 cultivated in different concentration of nitrogen with and without air pump supplied. Bars are ± SD (n = 3).

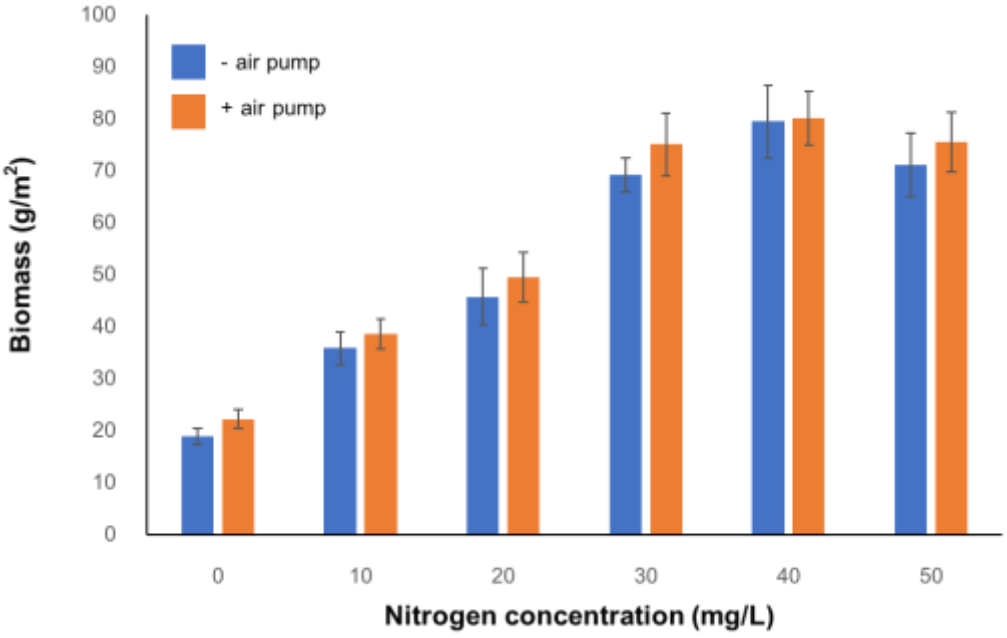

Figure 7: Biomass production of *Lemna perpusilla* SC024 cultivated in different concentration of nitrogen with and without air pump supplied. Bars are ± SD (n = 3).

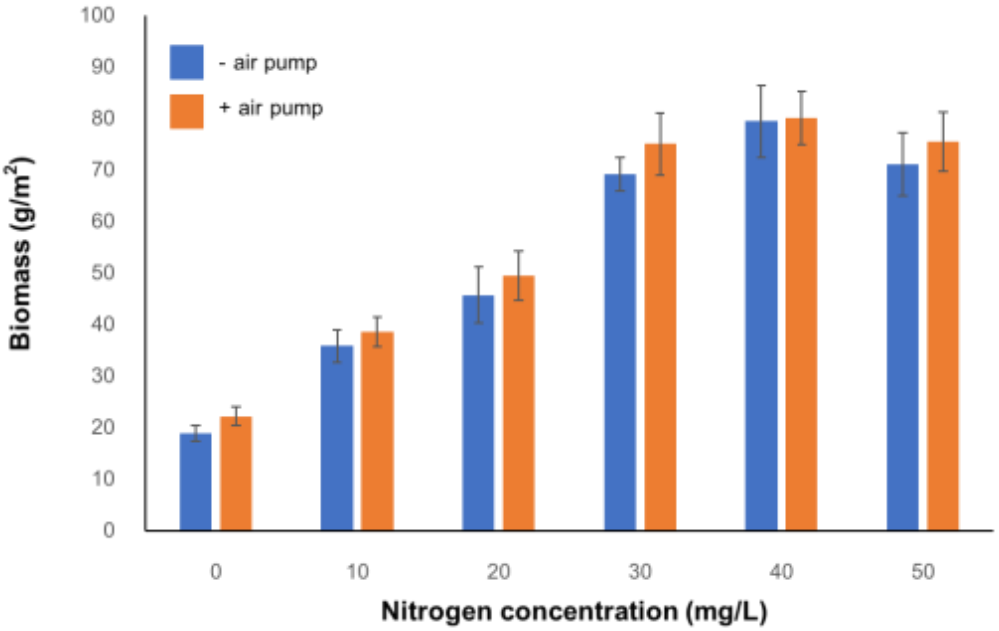

Figure 8: Biomass production of *Landoltia punctata* SC016 cultivated in different concentration of nitrogen with and without air pump supplied. Bars are ± SD (n = 3).

2) Phosphorus

The selected duckweed of each specie was grown in different concentration of phosphorus (0, 1, 2, 3, 4 and 5 mg/L). All tested duckweed samples showed similar response to phosphorus concentration. Higher concentration could increase biomass production of those duckweed samples. The optimal concentration for all duckweed samples was 5 mg/L. Moreover, when the cultivations were supplied with air pump, the biomass was increased in all treatments tested. From this experiment, the maximum of biomass production of *Wolffia arrhiza* SC004, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 and *Landoltia punctata* SC016 were 12.7 g/m^2, 81.2 g/m^2, 78.6 g/m^2 and 129.2 g/m^2 respectively when cultivated with 5 mg/L of phosphorus with air pump.

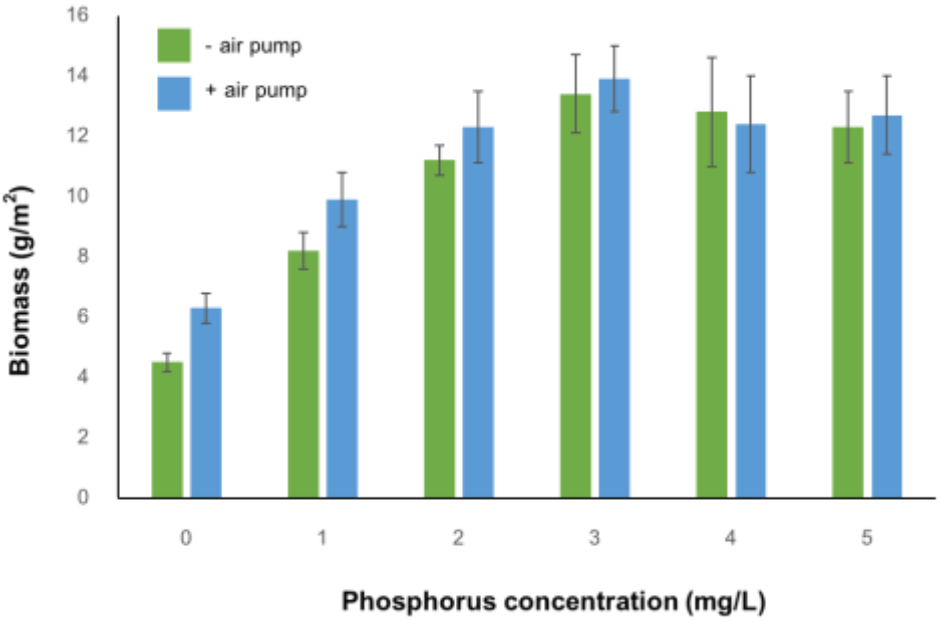

Figure 9: Biomass production of *Wolffia arrhiza* SC002 cultivated in different concentration of phosphorus with and without air pump supplied. Bars are ± SD (n = 3).

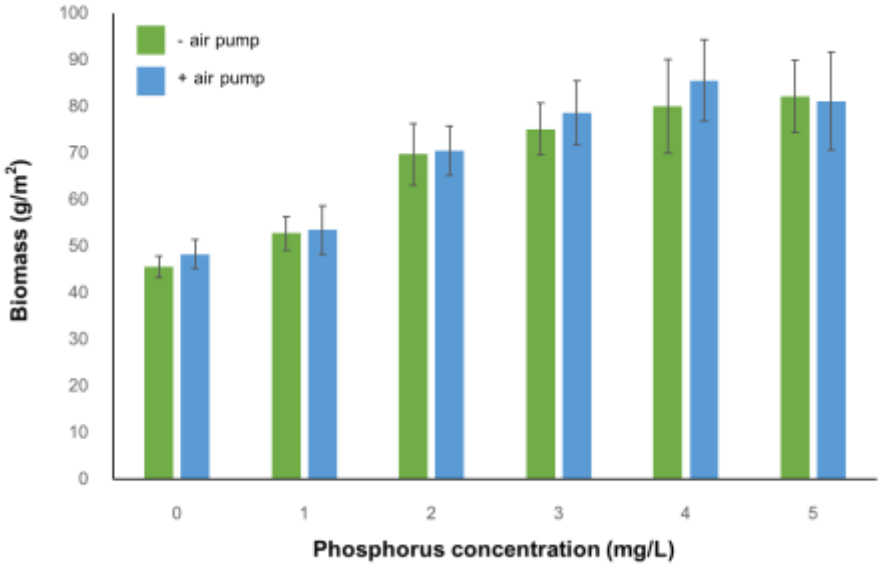

Figure 10: Biomass production of *Lemna aequinoctialis* SC022 cultivated in different concentration of phosphorus with and without air pump supplied. Bars are ± SD (n = 3).

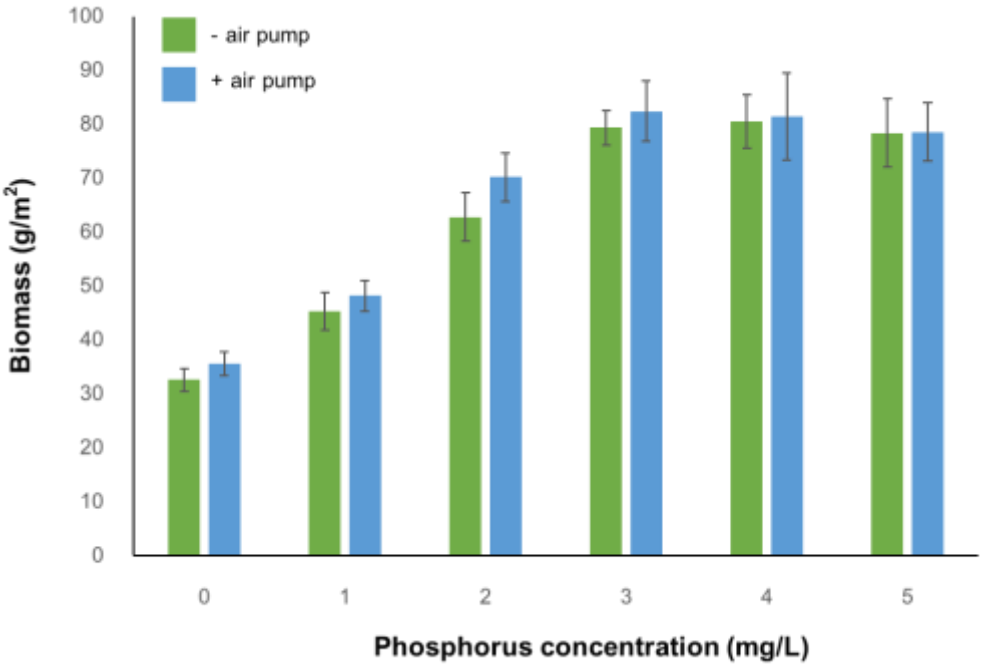

Figure 11: Biomass production of *Lemna perpusilla* SC024 cultivated in different concentration of phosphorus with and without air pump supplied. Bars are ± SD (n = 3).

