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Effects of Wastewater Strength and Salt Stress on Microalgal Biomass Production and Lipid Accumulation

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***Abstract*—**This work aims to investigate a potential of microalgae for utilizing industrial wastewater as a cheap nutrient for their growth and oil accumulation. Wastewater was collected from the effluent ponds of agro-industrial factories (cassava and ethanol production plants). Only 2 microalgal strains were isolated and identified as *Scenedesmus quadricauda* and *Chlorella* sp.. However, only *S. quadricauda* was selected to cultivate in various wastewater concentrations (10%, 20%, 40%, 60%, 80% and 100%). The highest biomass obtained at 6.6×10^6 and 6.27×10^6 cells/ml when 60% wastewater was used in flask and photo-bioreactor. The cultures gave the highest lipid content at 18.58 % and 42.86% in cases of *S. quadricauda* and *S. obliquus*. In addition, under salt stress (1.0 M NaCl), *S. obliquus* demonstrated the highest lipid content at 50% which was much more than the case of no NaCl adding. However, the concentration of NaCl does not affect on lipid accumulation in case of *S. quadricauda.*

***Keywords*—**Cassava wastewater, cultivation, lipid accumulation, microalgae

I. Introduction

NOWADAYS, the consumptions of energy around the world are increase. It causes the cost of fuel is more expensive. Moreover, petroleum fuel has the bad results for the environment especially air pollution and global warming which it has become an important environmental concern [1]. Therefore, the use of others energy such as ethanol, natural gas and biodiesel could be alternative choices to reduce the petroleum fuel demand.

Biodiesel has received much attention in recent years. It is the renewable energy and has a high potential to replace diesel fuel without causing damage of the engine. It was produced from vegetable oils, animal fats and used cooking oil via transesterification reaction. Generally, biodiesel made from vegetable oil are better than animal fat in quality. Since, it contains more saturated fatty acid that resulting to low iodine value in biodiesel and reduce polymerization reaction cause of stickiness in fuel. So, biodiesel made from vegetable oils have been lubrication property above animal fats [2]. However, vegetable oils are also use for human consumption, causing the argument to use these vegetable oils for food or energy.

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Moreover, oils crops are needed more area for cultivation and take long time to grow. Afterward, the scientists were found some microalgae strains had the lipid component in their cell called as microalgal oil. This oil can be used as a raw material for biodiesel production like vegetable oils. In the optimal condition the microalgae can accumulated the lipids in their cells more than protein carbohydrate or other components. Compared with other plant oils, the microalgae have a short growth, needed small area and high lipid content.

II. Materials and Methods

A. Collection, Isolation, and Identification of microalgae

The wastewater samples from agricultural manufactories located in the northeast of Thailand (Cassava Starch production, Kalasin province and Alcoholic beverage production, Khon Kaen province were collected by using 60 μm plankton net. Then, the collected samples were added into 250 mL conical flask containing 100 mL of Blue-Green 11 Medium (BG-11 medium) [3]-[5] for enrichment of the algal cells for7 days.

Then, it was picked to isolate by Manipulative method under optical microscope (Primo Star, Carl Zeiss, and Germany) and the single cell was obtained. Two isolates were identified their morphology under microscope observation and certified by Thailand Institute of Science and Technology Research (TISTR).

After enrichment, microalgal cells were considered on the lipid accumulation in their cells by Sudan Black B staining [6] and lipid extraction following the method explained by Folch [7]. Only, the strain that gives the highest lipid accumulation will be selected to further study.

B. Starter preparation

After screening and enriching the microalgae, the algal biomass (20% inoculums) as a seed starter [8] was added into 100 mL BG-11 medium in 250 mL Erlenmeyer flask. The flasks were incubated on orbital shaker (140 rpm) with the light: dark cycle (16:8 h) [9] and controlled temperature of 28±2°C.

