CHARACTERIZATION OF STARCH-ACCUMULATING DUCKWEEDS, WOLFFIA GLOBOSA, AND OLEAGINOUS FUNGUS, MORTIERELLA ALPINA, AS RENEWABLE CARBON SOURCE PRODUCERS

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INTRODUCTION

Every agricultural practice is an economic activity that inherently impacts the ecosystem. It adds extra energy and physical substances into the material circulation of an ecosystem.

In the conventional agricultural practices, soil is an essential element while arable soil is a limited resource (Fig. 1). Even the existing farm land has been turned to desert by plantation agriculture (Fig. 2). In order for humankind to continue living on Earth, it is critical to conduct research and development on 1. preservation of soil and 2. agricultural technologies that do not require soil. The examples of the latter task are production of algae that produce industrially important substances and the organic hydroponic culture which Chitose Laboratory Corporation, the company that the author founded and leads, has been engaged in.

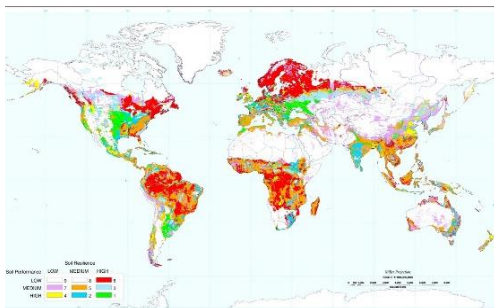

Fig. 1 Limit of arable soil.

Fig. 2 Desertification by plantation.

The effective approach to tackling the first task is preventing soil-loss in palm

plantations, mostly practiced in Malaysia and Indonesia, due to its size and impact on the ecosystem. Unfortunately, soil-loss is proliferated by strong economic interests within the industry. The size of the industry exceeds ¥7 trillion and the area expands to 14 million ha.

Among numbers of impacts on the environment, including air pollution through open burning, soil erosion caused by loss of biodiversity is a serious problem. This loss of biodiversity is related to water pollution caused by untreated or insufficiently treated palm oil mill effluent (POME) in addition to excessive usage of chemical fertilizer. There are about 1,000 oil mills, and every mill releases 500 t of POME a day (Fig. 3). POME is commonly treated in open lakes each of which is one hectare in size located near a mill and then released to rivers and seas. Owners of mills claim anaerobic and aerobic fermentation processes that occurred in lakes reduce BOD under 100 mg/L (Fig.4).

Fig. 3 Releasing POME from mill.

Fig. 4 Releasing POME to river.

The major problem in this “treatment” is that using BOD as an evaluation criteria is too simplistic; while the government promotes the regulatory requirement initiative for mill owners to reduce BOD under 20 mg/L, the amount of POME is so large and contains phosphorus and nitrogen so much that it still causes a state of overnutrition.

Chitose Laboratory Corporation developed an innovative POME treatment facility that utilizes duckweed, which is 1 ha in size and in full-fledge operation at a mill. In the facility, POME is used to grow duckweed and the microorganisms nurtured in the ecosystem created by duckweed reduce BOD level under 20 mg/L. Furthermore, duckweed absorbs phosphorus and nitrogen, preventing overnutrition of rivers and seas (Fig. 5).

Fig. 5 Duckweed field in Malaysia.

Duckweed is not only a useful organism to treat POME, it can also be harvested and converted to industrially useful substances. Its high productivity and nutrient contents make it commercially advantageous. According to the preliminary study (SITATION),

productivity of duckweed grown on POME is as high as 48 t/ha/month in dry weight. In addition, 35% of its weight is carbohydrate and 40% is protein in certain conditions. The productivity of carbohydrate is as large as that of corn and protein is about 10 times as large as soybeans.

Some of the potential applications are fertilizer and food. Chitose Laboratory Corporation has developed organic fertilizer from duckweed and various kinds of organic components discharged from plantations. It is specifically designed for prevention of "genodama", a fungi that damages palm trees, by taking advantage of high content rate of protein of duckweed.

It is now engaged in research and development on high-value fat for the food industry, taking advantage of its high content rate of carbohydrate. Carbohydrate is extracted first and then converted to high-value fat through fermentation. High-value fat is targeted due to its large market size and its potential to be converted/fermented by *Mortierella*, filamentous fungi directly from whole duckweed without first extracting carbohydrate.

This thesis confirms the potential of carbohydrate extracted from duckweed as fermented raw material for food by comparing the results of the ethanol fermentation performance test of duckweed grown in Japan and in Borneo, Malaysia using yeast. What

is more, it suggests a high potential of production of high-value fat directly from duckweed, using *Mortierella* as a converter.

CHAPTER 1

Characterization of starch-accumulating duckweeds, *Wolffia globosa*, as renewable carbon source for bioethanol production

Abstract

The growth and starch accumulation ability of two types of duckweeds (*Wolffia globosa*), designated as duckweeds J and B, respectively, were investigated under different nutrition conditions using HYPONeX. Both duckweeds J and B grew better in rich nutrient condition (5,000-fold diluted HYPONeX solution) than in poor nutrient condition (80,000-fold diluted HYPONeX solution). In terms of starch accumulation, duckweed J accumulated more starch in the rich nutrient condition, whereas duckweed B accumulated more starch in the poor nutrient condition. In the rich nutrient condition, the dry weight of duckweed J increased by about 5.1 folds and the accumulated starch content was about 22% (w/w) of dry duckweed after one week cultivation. In the poor nutrient condition, the dry weight of duckweed B increased by about 5.0 folds and the accumulated starch content was about 28% (w/w) of dry duckweed after one week cultivation. Furthermore, ethanol production from the duckweeds was investigated using *Saccharomyces cerevisiae* NBRC0224. The most effective pretreatment of duckweeds for ethanol production was treatment with 1% hydrogen peroxide for 1 h, followed by

treatment with 1% sodium hydroxide for 1 h. In the case of the duckweed J, 70 g/L ethanol was produced from 30% (w/v) of the pretreated duckweed with 20 mM urea or 0.1% yeast extract and 30 mM ammonium sulfate. In the case of the duckweed B, 30 g/L ethanol was produced from 30% (w/v) of the non-pretreated duckweed without nitrogen source. In conclusion, the duckweeds, *Wolffia globosa*, were found to be a promising renewable carbon source for the production of third-generation bioethanol.