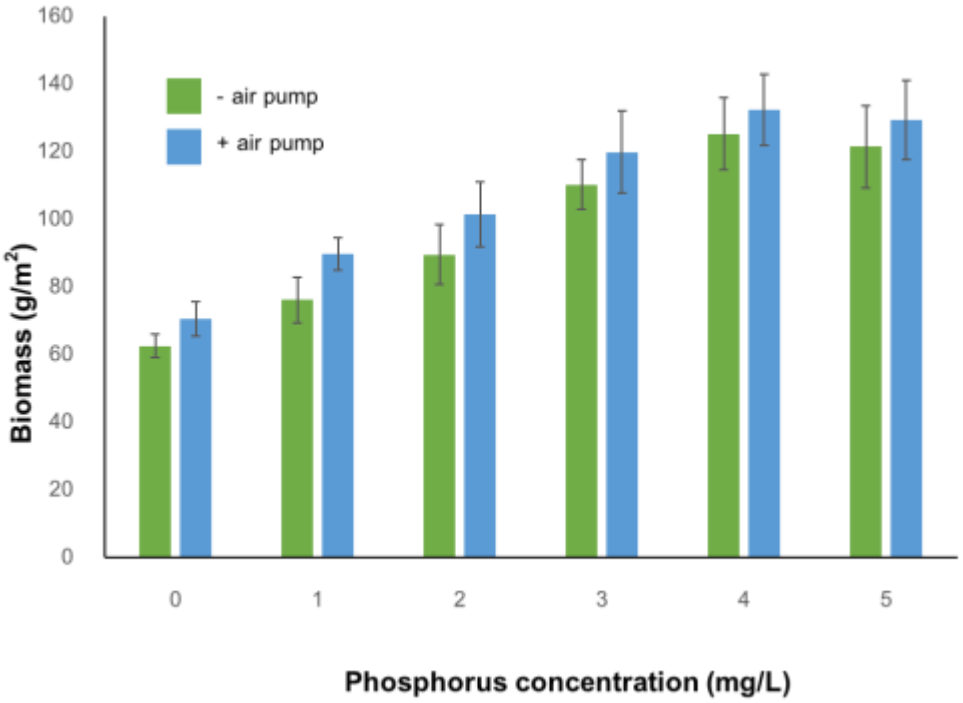

Figure 12: Biomass production of *Landoltia punctata* SC016 cultivated in different concentration of phosphorus with and without air pump supplied. Bars are ± SD (n = 3).

4. Duckweed and starch accumulation

Duck weed samples were analyzed for chemical compositions. The analysis showed that protein was the most abundant content for all duckweed samples, which were 37.8%, 30.1%, 27.8% and 25.3% for *Wolffia arrhiza* SC002, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 and *Landoltia punctata* SC016 accordingly (Table 3).

The starch contents were varied. *Lemna perpusilla* SC024 has the highest starch content, which was 26.2%, whereas *Wolffia arrhizal* SC002, *Lemna aequinoctialis* SC022 and *Landoltia punctata* SC016 has the starch content of 9.7%, 21.4% and 17.8% respectively (Table 3). However, when the starch was considered, *Landoltia punctata* SC016 showed the highest productivity, which was average of 16.5 g/m^2. *Lemna perpusilla* SC024 and *Lemna aequinoctialis* SC022 have similar starch productivity, which were 15.1 g/m^2 and 13.5 g/m^2 respectively.

This experiment wanted to induce stress in duckweed samples before the harvest period by nutrient starving. This stress could enhance starch production in all duckweed samples. The starch contents were increased by 7.3, 4.2, 2.4 and 3.7 fold change for *Wolffia arrhiza* SC002, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 and *Landoltia punctata* SC016 accordingly, compared to non-stress duckweed (Figure 13 – 16). The stressed *Landoltia punctata* SC016 was still able to produce the highest starch content (61.6 g/m^2). Under stressed condition, *Wolffia arrhiza* SC002, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 produced starch content of 8.2 g/m^2, 56.3 g/m^2 and 37.0 g/m^2 respectively.

Table 2: Chemical compositions of duckweed

	W.arrhiza **SC002**	***L. aequinoctialis*** **SC022**	***L. perpusilla*** **SC024**	***L. punctata*** **SC016**
Starch (% w/w – DM)	9.7	21.4	26.2	17.8
Protein (% w/w – DM)	37.8	30.1	27.8	25.3
Lipid (% w/w – DM)	6.9	5.3	5.2	4.5
Fiber (% w/w – DM	9.4	9.2	8.9	8.7
Ash (% w/w – DM)	15.2	14.8	13.7	14.2
Lignin (% w/w – DM)	4.8	5.1	4.7	4.2

* DM = dry matter

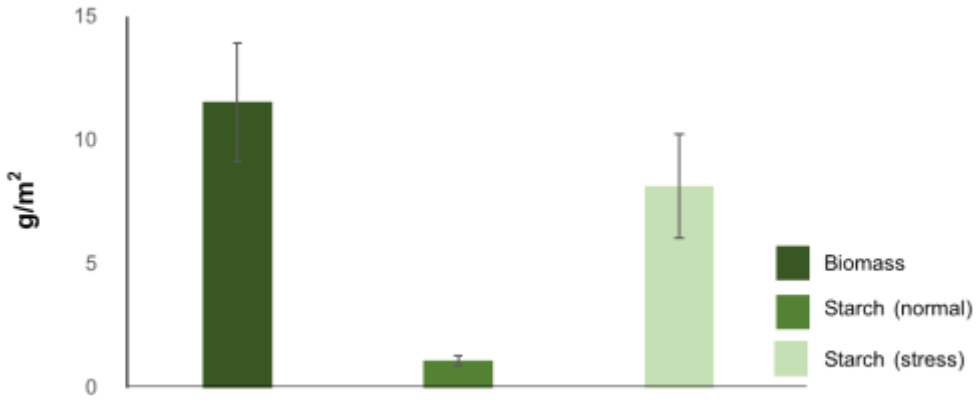

Figure 13: Biomass and starch production of *Wolffia arrhiza* SC002 cultivated for 9 days in laboratory condition. Stress induction was performed by cultivated duckweed in minimal medium for 5 days before harvest. Bars are ± SD (n = 3).

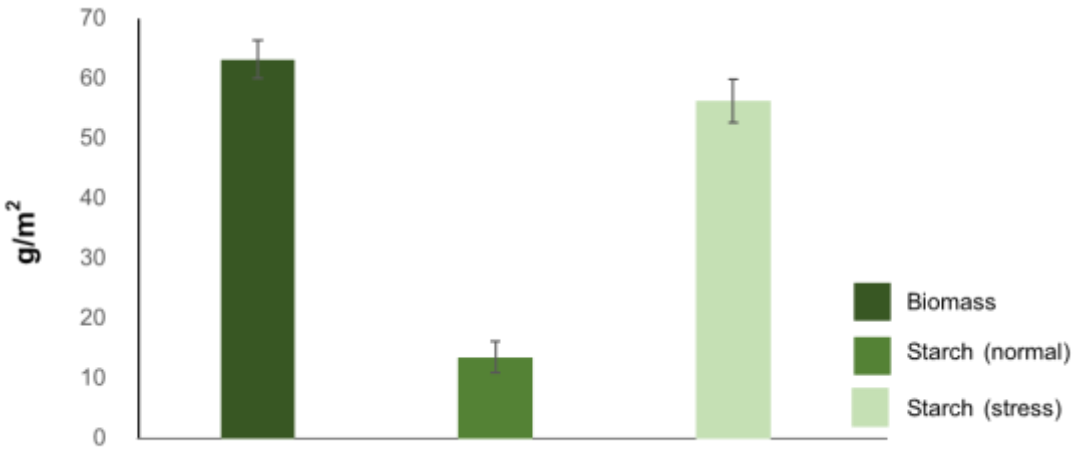

Figure 14: Biomass and starch production of *Lemna aequinoctialis* SC022 cultivated for 9 days in laboratory condition. Stress induction was performed by cultivated duckweed in minimal medium for 5 days before harvest. Bars are ± SD (n = 3).

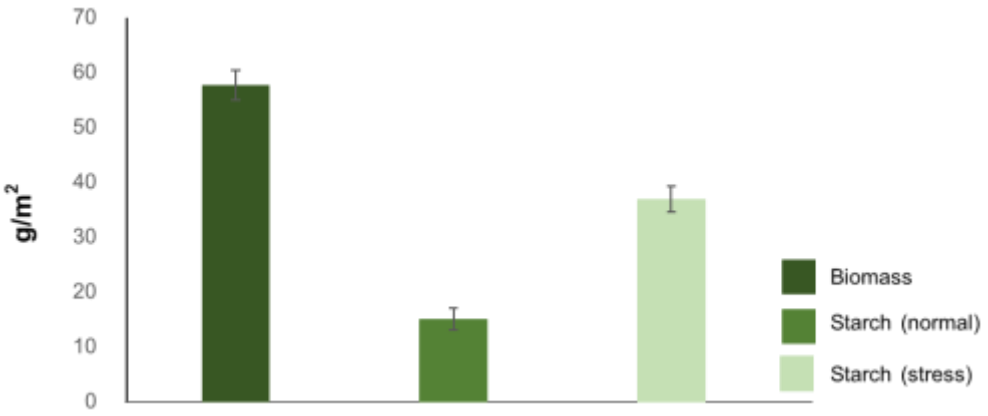

Figure 15: Biomass and starch production of *Lemna perpusilla* SC024 cultivated for 9 days in laboratory condition. Stress induction was performed by cultivated duckweed in minimal medium for 5 days before harvest. Bars are ± SD (n = 3).