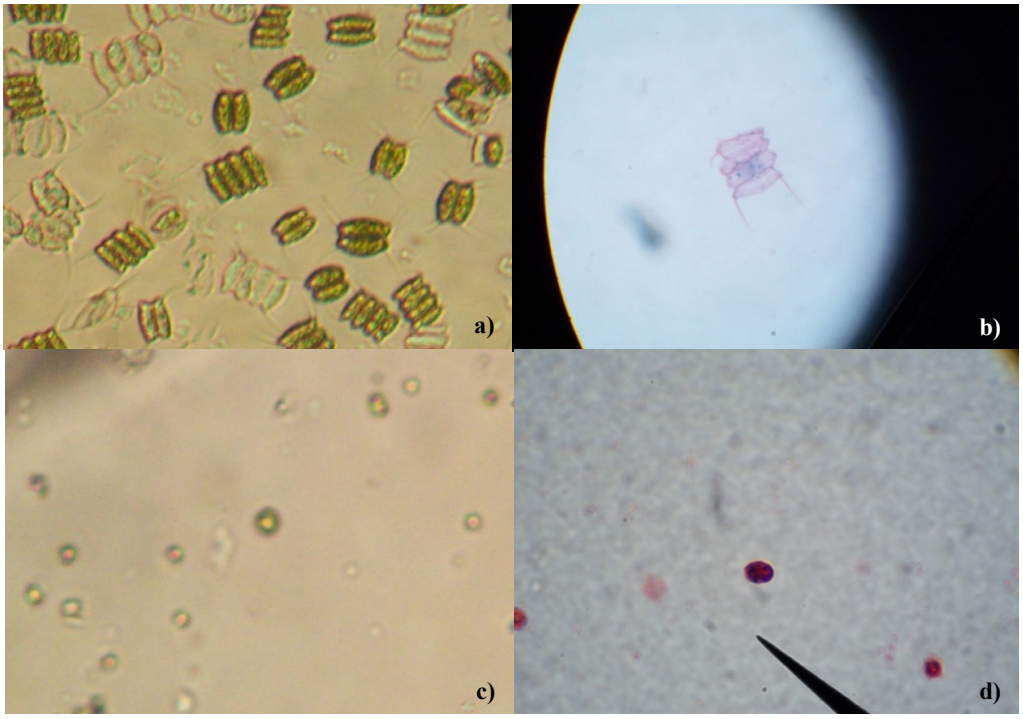

Fig. 1 Morphology of isolated microalgal strains; a) and c) the morphology of isolate I and isolate II, c) and d) isolate I and isolate II after staining by Sudan Black B

C. Characterization of cassava wastewater

The cassava wastewater, used as a nutrient for growth cultures throughout this study, was collected from effluent pond. In this case, the wastewater was primary treated by Upflow Anaerobic Sludge Blanket (UASB). The Chemical Oxygen Demand (COD) showed in a range 1,000-1,500 mL^{-1}. However, to obtain the desired values, variations of wastewater concentration were diluted with distilled water (10% 20% 40% 60% 80% and 100%). Before cultivation, the COD and Total Nitrogen (TN) in each concentration were measurement.

D. Cultivation of microalgae in cassava wastewater

The 6 variations of cassava wastewater were applied as substrate for microalgae cultivation. The experiments were performed in both flask scale and photobioreactor.

1) Flask scale

A volume of 300 mL wastewater in each 6 dilution levels were separately added into 500 mL Erlenmeyer flask and 100 % distilled water was used as a control set. Then, the microalgal starter (20% inoculums containing of algal biomass at 1×10^6 cells/ml) [10] was added into the culture flask. Air supply was used via air pump with a flow rate of 2.75 $Lmin^{-1}$ and illumination was also applied with an intensity of 4 klux. The duration of light: dark cycles was also controlled at 16:8 h under temperature at about 28-30°C. The culture was withdrawn to monitor growth rate every 24 h.

2) Photobioreactor

Only three dilutions of 40%, 60% and 80% wastewater obtained from flask scale were used to extend the cultivation in a large scale of 3glass tubular photo-bioreactors containing 2 L working volume. In addition, the distilled water was used as a control set. Then, the cultivation was performed by adding microalgae with 20% inoculums that contained of 1×10^6 cells/ml biomass. The controlled condition of airflow rate at 2.75 $Lmin^{-1}$ and supply with 7.5 Klux illumination at 16:8 h light: dark cycles under temperature at about 29-35°C. The culture was withdrawn to monitor growth rate every 24 h.

E. Optimal condition for lipid accumulation in microalgal cell

Only the optimal wastewater concentration obtained from flask and photo-reactor was used to further investigate lipid accumulation under different culture conditions (heterotrophic and mixotrophic cultures) and salt stress [11]. In this study, the reference strain of *Scenedesmus obliquus* [12], obtained from Thailand Institute of Scientific and Technological Research (TISTR), was used to compare the lipid accumulation with the highest oil accumulating isolated strain.

The cultivated condition for both strains were carried out that was explained as previous experiment. After cultivation until 6 days and 10 days for *S. obliquus* and *S. quadricauda,* the highest oil accumulating isolated strain (early stationary phase), the illumination will be stopped. To obtain the culture condition as heterotrophic system, a black plastic bag was covered photo-bioreactor to control as a dark condition. In

case of mixotrophic cultivation, the 16:8 h light: dark cycles were continually supplied. In Both culture conditions were carried out in duplication. The samples were withdrawn every 5 day (during stress period) for measurement of the lipid content.