1. Introduction

Nowadays, there is a global increase in the development of techniques that can produce useful materials from renewable biomass. In particular, owing to the depletion of fossil fuels and global warming, various methods that can produce biofuels from renewable biomass are being established. Bioethanol, a representative biofuel, is produced from different kinds of renewable feedstocks such as foodstuffs, including corn and cassava (first generation), cellulose biomass (second generation), and algal biomass (third generation) (Baeyens et al., 2015; Maity et al., 2014). In addition to first generation bioethanol, various other useful products are also being produced from foodstuffs. Nevertheless, the use of foodstuffs as feedstock may result in the shortage of food for human consumption and an increase in food prices. Moreover, the production of

second-generation bioethanol has disadvantages such as high cost owing to degradation difficulties and utilization of more land area, thus, decreasing the cultivable land available for agriculture. Therefore, in the present study, the author focused on the use of duckweed (*Wolffia globosa*), a freshwater plant that accumulates starch, as a renewable biomass for the production of third-generation biofuels.

The duckweed is the smallest flowering plant that does not have a root and purifies wastewater (Fujita et al., 1999). It thrives well on the surface of fresh waters and accumulates high amount of starch (>50% of dry weight). Because of its ability to accumulate high quantity of starch, the duckweed can not only be used in biofuel production but also in the production of foods and other high value-added materials. In the present study, the author attempted to develop a bioethanol production system with duckweeds using simultaneous saccharification and fermentation (SSF) and very high gravity (VHG) technology, which involved fermentation of considerably high amounts of mashed duckweed powder (>300 g/L) to yield high concentration of ethanol (Pereira et al., 2010; Srichuwong et al., 2009; Yingling et al., 2011).

2. Materials and Methods

2.1. Chemicals

α-Amylase from *Bacillus* sp. was purchased from Sigma–Aldrich (St. Louis, USA). Glucoamylase from *Rhizopus* sp. was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). All of the other chemicals were of analytical grade and were obtained commercially.

2.2. Duckweeds and microorganisms

The duckweeds, *Wolffia globosa*, designated as duckweed B and duckweed J, were obtained from Chitose Laboratory Corp. (Kanagawa, Japan). *Saccharomyces cerevisiae* NBRC0224 was obtained from NITE Biological Resource Center (NBRC, Chiba, Japan).

2.3. Cultivation media

The medium for the cultivation of duckweeds was diluted HYPONeX stock solution 6-10-5 (N (6%), P (10%), K (5%), Mg (0.05%), Mn (0.001%), and B (0.005%)). HYPONeX stock solution 6-10-5 was purchased from HYPONeX JAPAN (Osaka, Japan). The medium for the preculture of *Saccharomyces cerevisiae* comprised 20 g/L peptone, 10 g/L yeast extract, and 20 g/L glucose.

2.4. Cultivation of duckweeds

To prepare the stock culture of duckweeds, duckweed B was cultivated in 80,000-fold diluted HYPONeX solution and duckweed J was cultivated in 5,000-fold

diluted HYPONeX solution. Subsequently, these stock cultures were transferred to freshly diluted HYPONeX solution every 2 weeks, and contaminants, including algae, in the culture were removed using a mesh sieve. For starch accumulation test, 200 mg of wet duckweed J (dry weight, 12 mg) or duckweed B (dry weight, 8 mg) from the stock culture were inoculated into 100 mL of 5,000-fold diluted HYPONeX solution, 80,000-fold diluted HYPONeX solution, or Milli-Q water and cultivated at 30°C for 1 week.

2.5. Harvest and pretreatment of duckweeds

For starch accumulation analysis, the duckweeds cultivated in 80,000-fold diluted HYPONeX solution for 2 weeks were harvested using 212-μm mesh sieve. The collected duckweeds were washed using tap water, dried at 60°C, and powdered using mortar. The powdered duckweeds were treated with 1% sodium hydroxide and 1% hydrogen peroxide for 12 h (Mishima et al. 2008; Mishima et al. 2006; Srichuwong et al. 2010). Subsequently, the treated duckweed powder was collected, washed with tap water using 38-μm mesh sieve until the pH of the lavage fluid became neutral, dried at 60°C, and then powdered using mortar.

2.6. Comparison of pretreatment conditions

Fermentation tests were performed with duckweed J pretreated under different conditions. The pretreated duckweed J was converted into glucose using α-amylase and

glucoamylase (Choi et al., 2010). The reaction mixtures comprised filter-sterilized α-amylase (18 U/g duckweed powder), glucoamylase (100 U/g duckweed powder), and 50 mM sodium citrate buffer (pH 5.0). The reaction was conducted at 60°C for 2 h. Subsequently, the reaction mixtures were cooled to room temperature and *S. cerevisiae* preculture was added to them. The preculture of *S. cerevisiae* was prepared by cultivating the organism in YPD medium at 28°C for 18 h and then washing the culture twice with 0.9% (w/v) NaCl.

2.7. SSF

Non-pretreated or pretreated dry duckweed powder with high amount of accumulated starch (about 30% (w/w)) was dispensed into 50 mM sodium citrate buffer (pH 5.0) containing 20 mM urea, 30 mM ammonium sulfate, or 0.1% yeast extract and 30 mM ammonium sulfate. To gelatinize the starch accumulated in the duckweeds, filter-sterilized α-amylase (18 U/g duckweed powder) was added to the mixture. The reaction was conducted at 60°C for 2 h. After the reaction, *S. cerevisiae* preculture (prepared as described above) and glucoamylase (100 U/g duckweed powder) were added to the mixture to commence SSF and performed at 30°C.