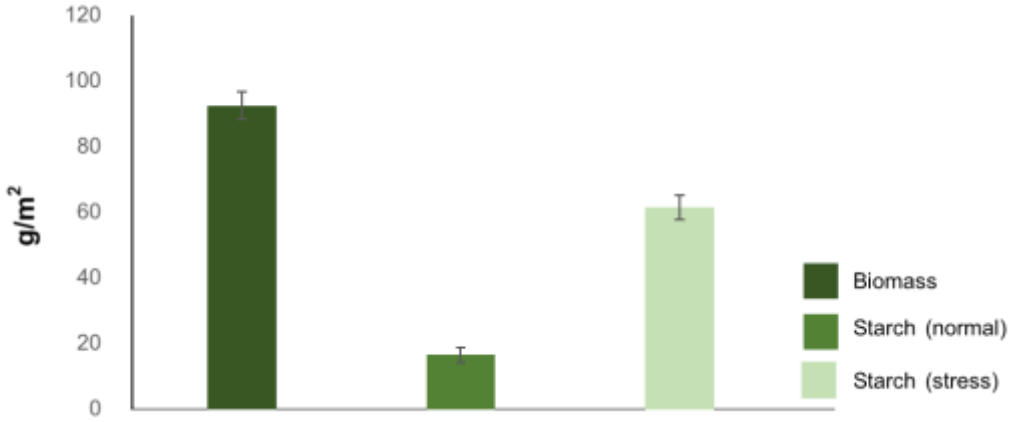

Figure 16: Biomass and starch production of *Landoltia punctata* SC016 cultivated for 9 days in laboratory condition. Stress induction was performed by cultivated duckweed in minimal medium for 5 days before harvest. Bars are ± SD (n = 3).

Figure 19: Starch accumulation *Lemna perpusilla* SC024: KI staining of low starch in non-stress duckweed (Left) and high starch (Right) in stressed (nutrient starving) duckweed. Scale bar = 5 mm.

5. Duckweed and Plant Hormones

***Wolffia arrhiza* SC002**

Wolffia arrhiza SC002 responded to plant hormones differently. IAA at high dose (more than 10^{-5} mM) showed negative effect on biomass production. The biomass of *Wolffia arrhiza* SC002 was reduced to 31.8% when cultivated in 10^{-1} mM of IAA compared with control treatment (no IAA). Moreover, IAA at 10^{-2} mM and 10^{-3} mM could increase starch content to 5.5 g/m^2 and 6.3 g/m^2, whereas the control treatment (no IAA) produced 2.7 g/m^2 of starch (Figure 20). Cytokinin (6-BA) at low concentration (10^{-4}- 10^{-5} mM) showed no effect on biomass and starch productivity, but 6-BA at 10^{-3} mM and 10^{-4} mM could enhance biomass to 14.2 and 14.7 g/m^2 compared to the control treatment's biomass, which was 11.9 g/m^2, and these concentration could enhance starch accumulations to 5.1 g/m^2 and 5.7 g/m^2, while the control treatment's starch content was 3.1 g/m^2 (Figure 21). ABA hormone (10^{-5} - 10^{-1}mM) had no effect on biomass production, but ABA could enhance the starch accumulation, the highest starch content was 7.8 g/m^2 at concentration of ABA at 10^{-3} mM (Figure 22). GA hormone (GA_3) (10^{-1}- 10^{-5} mM) showed no effect on biomass and starch production for *Wolffia arrhiza* SC002 (Figure 23).

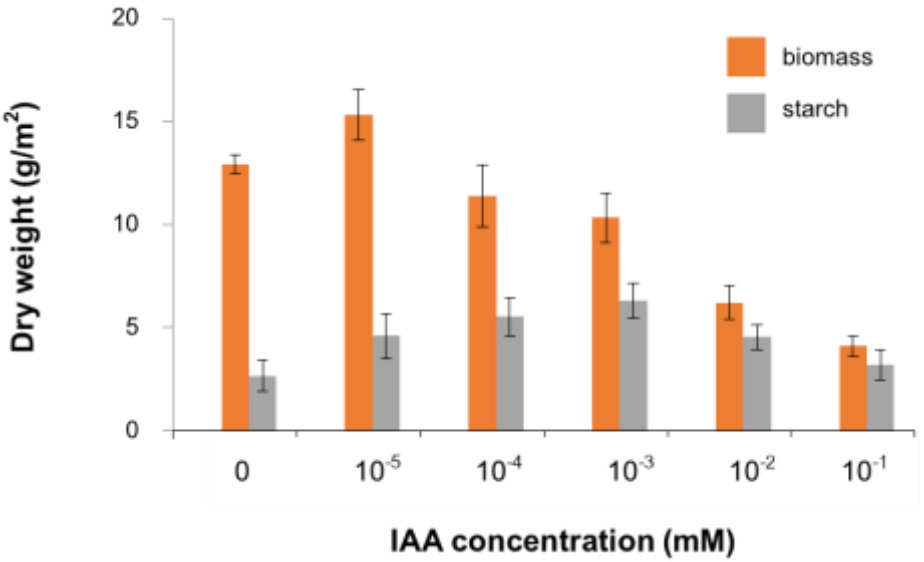

Figure 20: Biomass and starch production of *Wolffia arrhiza* SC002 cultivated with different concentration of auxin (IAA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

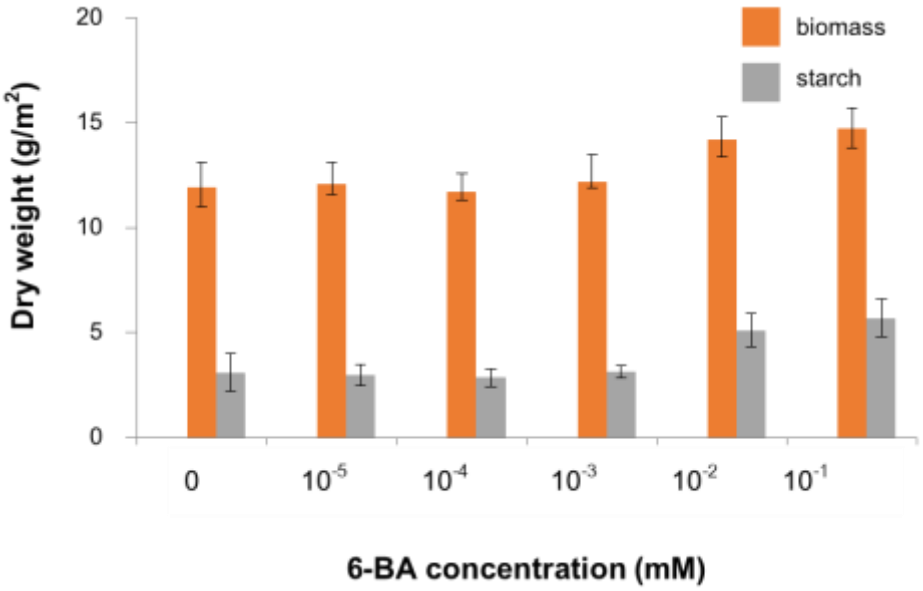

Figure 21: Biomass and starch production of *Wolffia arrhiza* SC002 cultivated with different concentration of cytokinin (6-BA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

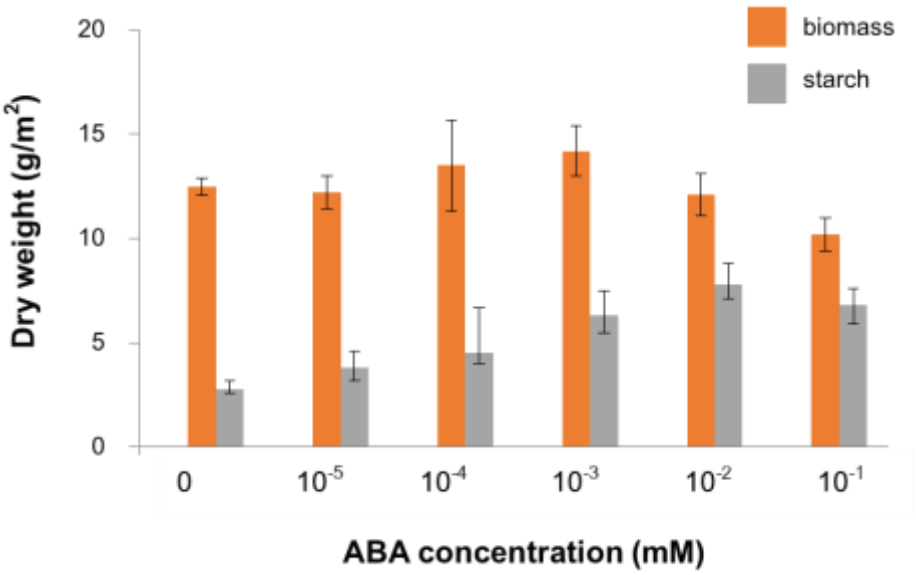

Figure 22: Biomass and starch production of *Wolffia arrhiza* SC002 cultivated with different concentration of ABA for 9 days in laboratory condition. Bars are ± SD (n = 3).

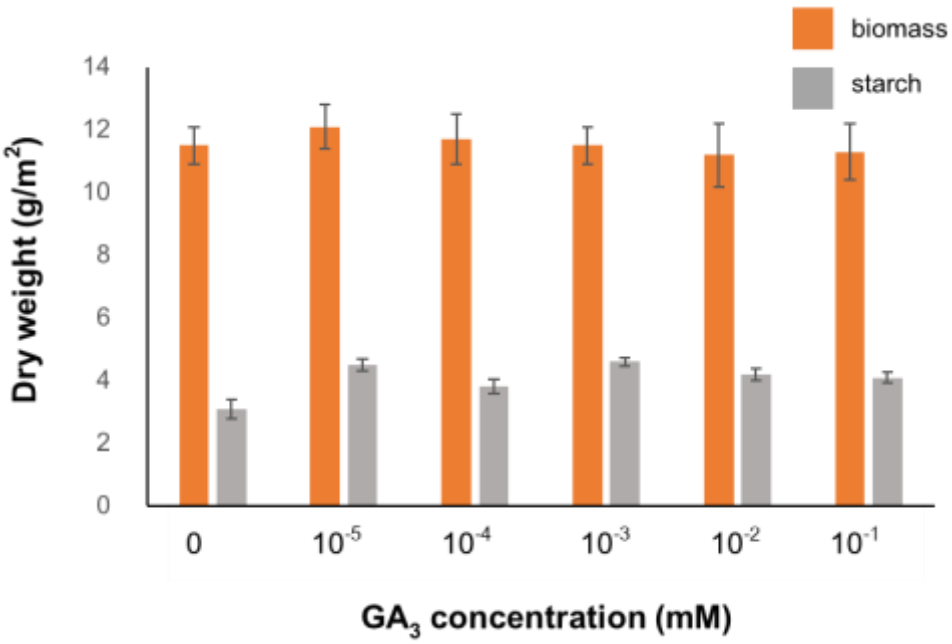

Figure 23: Biomass and starch production of *Wolffia arrhiza* SC002 cultivated with different concentration of GA (GA_3) for 9 days in laboratory condition. Bars are ± SD (n = 3).