The optimal culture condition that gave the highest lipid content was chosen to investigate the effect of salt stress. The concentration of NaCl was varied in 3 levels (0.3 M, 0.6 M and 1.0 M NaCl). The desired salt concentrations were added at early stationary phase. In the same way, the cultures were withdrawn every 5 day for measurement of the lipid content.

F. Analytical technic

Growth of starter was measured by optical density (T60 V spectrophotometer, PG Instruments limited, United Kingdom) at wavelength 665 nm, cell count was determined by haemacytometer (BOECO, Germany) and dry cell weight was done following [8]. Growth of microalgae cultivated in cassava wastewater was measured by haemacytometer and specific growth rate [13]. COD and Total N followed by APHA, AWWA [14]. For lipid measurement was modified from Folch method [7].

III. Results and Discussion

A. Isolation and Identification of microalgae

Only 2 microalgal strains were isolated from effluent pond of cassava starch production wastewater treatment plant and coded as Isolate I and Isolate II. The morphology of Isolate I is a flat colony, ovoid or cylindrical in shape and formed in single row or two rows in lateral contact (See Fig.1 a). Meanwhile, Isolate II showed a small green cell with spherical or ellipsoidal shape (See Fig.1 c). However, both isolated were identified by observation of morphology under microscopic technique and certified by Thailand Institute of Science and Technology Research (TISTR). Table I shows the result of both isolated strains identification

However, when both microalgal strains were estimated lipid accumulation by Sudan Black B staining. In Fig. 1 b) and d) showed the Isolate I contained of lipid in its cell that was more than the Isolate II (See the black point in their cell). Furthermore, after the Folch's method was used for lipid extraction in both isolated strains to confirm the lipid accumulation in their cell. The result showed that, the lipid content obtained from *S.quadricauda* (6.47%) was higher than *Chlorella sp*. (2.67%) as shown in Fig.2. Thus, only the Isolate I was selected to further study.

TABLE I
Microalgal strain identification

Code	Microalgal strain
Isolate I	*Scenedesmus quadricauda*
Isolate II	*Chlorella sp.*

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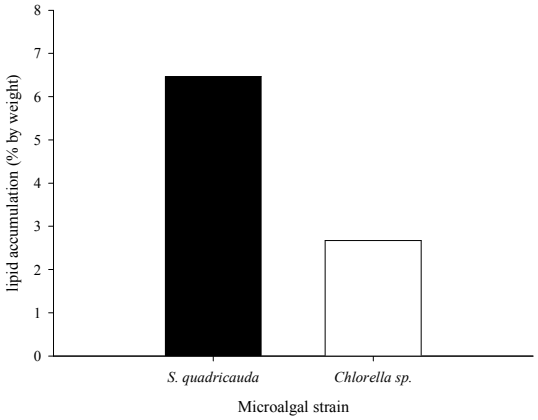

Fig. 2 Lipid accumulation in *S.quadricauda* and *Chlorella sp*

B. Cassava wastewater characteristics

Cassava wastewater was varied at 6 strengthen levels (20%, 40%, 60%, 80% and 100%) to cultivate microalgae. It noted that for the control set, distilled water was used without adding wastewater. The wastewater was characterized in terms of chemical oxygen demand (COD) and Total N as shown in Table II

TABLE II
The COD and total N of 6 different wastewater concentrations

Wastewater concentration (%)	COD (mgL^{-1})	Total N (%)
0 (control)*	73.44	0.0002
10	141.44	0.0007
20	353.60	0.0016
40	935.00	0.0034
60	992.80	0.0051
80	1327.26	0.0067
100	1502.80	0.0085

*Control: distilled water

C. Cultivation of microalgae in cassava wastewater

1) Flask scale

In Fig.3 shows growth profile of *S. quadricauda* under variations of wastewater. It was that the profile was fluctuated for all conditions. It can be concluded that the wastewater used was not homogeneous. Furthermore, during the cultivation was not employed aseptic technique. Therefore, native microorganisms and other microalgal strains can be grown in the wastewater as well. The competition condition among them could be happened in the cultivation system. In this case, at the strengthen wastewater at 40%, 60% and 80%, the highest cell growth obtained at 60×10^5, 66×10^5 and 48×10^5 cells/ml, respectively.