2.8. Analytical techniques

The ethanol and sugar contents were measured using Shimadzu (Kyoto, Japan)

HPLC equipped with an Aminex Fermentation Monitoring Column (Bio-Rad Laboratories, Hercules, CA, USA) and refractive-index detector. Elution was achieved at 60°C with 5 mM hydrogen sulphate at a flow rate of 0.6 mL/min. The concentrations of ethanol and glucose were calculated from the peak areas.

3. Results and Discussion

3.1. Growth of duckweeds and their starch accumulation ability

The effects of nutritional condition on the growth of duckweeds and their starch accumulation ability were investigated using diluted HYPONeX solution and Milli-Q water (Fig. 1). Both duckweeds J and B exhibited high growth rate when cultivated in rich nutrient condition (5,000-fold diluted HYPONeX solution). In case of duckweed J, the total starch accumulation depended on the growth rate, and higher amount of starch (13.5 mg) was noted in samples cultured in 5,000-fold diluted HYPONeX solution than that noted in samples cultivated in 80,000-fold diluted HYPONeX solution and Milli-Q water (Fig. 1A). Conversely, in case of duckweed B, the total starch accumulation did not depend on the growth rate, and higher amount of starch (11.4 mg) was observed in samples cultured in 80,000-fold diluted HYPONeX solution than that observed in samples incubated in 5,000-fold diluted HYPONeX solution and Milli-Q water (Fig. 1B).

This could be probably because of the harvesting of duckweed B cultured in 5,000-fold diluted HYPONeX solution before starch accumulation caused by nitrogen depletion. It is believed that duckweeds J and B exposed to poor nutrient condition could have accumulated starch as a survival strategy via a mechanism similar to glycogen accumulation in *Arthrospira platensis* under condition of nitrogen depletion (Hasunuma et al., 2013). Thus, it can be concluded that duckweed B is more suitable for starch production than duckweed J owing to the higher

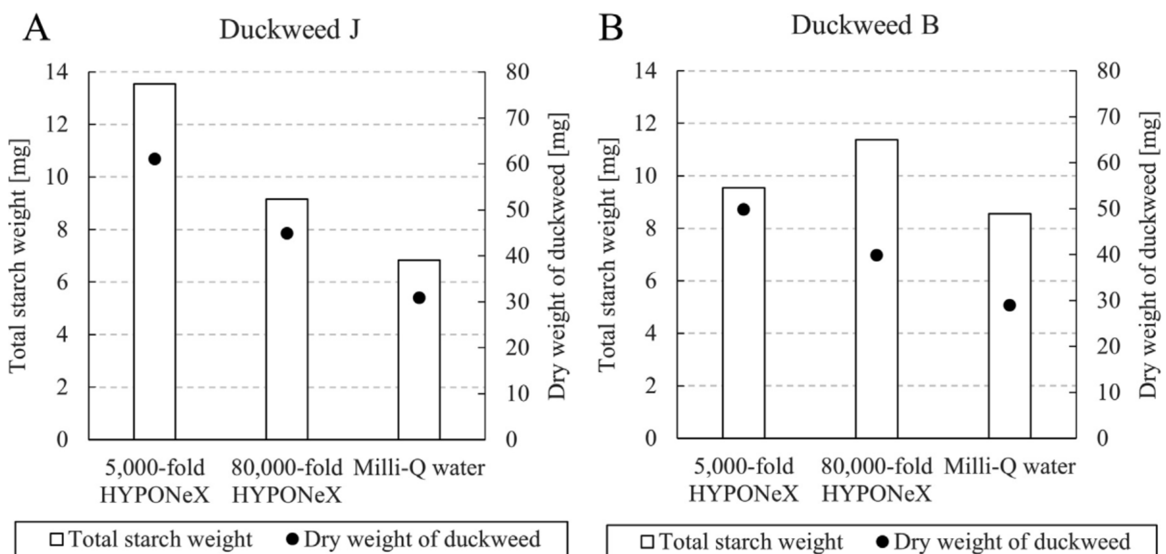

Fig. 1-1. Total starch weight of (A) duckweed J and (B) duckweed B. Two hundred mg of wet duckweed J (12 mg dry weight) or B (8 mg dry weight) from stock culture was inoculated into 100 mL of 5,000-fold diluted HYPONeX, 80,000-fold HYPONeX, or milli-Q water and cultivated at 30°C for 1 week.

amount of starch accumulation under nitrogen depletion condition (80,000-fold diluted HYPONeX solution).

3.2. Comparison of pretreatment conditions

The pretreatment conditions for duckweeds were examined using sodium hydroxide and hydrogen peroxide. The effects of pretreatment on the saccharification efficiency of the duckweeds are shown in Fig. 2. When the duckweeds were pretreated with condition 6, the saccharification efficiency was defined as 100%. When the duckweeds were pretreated with conditions 1, 2, and 3, the relative efficiency of saccharification was 87%, 90%, and 88%, respectively. When the duckweeds were

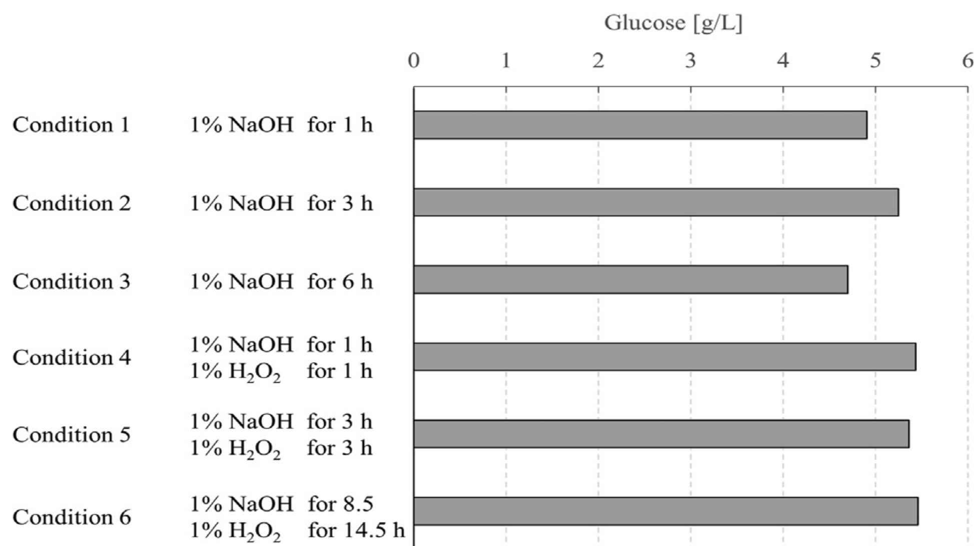

Fig. 1-2. Effects of pretreatment on saccharification efficiency. The powdered duckweeds were reacted under six different conditions and hydrolyzed by α-amylase and glucoamylase.

pretreated with conditions 4 and 5, the relative efficiency of saccharification was 99% and 98%, respectively.