***Lemna aequinoctialis* SC022**

The auxin (IAA) (10^{-5} - 10^{-1} mM) showed no effect on biomass and starch production for *Lemna aequinoctialis* SC022 (Figure 24). Cytokinin (6-BA) at concentration of 10^{-3} mM slightly increased biomass production to 91.4 g/m^2 compared to the biomass of control treatment (no 6-BA), which was 78.2 g/m^2. High 6-BA concentration (10^{-3} – 10^{-1} mM) could enhance starch accumulation to 19.7 g/m^2, 25.3 g/m^2 and 20.1 g/m^2 compared to the starch of control treatment (no 6-BA), which was 10.5 g/m^2 (Figure 25). ABA hormone at high concentration (10^{-3} – 10^{-1} mM) had negative effect on biomass production, which those biomasses were decreased to 72.1 g/m^2, 65.3 g/m^2 and 60.1 g/m^2 compared to the biomass of control treatment (no ABA), which was 81.2 g/m^2. However, ABA at those concentrations could enhance starch accumulation despite of decreasing biomass production. The starch contents were increased up to 25.6 g/m^2, 32.4 g/m^2 and 45.3 g/m^2 for ABA concentration of 10^{-3} mM, 10^{-2} mM and 10^{-1} mM respectively, while the control treatment produced starch at 12.4 g/m^2 (Figure 26). GA hormone (GA_3) (10^{-5} - 10^{-2} mM) showed no effect on biomass and starch production for *Lemna aequinoctialis* SC022 (Figure 27).

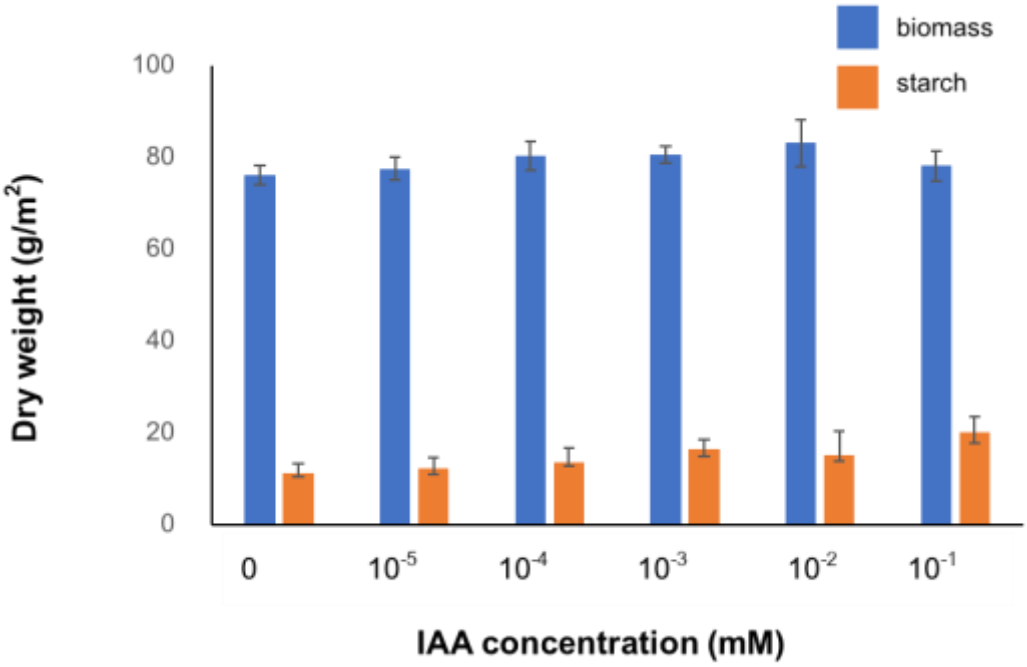

Figure 24: Biomass and starch production of *Lemna aequinoctialis* SC022 cultivated with different concentration of auxin (IAA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

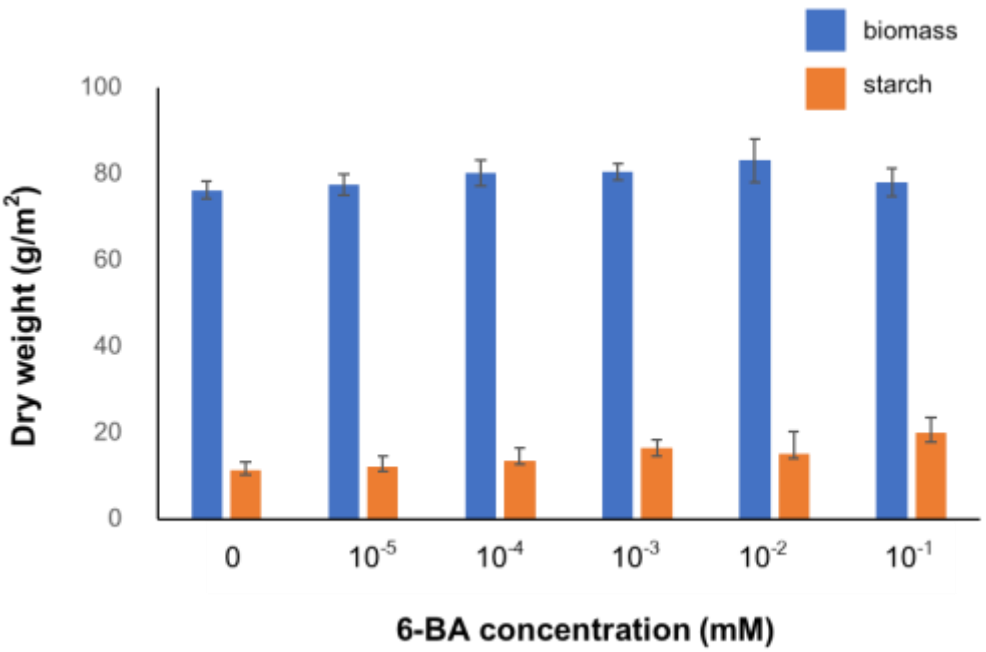

Figure 25: Biomass and starch production of *Lemna aequinoctialis* SC022 cultivated with different concentration of cytokinin (6-BA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

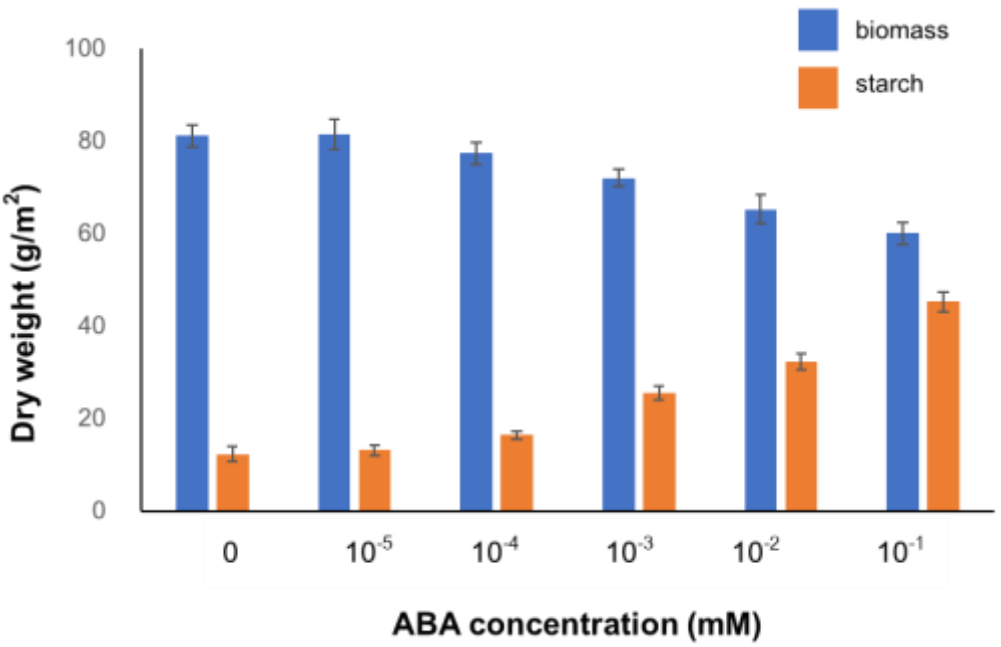

Figure 26: Biomass and starch production of *Lemna aequinoctialis* SC022 cultivated with different concentration of ABA for 9 days in laboratory condition. Bars are ± SD (n = 3).

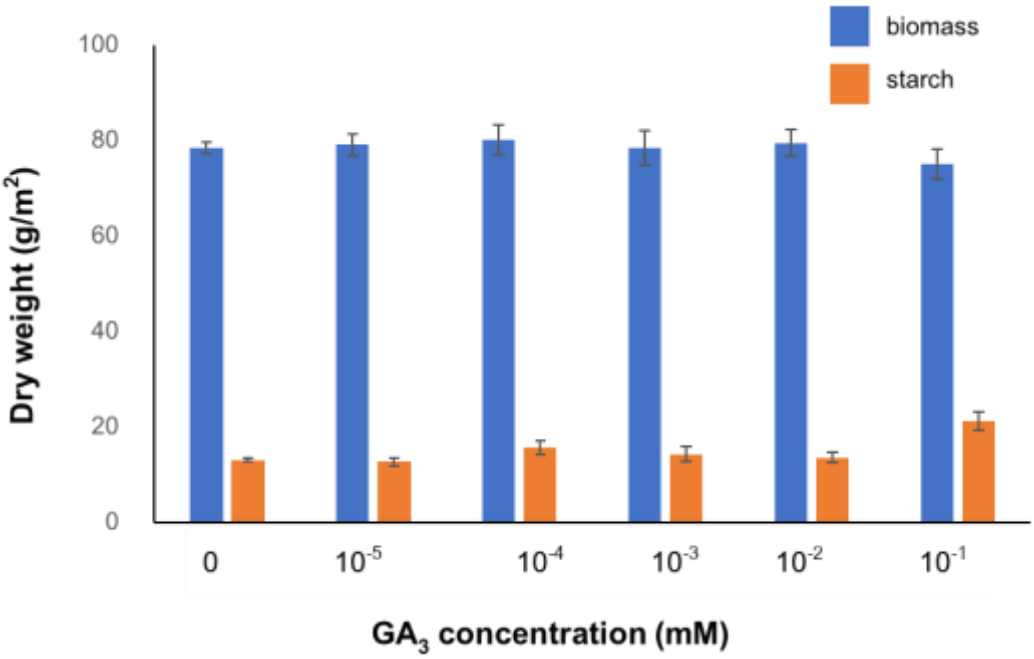

Figure 27: Biomass and starch production of *Lemna aequinoctialis* SC022 cultivated with different concentration of GA (GA_3) for 9 days in laboratory condition. Bars are ± SD (n = 3).