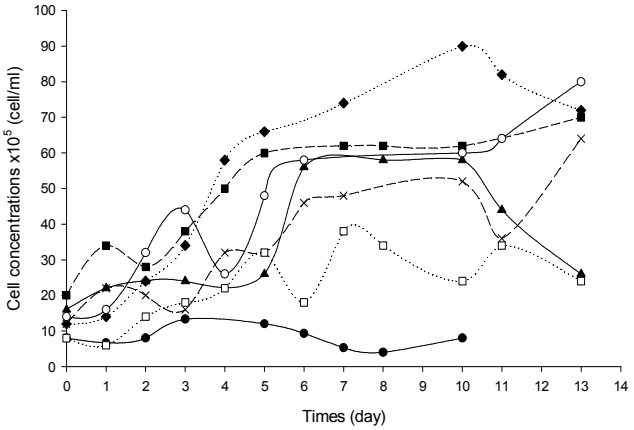

Fig. 3 Growth profile of *S. quadricauda* under different wastewater strengths in flask scale. Control (—●—), 10% (—▲—),20% (- -×- -), 40% (- -■- -), 60% (···♦···), 80% (—○—) and 100% (···□···).

2) Photobioreactor

In Fig.4 shows growth profile of *S. quadricauda* obtained from the reactors under 3 different concentrations (40%, 60%, and 80%). It was found that the highest biomass and specific growth rate obtained at 6.27×10^6cells/ml and 1.3667 d^{-1}. Meanwhile, at the strength of 40% and 80% wastewater gave biomass at 5.33×10^6 and 1.80×10^6 cells/ml, and specific growth at 0.7714 d^{-1} and 0.2120 d^{-1}. It should be noted that all three concentrations were reached early stationary phase in 10 day that was an optimal condition for salt stress in further experiment.

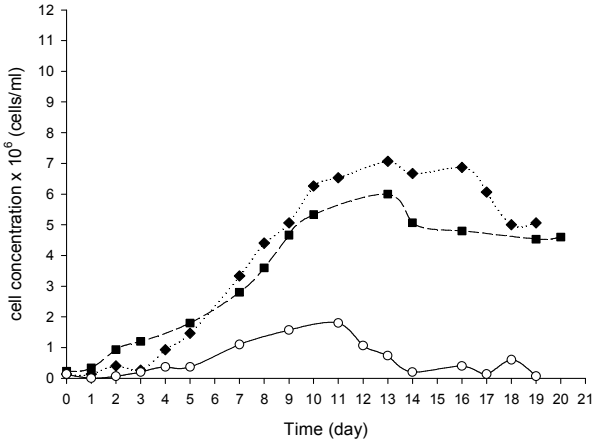

Fig. 4 Growth profile of *S. quadricauda* in 40%, 60% and 80% wastewater concentrations during cultivation in photobioreactor. 40% (- -■- -), 60% (···♦···) and 80% (—○—)

D. Effect of mixotrophic and heterotrophic cultivations on lipid accumulation

As previous experiment, at 60% wastewater concentration showed the optimum condition for micro-algal growth. After both strains of *S. quadricauda* and *S. obliquus* reached to early stationary phase. Two different cultivations as mixotrophic and heterotrophic were performed. The results obtained are shown as Figs. 5-6. Under mixotrophic culture of *S. quadricauda* and *S. obliquus* the highest lipid content obtained at 18.58 % and 42.86% within 10 day. However, under heterotrophic culture of *S. quadricauda* showed the highest lipid content at 5 day (17.87%) and after that it was slightly decreased.

E. Effect of NaCl concentration on lipid accumulation

Fig. 7 presents the lipid content of *S. quadricauda* culture under different NaCl concentrations. It was found that NaCl does not affect lipid accumulation in this microalgal strain. Since, the control condition (0 M NaCl) gave the highest lipid content at 10 day stress period. Meanwhile, in case of the reference strain of *S. obliquus*, the lipid content was increased as NaCl concentration increased as showed in Fig.8. In addition, the highest lipid content (50%) obtained at 10 day stress periods when NaCl at 1.0 M was used. It noted that the lipid content under NaCl stress (1.0 M) gave 0.38 fold that was higher than in case of no addition of NaCl.

IV. Conclusion

The isolated microalgal strain of *Scenedesmus quadricauda* showed in its potential for growth as well as for lipid accumulation under variations of wastewater strength and salt stress. The wastewater obtained from cassava production plant showed a potential of 60% in dilution strength for using as a cheap nutrient during cultivation. The highest algal biomass was obtained at 6.27×10^6 cells/ml. Under mixotrophic cultivation, the highest lipid content obtained at 18.58 % and 42.86% in cases of *S. quadricauda* and *S. obliquus*. In addition, under salt stress condition (1.0 M NaCl); *S. obliquus* gave 50% lipid content that was 0.38 times higher than that no NaCl adding. However, in case of *S. quadricauda,* the NaCl does not affect on lipid accumulation.