Fermentation test was performed using duckweeds pretreated under different conditions. The final ethanol concentration obtained following fermentation using duckweeds that were pretreated with condition 6 was 2 g/L (data not shown). Therefore, it can be presumed that pretreatment conditions 4 and 5 did not inhibit ethanol fermentation by *S. cerevisiae* (Fig. 3). These findings suggested that the duration of pretreatment is not critical for saccharification by duckweeds, but pretreatment in combination with sodium hydroxide and hydrogen peroxide is important. Based on

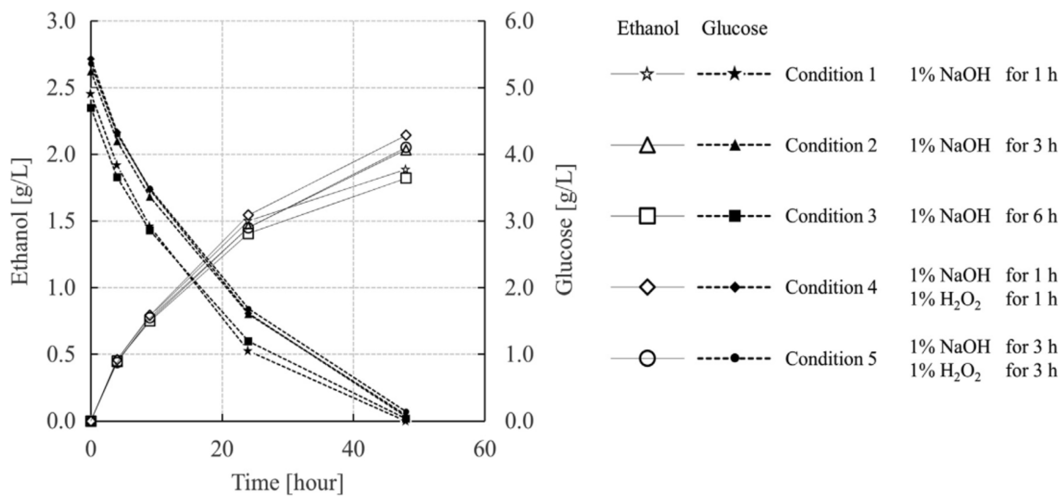

Fig. 1-3. Effects of pretreatment on ethanol production. Duckweed J was pretreated under condition 1 (stars), condition 2 (triangles), condition 3 (squares), condition 4 (diamonds), or condition 5 (circles) and used for ethanol fermentation tests with *S. cerevisiae* NBRC0224. Open and closed symbols represent ethanol and glucose concentration, respectively.

these results, it can be concluded that pretreatment condition 4 may be more suitable to

achieve high saccharification efficiency in duckweeds.

3.3. Bioethanol production by SSF of VHG duckweeds

Figure 4 shows the results of SSF using duckweed J. Maximum ethanol

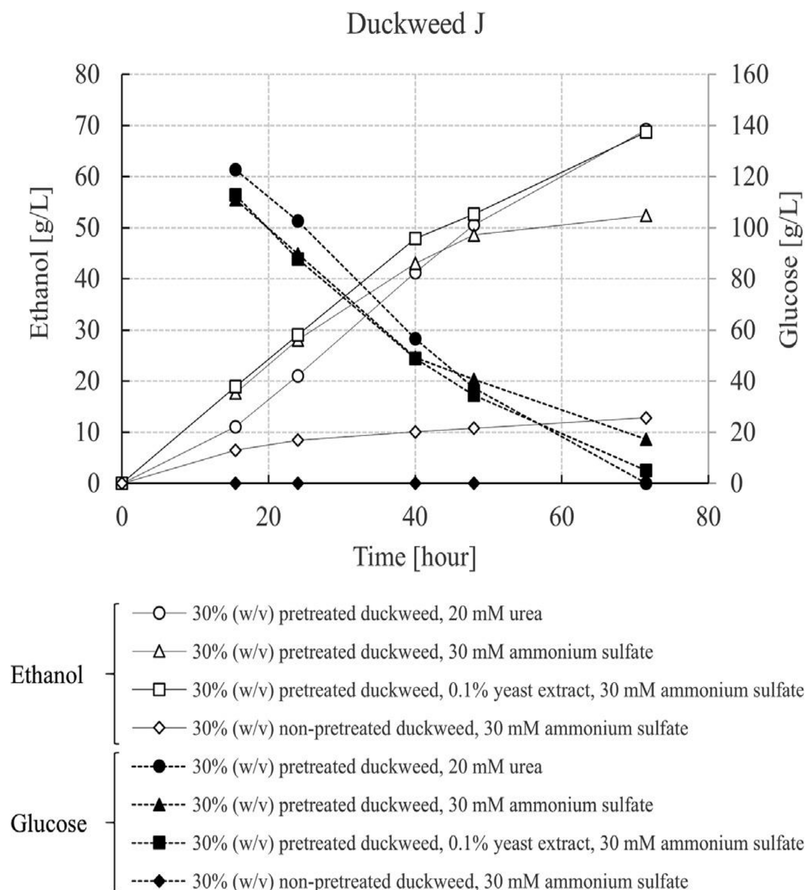

Fig. 1-4. Ethanol production from duckweed J by SSF. SSF was performed under four different conditions such as 30% (w/v) pretreated duckweed and 20 mM urea (circles), 30% (w/v) pretreated duckweed and 30 mM ammonium sulfate (triangles), 30% (w/v) pretreated duckweed, 0.1% yeast extract, and 30 mM ammonium sulfate (squares), and 30% (w/v) non-pretreated duckweed and 30 mM ammonium sulfate (diamonds). Open and closed symbols represent ethanol and glucose concentrations, respectively.

concentration(69g/L) was obtained when SSF was performed using a combination of

30% (w/v) pretreated duckweed J, 20 mM urea or 0.1% yeast extract, and 30 mM ammonium sulfate. Conversely, the ethanol concentration was low (13 g/L) when SSF was performed using non-pretreated duckweed J.