***Lemna perpusilla* SC024**

Auxin (IAA) at high concentration increase biomass and starch production of *Lemna perpusilla* SC024. Duckweed grown at 10^{-4} mM, 10^{-3} mM, 10^{-2} mM and 10^{-1} mM of IAA produced biomass of 69.5 g/m^2, 72.1 g/m^2, 80.1 g/m^2 and 68.9 g/m^2 respectively and generated starch of 15.6 g/m^2, 20.5 g/m^2, 22.8 g/m^2 and 21.1 68.9 g/m^2 respectively. Duckweed grown in nutrient without IAA supplement produced biomass and starch of 62.3 g/m^2 and 12.3 g/m^2 respectively (Figure 28). Cytokinin (6-BA) showed similar effect of IAA on biomass and starch production. Duckweed grown at 10^{-4} mM, 10^{-3} mM, 10^{-2} mM and 10^{-1} mM of IAA produced biomass of 75.6 g/m^2, 82.1 g/m^2, 89.1 g/m^2 and 76.8 g/m^2 respectively and made starch of 17.2 g/m^2, 19.3 g/m^2, 19.8 g/m^2 and 16.5 g/m^2 respectively (Figure 29). ABA hormone at high concentration (10^{-3} – 10^{-1} mM) had negative effect on biomass production, which those biomasses were decreased to 56.7 g/m^2, 54.1 g/m^2 and 45.6 g/m^2 compared to the biomass of control treatment (no ABA), which was 60.1 g/m^2. However, ABA at those concentrations could enhance starch accumulation despite of decreasing biomass production. The starch contents were increased up to 25.6 g/m^2, 26.4 g/m^2, 30.1 g/m^2 and 22.3 g/m^2 for ABA concentration of 10^{-4} mM, 10^{-3} mM, 10^{-2} mM and 10^{-1} mM respectively, while the control treatment produced starch at 13.4 g/m^2 (Figure 30). GA hormone (GA_3) (10^{-6} - 10^{-1} mM) showed no effect on biomass and starch production for *Lemna perpusilla* SC024 (Figure 31).

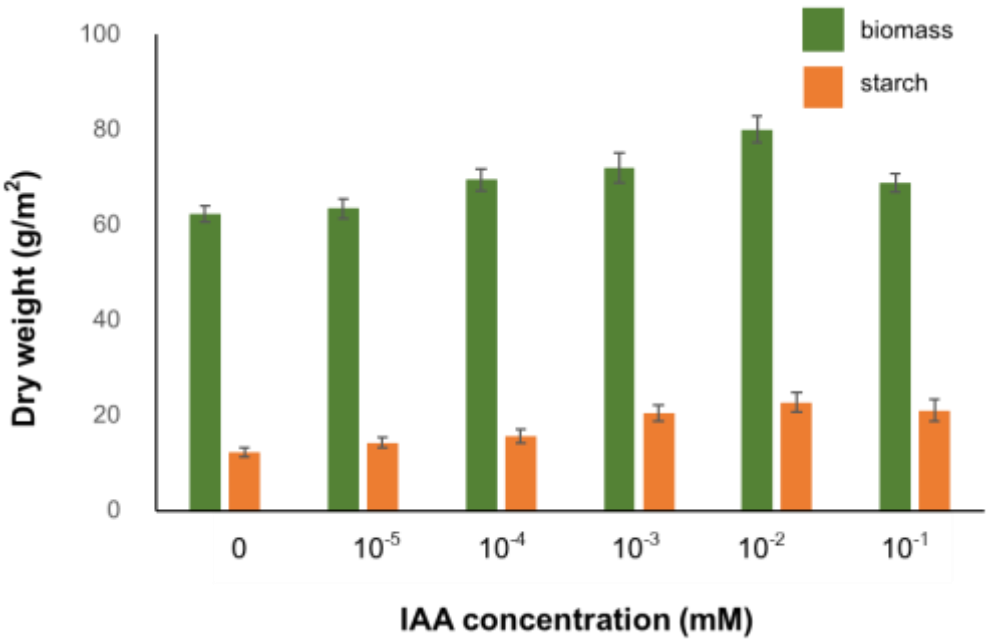

Figure 28: Biomass and starch production of *Lemna perpusilla* SC024 cultivated with different concentration of auxin (IAA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

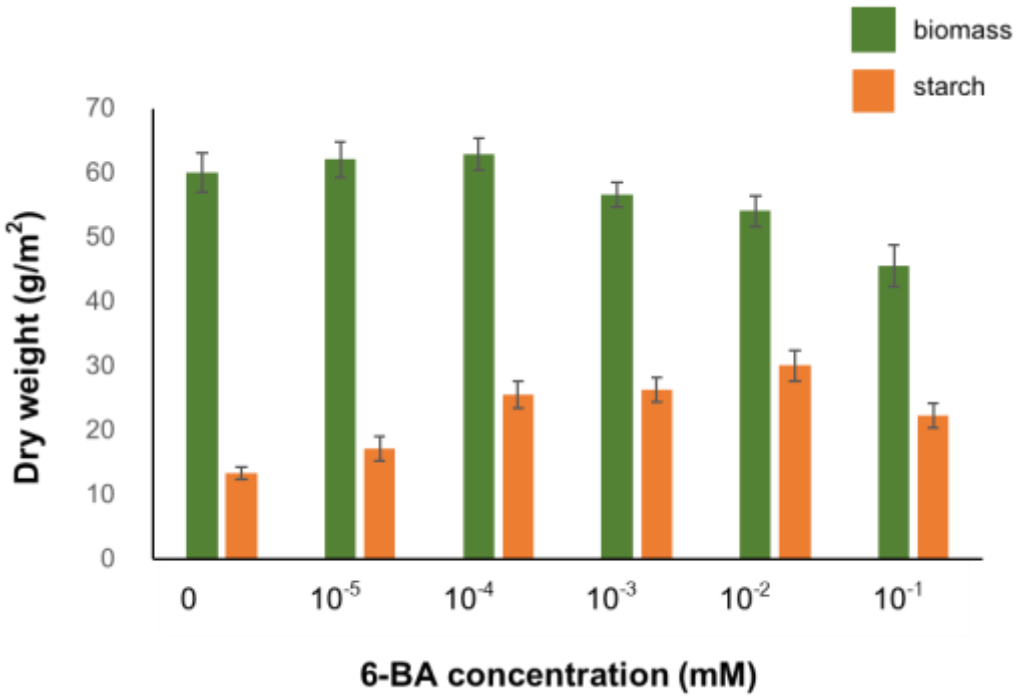

Figure 29: Biomass and starch production of *Lemna perpusilla* SC024 cultivated with different concentration of cytokinin (6-BA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

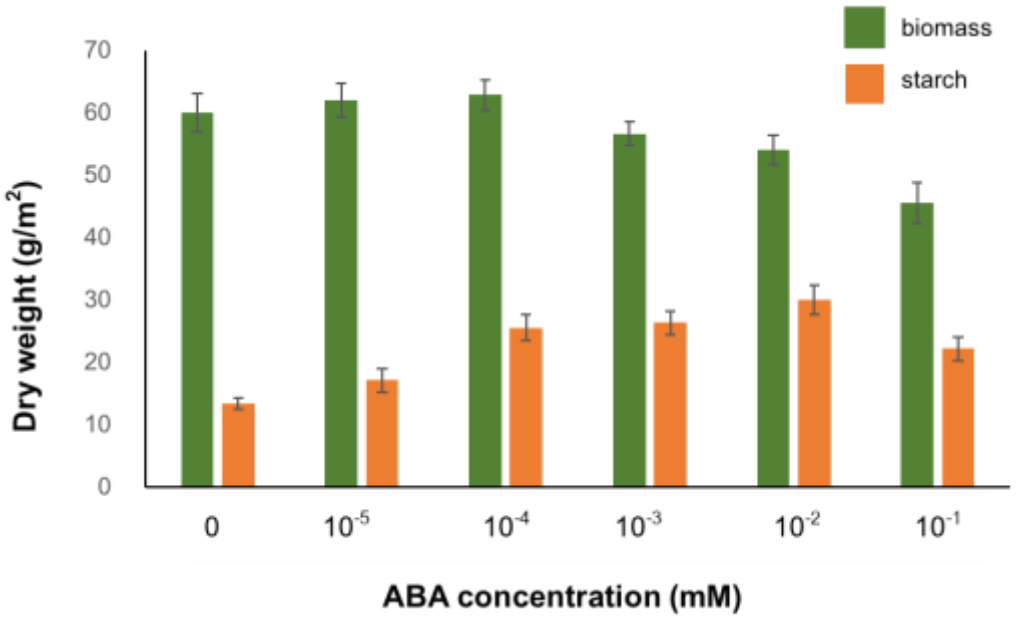

Figure 30: Biomass and starch production of *Lemna perpusilla* SC024 cultivated with different concentration of ABA for 9 days in laboratory condition. Bars are ± SD (n = 3).

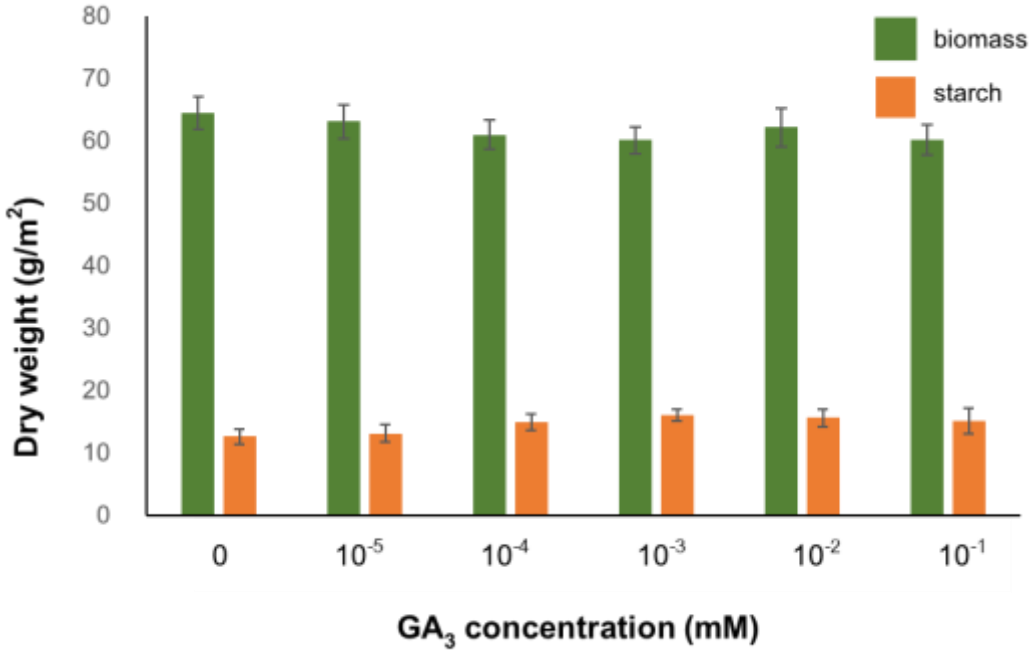

Figure 31: Biomass and starch production of *Lemna perpusilla* SC024 cultivated with different concentration of GA (GA_3) for 9 days in laboratory condition. Bars are ± SD (n = 3).