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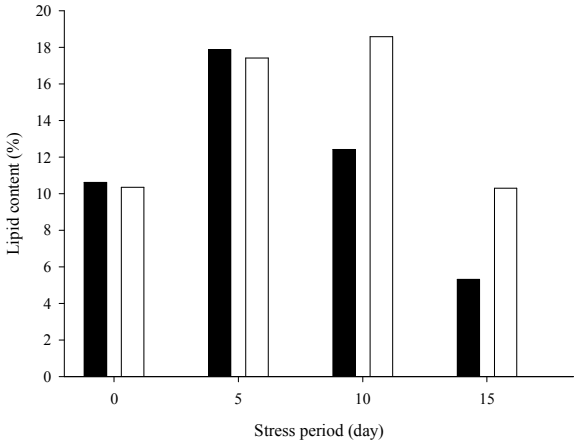

Fig. 5 Lipid accumulation compared between heterotrophic culture and mixotrophic culture in *S. quadricauda*. Heterotrophic (■) and Mixotrophic (□)

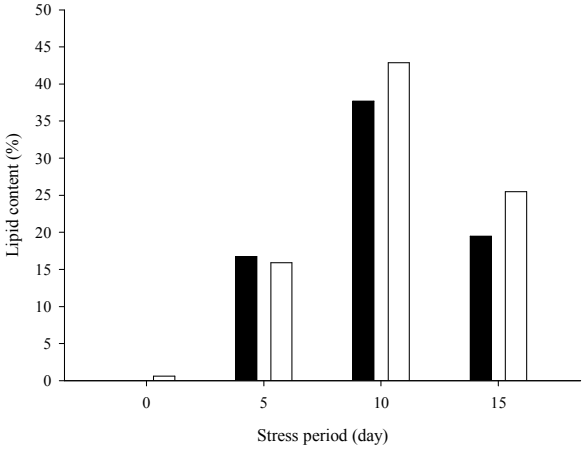

Fig. 6 Lipid accumulation compared between heterotrophic and mixotrophic cultures in *S. obliquus*.Heterotrophic (■) and Mixotrophic (□)

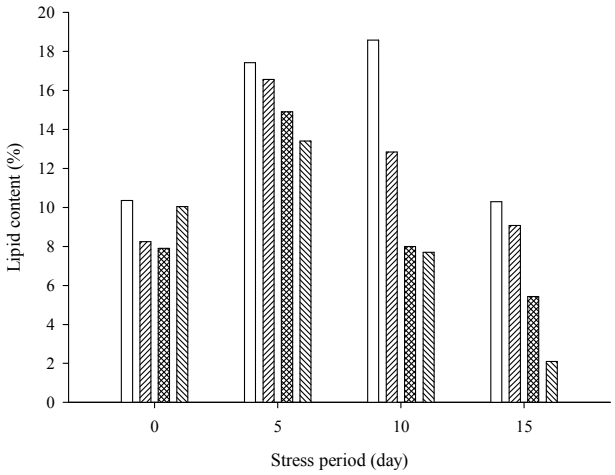

Fig. 7 Lipid content of *S. quadricauda* as a function of salt stress under different concentrations.0 M NaCl (□), 0.3 M NaCl (▨), 0.6 M NaCl (⊠) and 1.0 M NaCl (▧)

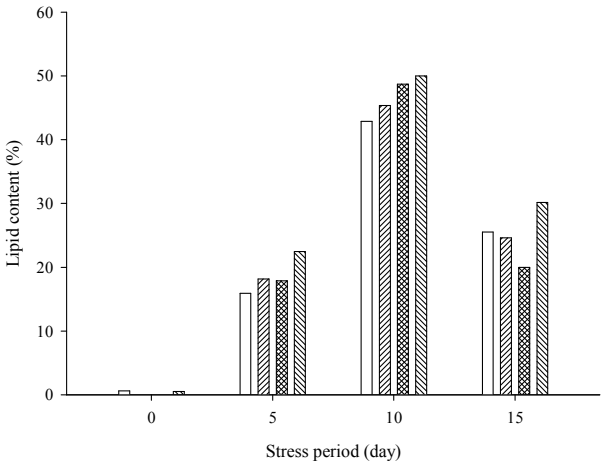

Fig. 8 Lipid content of *S. obliquus* as a function of salt stress period under different NaCl concentrations.0 M NaCl (□), 0.3 M NaCl (▨), 0.6 M NaCl (⊠) and 1.0 M NaCl (▧).

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