Figure 5 presents the findings of SSF using duckweed B. Ethanol production was inhibited when pretreated duckweed B was used as the carbon source. However, when SSF was performed using 30% (w/v) non-pretreated duckweed B and 30 mM ammonium sulfate, maximum ethanol production (34 g/L) and yield were achieved. Furthermore, 32 g/L ethanol was obtained from non-pretreated duckweed B without nitrogen source and with 20 mM urea. These results suggested that pretreatment of duckweed B is not necessary for ethanol production. Nevertheless, ethanol production using non-pretreated duckweed B stopped at 30 g/L compared with that using duckweed J (69 g/L), which may be because of the incomplete hydrolysis of starch by α-amylase and glucoamylase in the non-pretreated duckweed B. Despite the advantages such as not requiring pretreatment and a nitrogen source for use in SSF, duckweed B must be examined further to increase its ethanol production.

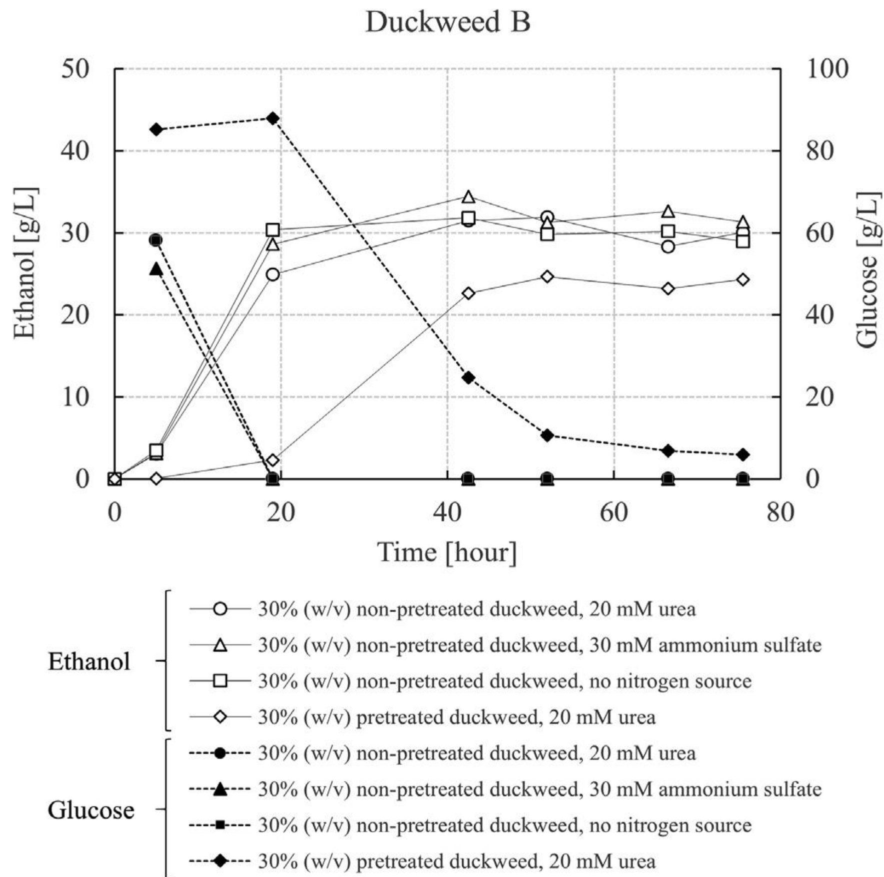

Fig. 1-5. Ethanol production from duckweed B by SSF. SSF was performed under four different conditions such as 30% (w/v) non-pretreated duckweed and 20 mM urea (circles), 30% (w/v) non-pretreated duckweed and 30 mM ammonium sulfate (tri-angles), non-pretreated duckweed without nitrogen source (squares), and 30% (w/v) pretreated duckweed and 20 mM urea (diamonds). Open and closed symbols represent ethanol and glucose concentrations, respectively.

4. Conclusion

The optimal medium composition for the growth of duckweeds J and B and starch accumulation was 5,000- and 80,000-fold diluted HYPONeX solution, respectively. Furthermore, pretreatment of duckweeds with 1% sodium hydroxide for 1 h, followed by

1% hydrogen peroxide for 1 h was found to be most suitable. When SSF was performed with 30% (w/v) pretreated duckweed J, 20 mM urea or 0.1% yeast extract, and 30 mM ammonium sulfate as nitrogen source, ethanol production reached 69 g/L after 70 h. Conversely, when SSF was conducted with 30% (w/v) non-pretreated duckweed B without nitrogen source, ethanol production reached 30 g/L after 70 h. The ethanol concentration in the case of duckweed J were higher than that in the case of Landoltia punctate (30.8 g/L) (Chen et al., 2012). Thus, duckweeds, rich in starch, are a promising renewable carbon source for bioethanol production. In addition, they can be further exploited for use as a foodstuff and starting material for obtaining valuable fermentation products.