***Landoltia punctata* SC016**

IAA at high level could enhance biomass production. Biomass of duckweed grown in nutrients supplemented with 10^{-3} mM, 10^{-2} mM and 10^{-1} mM IAA were 140.5 g/m^2, 155.7 g/m^2 and 132.6 g/m^2, where the biomass of control (no IAA) was 110.5 g/m^2. However, IAA showed no effect on starch production (Figure 32). Cytokinin (6-BA) at 10^{-4} mM, 10^{-3} mM, 10^{-2} mM and 10^{-1} mM enhanced biomass and starch production, the biomass production were 136.2 g/m^2, 145.6 g/m^2, 165.3 g/m^2 and 165.1 g/m^2, and the starch content were 36.3 g/m^2, 40.5 g/m^2, 60.3 g/m^2 and 63.6 g/m^2. Duckweed in the control treatment (no IAA) generated 109.3 g/m^{2}of biomass and 20.3 g/m^{2}of starch. (Figure 33). ABA at high level, which were 10^{-3} mM, 10^{-2} mM and 10^{-1} mM reduced biomass accumulation but increased starch content. Duckweed grown in 10^{-3} mM, 10^{-2} mM and 10^{-1} mM ABA made 110.6 g/m^2, 101.5 g/m^2 and 95.1 g/m^2 of biomass and 50.3 g/m^2, 60.1 g/m^2 and 62.3 g/m^2 (Figure 34). GA hormone (GA_3) (10^{-5} - 10^{-1} mM) showed no effect on biomass and starch production for *Landoltia punctata* SC016 (Figure 35).

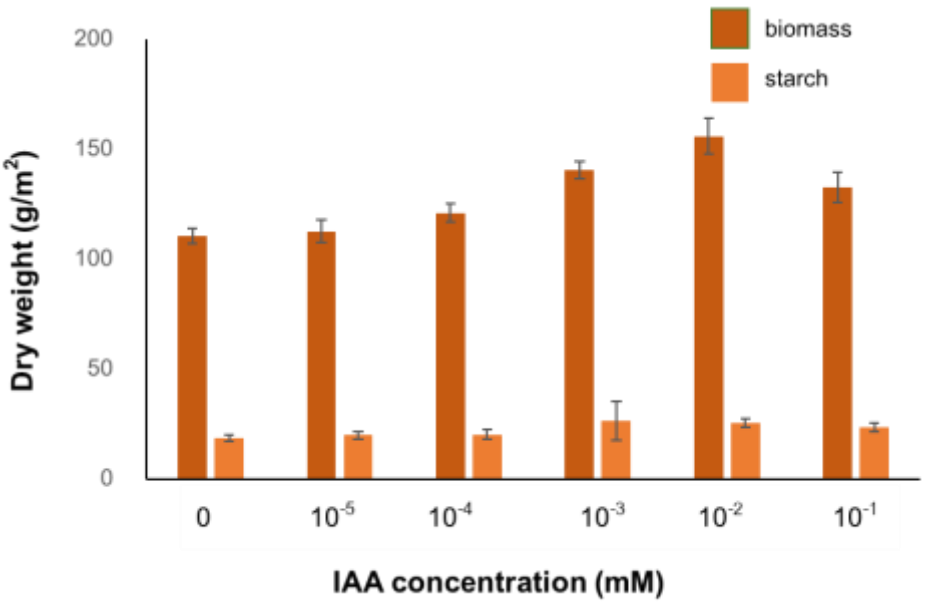

Figure 32: Biomass and starch production of *Landoltia punctata* SC016 cultivated with different concentration of auxin (IAA) for 9 days in laboratory condition. Bars are ± SD (n = 3)

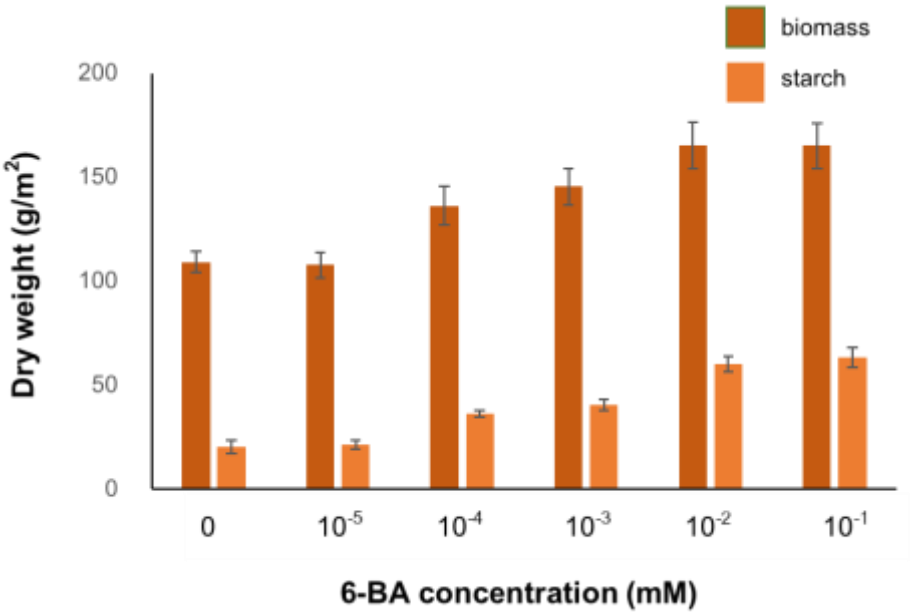

Figure 33: Biomass and starch production of *Landoltia punctata* SC016 cultivated with different concentration of cytokinin (6-BA) for 9 days in laboratory condition. Bars are ± SD (n = 3).

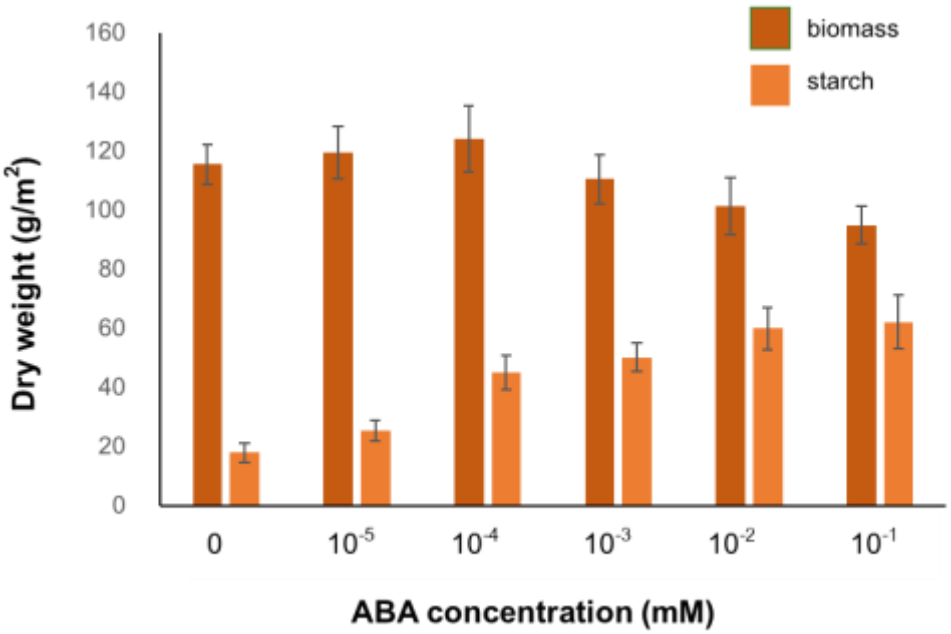

Figure 34: Biomass and starch production of *Landoltia punctata* SC016 cultivated with different concentration of ABA for 9 days in laboratory condition. Bars are ± SD (n = 3).

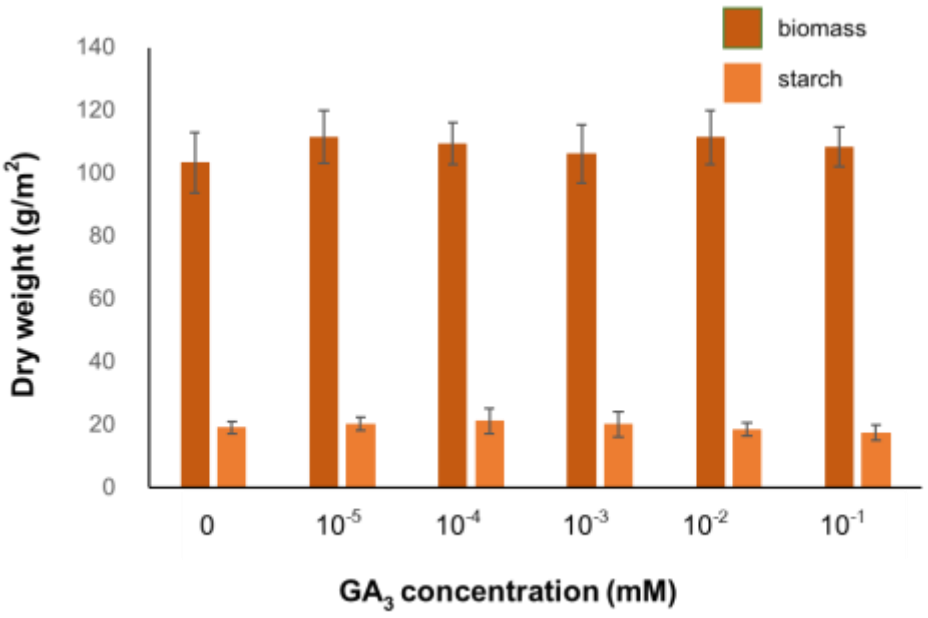

Figure 35: Biomass and starch production of *Landoltia punctata* SC016 cultivated with different concentration of GA (GA_3) for 9 days in laboratory condition. Bars are ± SD (n = 3).

6. Pilot test for duckweed biomass production and starch production

When duckweeds, *Wolffia arrhizal* SC002, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 and *Landoltia punctata* SC016, were cultivated outdoor in pilot scale, it was very difficult to maintain those cultures to be monoculture for each duckweed species. After 2 months of monoculture cultivation, other species were always observed in each pond. Therefore, mixed duckweed culture (1:1:1:1 biomass) or polyculture, which was more applicable for real production, was performed instead of monoculture.

Production of duckweed in pilot scale (outdoor condition) was determined by harvested twice a week for a year (October, 2014 – September, 2015) period. Production of biomass varied from 33.2 – 38.7 t/ha/year, 10.7– 22.1 t/ha/year and 20.4 – 26.7 t/ha/year during Thai summer, rainy and cool seasons (Figure 36). It was clearly that seasons effect the biomass productivity. Duckweed grew very well and showed the highest productivity during summer period. The weather condition (Table 4) also supported the results. During summer season showed the highest sun hour and the highest UV index, which might stimulate photosynthesis in duckweed. During rainy season, July, August and September had the highest rain fall, which were 117.68, 104.18 and 102.40 mm respectively, and the highest number of raining days, which were 27, 30, 36 days. Those months showed the lowest biomass production, which were 11.3, 12.6 and 10.7 t/ha/year.