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CHAPTER 2

Essential fatty acids for oleaginous fungus *Mortierella alpina*

Abstract

Oleaginous fungus *Mortierella alpina* 1S-4 accumulates arachidonic acid (20:4ω6) as a major polyunsaturated fatty acid. However, 20:4ω6 is not essential for the growth of *M. alpina* 1S-4, because various types of mutants that produce no 20:4ω6 were isolated. *M. alpina* JT-180 as a Δ6 desaturation-defective mutant that was derived from *M. alpina* 1S-4 by treatment of a chemical mutagen, accumulates Mead acid (20:3ω9) instead of 20:4ω6. *p*-Anisidine was found to be a Δ6 desaturation inhibitor in this study. The final concentration 0.1 mg/mL of *p*-anisidine had an inhibitory effects on growth of *M. alpina* JT-180. The addition of *p*-anisidine to the medium caused *M. alpina* JT-180 to produce only monoenoic acids such as oleic acid (18:1ω9) and eicosenoic acid (20:1ω9) as unsaturated fatty acids. The effects of exogenous fatty acids were investigated when *M. alpina* JT-180 was cultivated in the medium containing *p*-anisidine. The addition of linoleic acid (18:2ω6) and 6*Z*,9*Z*-octadecadienoic acid (18:2ω9) restored the growth of *M. alpina* JT-180 cultivated on the medium containing *p*-anisidine, but palmitic acid (16:0), 18:1ω9, and vaccenic acid (18:1ω7) had no effect. For the growth of oleaginous fungus *M. alpina*, 18-carbon length fatty acid with more

than two double bonds were considered to be essential.

1. Introduction

Filamentous fungus, *Mortierella alpina* (*M. alpina*) 1S-4, has been reported as a potential producer of single cell oil. The strain is now used for large-scale production of triacylglycerol containing arachidonic acid (20:4ω6) (Yamada et al., 1987; Sakuradani et al., 2013). This fatty acid is the most abundant C20 polyunsaturated fatty acid (PUFA) in humans, and not only exhibits various regulation effects and physiological activities but also plays important roles in infant nutrition (Carlson et al., 1993; Gill and Valivety 1997). However, the physiological function of 20:4ω6 for this fungus remains unclear. Thus far, various mutants with different fatty acid composition have been isolated from wild strain *M. alpina* 1S-4 by means of chemical mutagenesis (Jareonkitmongkol et al., 1992a). *M. alpina* JT-180 and *M. alpina* S14 are Δ12 fatty acid desaturation-defective and Δ5 fatty acid desaturation-defective mutants which accumulate mead acid (20:3ω9) and dihomo-γ-linolenic acid (20:3ω6), respectively. Neither mutants produces no 20:4ω6, which means that 20:4ω6 is non-essential for growth of *M. alpina* strains.

A different way to change the fatty acid composition in *M. alpina* is to use

inhibitors for fatty acid desaturation. We have reported three types of inhibitors i.e., lignan compounds (Shimizu et al., 1991), alkyl gallate derivatives (Kawashima et al., 1996a), and curcumin derivatives (Kawashima et al., 1996b). The lignin compounds in sesame seeds and oil are specific inhibitors of Δ5 desaturase, which catalyzes the conversion of 20:3ω6 to 20:4ω6 (Shimizu et al., 1991). Alkyl gallate derivatives, which are known to be antioxidants, show inhibitory effects on Δ6 desaturase as well as Δ5 desaturase (Kawashima et al., 1996a). The curucumin derivatives, which include the main component of the yellow spice turmeric, show a different inhibitory effect on Δ5 desaturase and a weak one on Δ6 desaturase (Kawashima et al., 1996b). In addition, 2,2-diphenyl-5-(4-{[(1*E*)-pyridin-3-yl-methylidene]amino}piperazin-1-yl)pentanenitrile (SC-26196) inhibits the Δ6 desaturation in isolated rat liver microsomes (Harmon et al., 2003). Treatment of the microalga *Porphyridium cruentum* with salicylhydroxamic acid (SHAM) inhibits growth and affects fatty acid composition due to Δ6 fatty acid desaturation (Khozin-Goldberg et al., 1999).

In this research, the author found a new Δ6 desaturation inhibitor, 4-methoxyaniline (*p*-anisidine), for *M. alpina* strains (Fig. 1). The author suggests essential fatty acid candidates for *M. alpina* strains based on comparison of growth and fatty acid composition between the wild strain *M. alpina* 1S-4 and Δ12 desaturation-

defective mutant JT-180 cultivated in the medium containing *p*-anisidine.

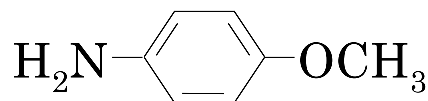

Fig. 2-1. Chemical structural formula of *p*-anisidine.

2. Materials and Methods

2.1. Chemicals

p-Anisidine was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Fatty acid methyl esters were purchased from Funakoshi (Tokyo, Japan). 6*Z*,9*Z*-octadecadienoic acid (18:2ω9) methyl ester were purified from fatty acid methyl esters prepared from microbial oil of Δ12 desaturation defective mutant *M. alpina* JT-180 (Sakuradani et al., 2003). All other reagents were of analytical grade.

2.2. Microorganisms and cultivation

M. alpina 1S-4 (wild strain) and its derivative mutant JT-180 were cultivated on a medium consisting of 2% glucose and 1% yeast extract (pH 6.0) with *p*-anisidine, as indicated, at 28°C with reciprocal shaking at 120 rpm for 7 d.

2.3. Fatty acid analysis

Mycelial cells were harvested by suction filtration, washed with water and then

dried at 100°C for 2 h for subsequent fatty acid analysis by gas-liquid chromatography after transmethylation with methanolic HC1 as described previously (Shimizu et al. 1988). Desaturation activity was expressed as the 'desaturation index', which is the ratio of the amount of the substrate of desaturase to that of the product and further metabolites. For example, Δ6 desaturation activity was expressed as the A6 desaturation index, i.e., the ratio of 18:2ω6 to γ-linolenic acid (18:3ω6) + 20:3ω6 + 20:4ω6.

3. Results and Discussion

3.1. Inhibition of fatty acid desaturase in M. alpina by p-anisidine

Δ6 desaturase activity was defined as a desaturase index. i.e., the ratio of 18:2 to 18:3ω6 + 20:3ω6 + 20:4ω6 in total mycelial fatty acids, since the desaturation reaction is not reversible and 20:4ω6 is not further metabolized in the fungus (Yamada et al., 1987; Sakuradani et al., 2013). Therefore, the desaturation index increases when the desaturase activity is reduced and the substrate 18:2ω6 for Δ6 desaturase is not consumed by Δ6 desaturation. Table 1 shows that *p*-anisidine supplementation caused increases in stearic acid (18:0), oleic acid (18:1ω9), and (18:2ω6), and decreases in 20:3ω6 and 20:4ω6. Addition of *p*-anisidine to the culture medium is considered to cause Δ6 desaturation inhibition through an increase in the Δ6 desaturase index. Similarly, the

supplementation of methyl *p*-coumarate and propyl gallate caused the inhibition Δ6 desaturase activities (Kawashima et al., 1996b). On the other hand, the supplementation of (+)-sesamine, which is the specific inhibitor of Δ5 desaturase, hardly influenced the fatty acid composition, except 20:3ω6 and 20:4ω6 and caused a remarkable decrease in only the Δ5 desaturase index (Shimizu et al., 1991).

Table 2-1
Effect of p-anisidine on Δ6 desaturase activity of *M. alpina* 1S-4 [a]

Inhibitor	Fatty acid composition (%) [b]								Δ6 Desaturase index [c]
	16:0	18:0	18:1ω9	18:2ω6	18:3ω6	20:3ω6	20:4ω6	others	
None	16.7	8.5	20.6	11.1	7.1	5.6	20.3	10.3	0.34
p-Anisidine	9.5	15.7	23.5	16.5	8.0	2.3	11.1	13.4	0.77

[a] *M. alpina* 1S-4 was grown in a medium containing 2% glucose and 1% yeast extract (pH 6.0) supplemented with *p*-anisidine (0.1 mg/mL).
[b] Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; 20:3ω6, dihomo-γ-linolenic acid; 20:4ω6, arachidonic acid.
[c] Δ6 Desaturase index, ratio of 18:2ω6/(18:3ω6 + 20:3ω6 + 20:4ω6) in total mycelial fatty acids.

3.2. Inhibitory effect of p-anisidine on growth of M. alpina mutants

The inhibitory effects of *p*-anisidine on the growth of various *M. alpina* mutants

on the agar medium were investigated. No inhibitory effect of *p*-anisidine was observed on the cultivation of Δ6 desaturation-defective mutant Mut49 (Jareonkitmongkol et al., 1993b), Δ5 desaturation-defective mutant S14 (Jareonkitmongkol et al., 2002), and Δ9 desaturation-defective mutant T4 (Jareonkitmongkol et al., 2002), accumulated 18:2ω6, 20:3ω6, 20:4ω6 and 18:0, respectively. On the other hand, the growth of Δ12 desaturation-defective mutants Mut48 and JT-180 (Jareonkitmongkol, 1992b; Sakuradani et al., 2003) were definitely inhibited on the agar medium containing *p*-anisidine. *p*-Anisidine (0.1 mg/mL) showed a low inhibitory effect on the growth of the wild strain (*M. alpina* 1S-4), but a significant inhibitory effect on the growth of JT-180. Strain JT-180 biosynthesizes several PUFAs such as 18:2ω9, 8*Z*,11*Z*-eicosadecadienoic acid (20:2ω9), and 20:3ω9 on the cultivation in the medium without *p*-anisidine. However, the addition of Δ6 desaturation-inhibitor *p*-anisidine prevented JT-180 from biosynthesizing such PUFAs (Fig. 2). Strain JT-180 accumulated 18:1ω9 and eicosenoic acid (20:1ω11) as unsaturated fatty acids on the cultivation in the medium containing *p*-anisidine through the route as shown in Fig. 3. Other Δ6 desaturation-inhibitors such as propyl gallate, sufrole, and methyl *p*-coumarate similarly prevented the growth of JT-180 (data not shown).

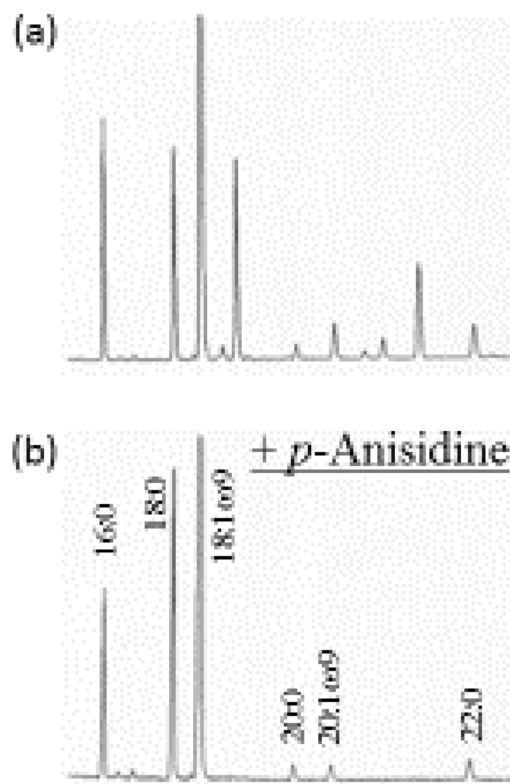

Fig. 2-2. GC chromatograms of the fatty acid methyl esters obtained from *M. alpina* JT-180 cultivated in a medium without (a) or with (b) *p*-anisidine.
Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 20:0, arachidic acid; 20:1ω9, eicosenoic acid; 20:2ω9, 8Z,11Z-eicosadecadienoic acid; 20:3ω9, mead acid; 22:0, behenic acid.

Fig. 2-3. Saturated and unsaturated fatty acid biosynthetic pathways in *M. alpina* JT-180.

p-Anisidine inhibited Δ6 desaturation in this pathways.
Abbreviations used are the same as those given in Fig. 2.