For starch production, the production varied from 12.5 – 18.6 t/ha/year, 6.3 – 10.7 t/ha/year and 8.3 – 19.6 t/ha/year during Thai summer, rainy and cool seasons (Figure 36). The highest starch production occurred during March, April, November and December, which yielded 18.6, 16.1, 16.5 and 19.6 t/ha/year of starch. However, when the ration between starch/biomass was considered, November and December showed the heist number, which were 0.77 and 0.74.

Table 4: Weather condition at the experiment site during October 2014 – September 2015.

Month	Max Temp.	Min Temp.	Avg. Temp.	Rain		Humidity	Cloud	Sun	UV
	(C°)	(C°)	(C°)	(mm)	days	(%)	(%)	(Hr)	index
January, 2015	27	15	20	22.41	4	60	20	264.5	6
February, 2015	31	19	25	1.76	1	47	11	280.0	7
March, 2015	35	24	29	13.87	12	42	20	286.0	8
April, 2015	36	31	26	28.00	15	44	21	304.5	8
May, 2015	35	30	26	30.08	13	55	30	364.0	7
June, 2015	32	24	28	86.35	19	70	40	304.5	7
July, 2015	30	23	26	117.68	27	80	52	242.0	6
August, 2015	31	23	26	104.18	30	77	41	251.5	6
September, 2015	30	23	26	102.40	26	80	42	240.0	7
October, 2014	29	21	25	39.76	15	77	31	232.0	7
November, 2014	30	21	24	4.20	4	75	20	220.5	6
December, 2014	28	18	22	15.49	10	72	28	210.0	6

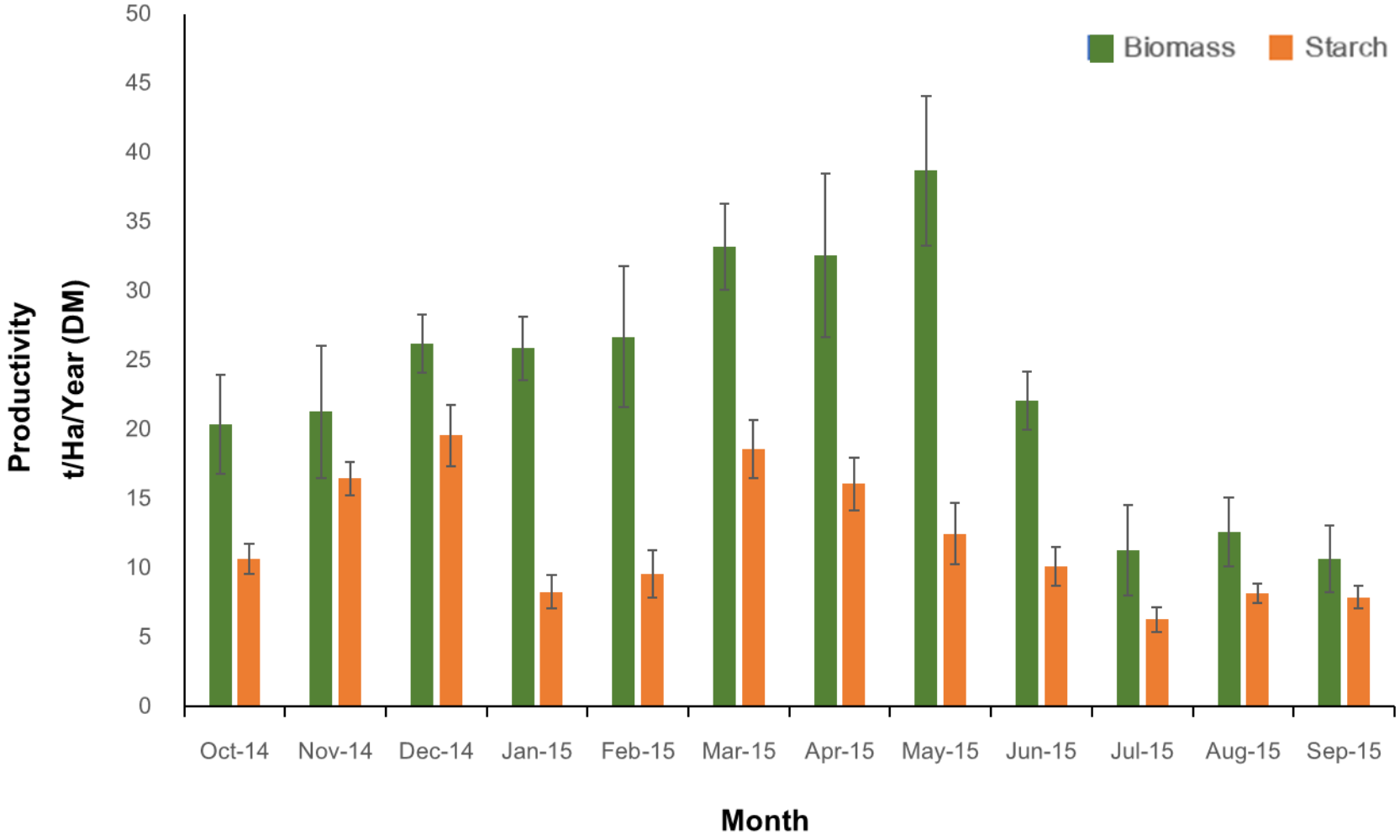

Figure 36: Biomass and starch production from mixed duckweed cultivation in pilot scale for 12 months (from October, 2014 – September, 2015)

7. Duckweed and Ethanol Production

Ethanol production from starch-rich duckweed was performed in SSF (simultaneous saccharification and fermentation). The ethanol concentration varied for each duckweed samples. *Lemna aequinoctialis* SC022 and *Lemna perpusilla* SC024 showed the highest concentration of ethanol (19.22 ± 0.6 and 20.76 ± 0.7 g/L respectively) (Table 5). For ethanol yield per unit biomass, *Lemna aequinoctialis* SC022 and *Lemna perpusilla* SC024 also showed the best result, which were 0.34 ± 0.012 and 0.37 ± 0.018 g/g respectively, which might due to they have high starch content.

Table 5: Ethanol yield per unit biomass from duckweeds in simultaneous saccharification and fermentation (SSF)

	Lignocellulose (g/g)	**Starch (g/g)**	**Ethanol**	
			Concentration (g/L)	**Yield (g/g)**
Wolffia arrhizal SC002	0.14	0.10	16.68 ± 0.8^{b}	0.12 ± 0.009^{c}
Lemna aequinoctialis SC022	0.14	0.21	19.22 ± 0.6^{a}	0.34 ± 0.012^{a}
Lemna perpusilla SC024	0.14	0.26	20.76 ± 0.7^{a}	0.37 ± 0.018^{a}
Landoltia punctata SC016	0.13	0.18	17.45 ± 0.8^{b}	0.28 ± 0.010^{b}

* Ethanol data are presented as the mean of triplicate measurements ± standard deviation. Different letters indicate significant differences between different conditions ($p < 0.05$).

8. Impact assessment of duckweed production

The function unit of assessment was area 1 ha that producing duckweed every day at the average biomass of 23.5 t DM/ha/year, which obtained from the pilot duckweed production tested. This assessment was performed as “Cradle-to-Gate”, which the ethanol production, the product usage stage and waste treatment are omitted because those activities were not performed in pilot scale in this experiment. Only cultivation and harvest of duckweed were used for calculation. All material and energy flows within the product system were shown in Table 6. The analysis showed

that major CO_2 emission came from N fertilizer, which similar to other works, which reported that N-fertilizer was the major source of GHG (greenhouse gas) of energy crop cultivation (Wang et al., 2012). When compared to other energy crops, duckweed production in this experiment released more GHG than the others. Duckweed produced GHG around 6,439.6 - 25,624.3 $kgCO_2eq/ha/yr$, where as other energy crops such as maize, sorghum, switchgrass and miscanthus produced 3,283, 2,265, 1,754 and 2,654 $kgCO_2eq/ha/yr$ (Camargo et al., 2013; Glab & Sowinski, 2019).

Table 6: Inputs and outputs for duckweed biomass production (per ha/yr)

Categories	Materials/Process	Amount	CO_2 ($kgCO_2eq$)
Input			
Raw material	Water (m^3)[a]	3,991.7	-
	Nitrogen (N) (kg)	3,285.0	10,852.3[e]
	Phosphate (P) (kg)	657.0	1032.5[e]
	Potassium (K) (kg)	657.0	1032.5[e]
	Trace elements (kg)	3,285.0	525.3
	Pesticides[b] (kg)	15.0	105.6
Energy	Pump[a] (kWh)	10.8	6.5
Output			
	Duckweed[c] (t DM)	23.5	-9,400.0
	CO_2[d] (kg)	2,284.9	2,284.9
	CH_4[d] (kg)	657.0	19,710.0
Net total			
	without CH_4[f]		6,439.6
	with CH_4[f]		25,624.3

[a] Water used in this experiment was from natural pond. For this purpose, we applied a physical relationship, which prescribes the energy required to lift 1 m^3 of water (with a density 1000 kg m^3) up 1 m at 100% efficiency is 0.0027 kWh (Rothausen and Conway 2011).

[b] Pesticides were used in this experiment were algicide (diuron - to eliminate microalgae) and insecticide (profenofos – to prevent insects)

[c] Biomass was dry mass, which was the average of biomass productivity from figure 36.

[d] Data from Dai et al., 2015

[e] Assumption of transportation from Bangkok with the distance of 700 km was made and incorporated into the calculation.

[f] Because the data from Dai et al. (2015) was obtained by producing duckweed biomass in wastewater, which generally made CH_4 by microorganisms in wastewater. This experiment didn't use wastewater, so that the assumption which CH_4 production were not occurred in this experiment could be made.

Conclusion and Discussion

1. Duckweed characterization

The 52 duckweed samples were collected from the different regions of the northern part of Thailand. There were 24 samples left for monoculture. Those sample can be characterized into 4 species, which were *Wolffia arrhiza* (2 samples), *Lemna aequinoctialis* (9 samples), *Lemna perpusilla* (8 samples) *and Landoltia punctata* (5 samples). Those 4 species were reported found in Thailand before. There were reports stated that 2 species of *Wolffia* spp. were found in Thailand, which were *Wolffia arrhiza* (L.) and *Wolffia globosa* (L.) (Rodroil et al., 2009; Rodroil et al., 2012; Ruekaewma et al., 2015). Bhanthumnavin and McGarry (1971) described that *Wolffia arrhizal* was one of the smallest duckweeds and it had been used as a nutritious vegetable by the people of Northern Thailand for generations. However, *Wolffia globose* was not found in the collected duckweed samples. *Lemna perpusilla* is reported as the most common species of the family Lemnaceae (Heuzé and Tran, 2015). It was also the most common duckweed in Thailand (Phewnil et al., 2012). *Lemna aequinoctialis* and *Landoltia punctata* were also common and popular for research in Thailand as shown in Jaiprasert (2018), Kittiwongwattana and Vuttipongchaikij (2013), Kittiwongwattana and Thawai (2014), Kittiwongwattana and Vuttipongchaikij (2015) and Kittiwongwattana (2019) and Xu et al. (2015).

2) Duckweed for biomass production

Wolffia arrhiza SC004*, Lemna aequinoctialis* SC022*, Lemna perpusilla* SC024 and *Landoltia punctata* SC016 were selected as the representative for each duckweed species because they showed the best growth performance in each group. With ample nutrients, the biomass productivities were 16.3 g/m^2, 93.5 g/m^2, 78.6 g/m^2 and 129.2 g/m^2 respectively for 12 days cultivation. The biomass production performance was in the order *L. punctata* SC016 > *L. aequinoctialis* SC022 > *L. perpusilla* SC024 > *W. arrhiza* SC004.

From the previous reports, the biomass production of *Wolffia arrhiza* varied. Chowhury et al. (2000) also reported the biomass production of 14.75 kg/ha/day or 1.4 g/m^2/d when cultivated with anaerobically fermented cow dung effluent. This report showed similar biomass production to this work. However, Soda et al. (2013) reported the biomass production of this

species at 23.3 t DW/ha/yr or 6.4 g/m^2/d when cultivated in continuous-flow mesocosms supplied with waste water, which suggested that not only nutrients that essential for biomass production but also the cultivation system as well.

Lemna aequinoctialis biomass production varied in previous works from 4.3 g/m^2/d – 19.4 g/m^2/d, and this work reported the biomass of this species within that range (8.2 g/m^2/d). Yu et al. (2014) reported the biomass production of 4.3 g/m^2/d (the lowest) when cultivated with sewage water and 10.0 4.3 g/m^2/d when cultivated in sufficient nutrients. Yin et al. (2015) described that maximum biomass production of this species was 8.9 g/m^2/d. Neto et al. (2019) showed that the best *Lemna aequinoctialis* strain could make biomass of 19.4 g/m^2/d, while the worst strain produced biomass of 4.5 g/m^2/d when cultivated with waste water. These works recommend that genetic factor and environmental factor have an effect on biomass production. To obtain maximum biomass production rate, duckweed strain, cultivation system and nutrients availability were important.

The previous works reported the range of biomass production of *Lemna perpusilla* was 1.5 g/m^2/d – 6.8 g/m^2/d when cultivated in waste water or lake. Edwards et al. (1990) reported the biomass production of *Lemna perpusilla* as 1.5 g/m^2/d. Chrismadha et al (2014) also reported the biomass production of 6.14 – 11.61 tDM/ha/y or 1.7 g/m^2/d – 3.2 tDM/ha/y. Chrismadha et al. (2019) also reported that the biomass production of *L. perpusilla* in natural lake could be as high as 6.8 g/m^2/d.

Mohedano et al. (2012) reported the biomass production of *Landoltia punctata* was 18.6 1.5 g/m^2/d. Cheng et al. (2002) also reported that *Landoltia punctata* could gave the highest biomass production as high as 32.1 g/m^2/d. Those reports indicated that this species gave higher biomass production than other species, which similar to this work showing that *Landoltia punctata* SC016 had the highest biomass production.

3) Duckweed for starch production

The starch contents were varied. *Lemna perpusilla* SC024 has the highest starch content, which was 26.2%, whereas *Wolffia arrhiza* SC002, *Lemna aequinoctialis* SC022 and *Landoltia punctate* SC016 has the starch content of 9.7%, 21.4% and 17.8% respectively (Table 2). However, when the starch was considered, *Landoltia punctate* SC016 showed the highest

productivity, which was average of 16.5 g/m^2. *Lemna perpusilla* SC024 and *Lemna aequinoctialis* SC022 have similar starch productivity, which were 15.1 g/m^2 and 13.5 g/m^2 respectively.

Based on the duckweed species and the growing conditions applied, duckweed starch contents ranging from 3% to 75% dry weight have been reported (Reid and Bieleski, 1970; Landolt and Kandeler, 1987). Yu et al. (2004) reported the starch content of *Lemna aequinoctialis* was about 25 - 27% w/w of dry mass, whereas Neto et al. (2019) reported that *Lemna aequinoctialis* had starch content of 2.9 – 6.10%. Yin et al. (2015) also showed that *Lemna aequinoctialis* had starch content of 42.3%. Soda et al. (2015) reported the starch content of *Wolffia arrhiza* as 17 – 20% w/w, and Takai et al. (2014) showed the starch content of *Wolffia arrhiza as* 40.0%. Chen et al. (2012) reported the content of starch was 24.59% in *Landoltia punctata*. The stressed *Landoltia punctata* SC016 was still able to produce the highest starch content (61.6 g/m^2). Under stressed condition, *Wolffia arrhiza* SC002, *Lemna aequinoctialis* SC022, *Lemna perpusilla* SC024 produced starch content of 8.2 g/m^2, 56.3 g/m^2 and 37.0 g/m^2 respectively.

Many previous works have shown that the starch content of duckweed can be substantially increased by manipulating growing conditions such as pH, phosphate concentration, and nutrient starvation (Cheng and Stomp, 2009; Tasseron-De Jong and Veldstra, 1971; McLaren and Smith, 1976), which makes duckweed a promising starch source and a potential feedstock for bioethanol production. Sree and Appenroth (2014) showed that the starch accumulation could be induced up to 50% in duckweed by application of cadmium ions and other heavy metals, application of NaCl and depletion of phosphate in the growth medium. Tao et al. (2017) also reported that nutrient starvation was the best option to obtain high starch and flavonoid accumulation simultaneously in a short time for biofuels fermentation and natural products isolation, and the content of starch was increased from 3.16% to 48.01%.

In this experiment, similar results were observed. When stress was induced in the duckweed samples by nutrient starving, the content of starch was increased significantly in all tested duckweed sample. Normal content of starch for *Wolffia arrhiza* SC004*, Lemna aequinoctialis* SC022*, Lemna perpusilla* SC024 and *Landoltia punctata* SC016 were 9.7%, 21.4%, 26.1% and 17.8% of dry mass respectively, and when the stress was induced, the new starch contents were 70.5%, 88.9%, 63.9% and 66.5 respectively. The nutrient starving might be able

to suppress growth more effectively than photosynthesis, resulting surplus of carbohydrates is then stored as starch.

4) Duckweed and plant Hormones

In this experiment, duckweed could response to plant hormones, which indicate that the duckweed might have plant hormones receptor and signaling cascade triggered by those plant hormones. IAA (Auxin) could enhance biomass accumulation at high dose in all duckweed species, except *Wolffia arrhiza* SC004, but the ratio between starch and biomass was still maintained. IAA had negative effect on biomass accumulation of *Wolffia arrhiza* SC004 but it has positive effect on starch accumulation. Starch content of *Wolffia arrhiza* SC004 was increased when high dose of IAA applied, despite the decreasing in biomass accumulation. This result is different from works of Liu et al. (2019), which showing that a “low dosage-promotion and high dosage-inhibition” effect on the biomass accumulation of *Landoltia punctata*.

In this study, cytokinin (6-BA) could stimulate biomass and starch accumulation for all duckweed samples, which is similar to works of Liu et al. (2019), which showing that exogenous application of cytokinin exhibited a positive effect on the growth of duckweed in terms of biomass production and starch accumulation.

ABA also exhibited ability to increase starch accumulation in this experiment despite of the negative effect on biomass accumulation. The result was similar to the results of Liu et al. (2019), which showing that ABA can dramatically promote starch accumulation. The total starch that accumulated in the ABA treated samples were 3.3 times higher than that in the control samples. However, GA show no effect on both, biomass and starch accumulation, of duckweeds.

Taken together, these findings indicated that 6-BA and ABA were the most effective plant hormones in terms of enhancing biomass and starch accumulation.

5) Duckweed for biomass and starch production

From pilot test for duckweed production, the result showed very promising sign because the polyculture showed high rate of biomass and starch production and it can compete with other cellulosic-ethanol crop. This work showed lower production compared to the previous works. It might be that this experiment was run all year round, while the other works were done in some period. Therefore, seasonal effect didn’t show in their works. Other aquatic weed such as *Azolla* spp. might yield more biomass per area, but the ethanol yield can’t compete with the duckweed

because the duckweed could accumulate more starch than *Azolla* does. The biomass and ethanol yield of duckweed were much lower than the microalgae's (Table 7). However, harvesting process for microalgae is very difficult and energy consuming (Chisti, 2017). The duckweed is much easier for harvest and drying. Other advantages of duckweed for being energy crop are the high protein content and less lignin, which requires less energy for pretreatment sample and the residue from fermentation process can be used for animal fed with high protein level.

The production of biomass and starch from duckweed in this experiment was not effective in term of input and GHG impact. Source of nutrients, N, P and K, should come from the cheaper sources such as waste water. There were many researchers reported the success of duckweed cultivation with wastewater rich with organic matter, N, P and K at lowest cost. Moreover, duckweed could reduce methane emission from waste water. Dai et al. (2015) showed that waste water with duckweed in pond system release methane at 180 – 299 $mg/m^2/day$, while waste water without duckweed released methane at 328 – 559 $mg/m^2/day$. Using waste water as nutrient source for duckweed could lower the production cost and give environmental benefits. Therefore, effective duckweed cultivation system with waste water should be developed and distributed to mitigate energy and environmental problems in the future.

Table 7: Comparison of dry biomass yield of duckweed with other commonly studied potential energy crops

Potential energy crop	Dry biomass yield (t/ha/yr)	Ethanol yield (l/ha)	Reference
Duckweed	23.5	6,521	This work
Duckweed	39.1– 105.9	6,420	Xu et al., 2011
Azolla sp.	93.4 – 100.00	1,205	Mishima et al., 2008 Miranda et al., 2016
Microalgae	39.1 – 105.9	46,760 – 140,290	Chisti, 2017 Lardon et al, 2009 Scott et al., 2010
Corn	9.4	3,751 - 4020	Mussato et al., 2010
Sweet sorghum	35	3,050 – 4,070	Mussato et al., 2010
Cassava	40	3,310 – 6,000	Mussato et al., 2010
Sugarcane	795	5,476L	Mussato et al., 2010
Switchgrass	5.2 -26.0	1,438-10,760	Ussiri and Lal, 2015 Mussato et al., 2010
Miscanthus	1.6 – 14.3	11,205	Lewandowski et al., 2003; Lewandowski et al., 2003 Himken et al., 1997

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