3.3. Effect of the supplementation of various fatty acids on the growth of M. alpina JT 180 mutant

The effects of addition of a variety of fatty acids on the growth of JT-180 on the agar medium containing *p*-anisidine were investigated as shown in Fig. 4. Control strain *M. alpina* 1S-4 formed colonies on the agar medium containing *p*-anisidine, which were similar to colonies on the medium without *p*-anisidine (Fig. 4). The growth of JT-180 was restored by the addition of 16:1ω7, 18:2ω6, or 18:2ω9. No effect of the addition of 16:0, 18:1ω9 or 18:1ω7 was observed on the growth of JT-180 on the agar medium

containing *p*-anisidine. The total fatty acid composition of strains

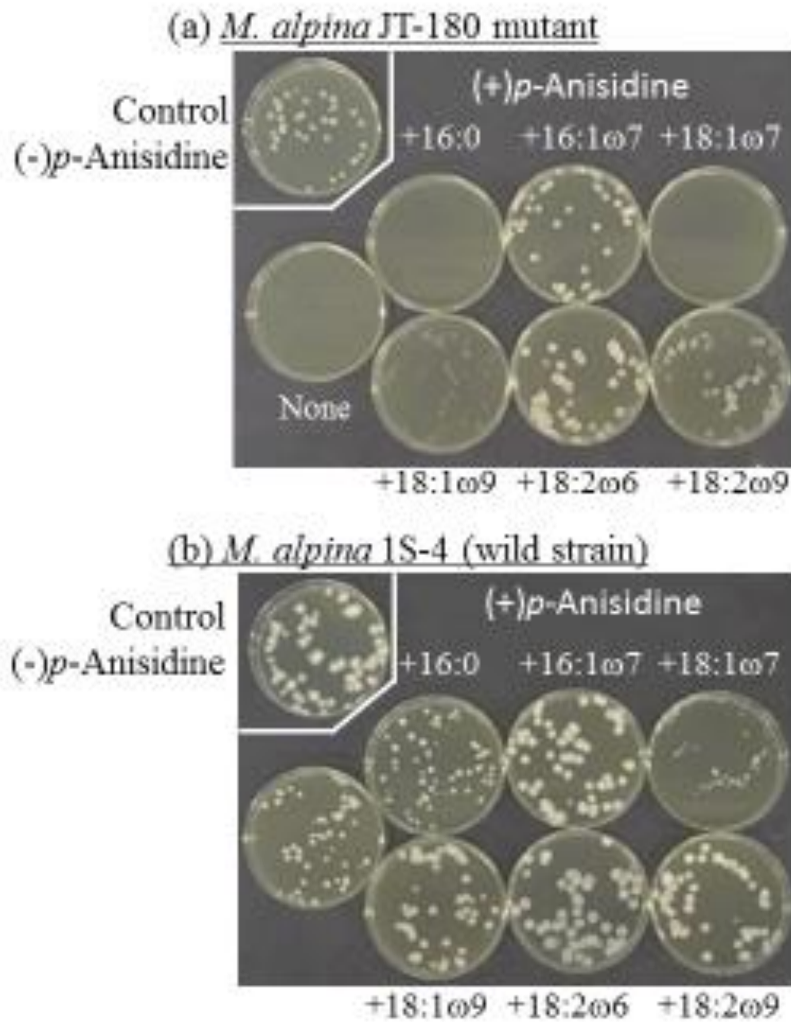

Fig. 2-4. Comparison of growth of *M. alpina* JT-180 (a) and wild strain *M. alpina* 1S-4 (b).

The strains were cultivated in agar medium containing 2% glucose, 1% yeast extract, and 0.01% *p*-anisidine (pH 6.0).

that were cultivated on the conditions of the addition of fatty acids to the agar medium were analyzed. Wild strain *M. alpina* 1S-4 accumulated 20:4ω6 as a major PUFA regardless of the type of the added fatty acids. The level of 20:4ω6 in the total fatty acids from *M. alpina* 1S-4 under the condition of the addition of 18:1ω7 was lowest among tested strains, which might mean that the addition of 18:1ω7 caused the slight inhibition of the growth of the wild strain. The addition of 18:2ω6 or 18:2ω9 to the medium induced JT-180 to accumulate 20:4ω6 or 20:3ω9, respectively. The melting points of 18:1ω7 and 18:1ω9 are 14°C and 13°C, respectively. On the other hand, the melting points of 18:2ω6, 18:3ω6, 20:4ω6 are -5°C, -11°C, and -50°C, respectively. *M. alpina* strains grow well at between 4-28°C. PUFAs that have more than two double bonds in their structure are considered to be essential for the growth of *M. alpina*.

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Perspective

The feasibility of water treatment technology using duckweeds has been proven ; duckweeds consume nitrogen and phosphorus contained in waste organic water emitted from palm plantations. However, alleviation of influence on environment by removing such organic substance will not be sufficient to motivate owners of plantations in Malaysia and Indonesia to implement the system; financial motivation is necessary. In other words, it is crucial to find high value-added applications that use a massive amount of duckweeds.

Our team has proved the feasibility of the system that uses duckweeds as a source for feed and fertilizer. This paper has investigated the possibility of utilizing carbohydrate extracted from duckweeds as fermented raw material for food.

In chapter 1, *Wolffia globosa*,a type of duckweed rich in hydrocarbon, is proven to be fermented. *Wolffia globosa* produced a high yield of ethanol based on sugar by simultaneous saccharifying fermentation.

In chapter 2, further research to turn duckweeds to high value-added products is conducted, that is, the possibility of producing unsaturated fatty acid, which is projected to be in high demand in the near future. The result suggests that combinations of gene-deficient strains and inhibitory substances play vital roles in producing various types of unsaturated fatty acid.

Further research on targeting a specific industrially important unsaturated fatty acid using duckweed as a raw material will be conducted in accordance to the state of progress of commercialization of water treatment technology.

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