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Improvement in Biomass Production of a Microalga *Chlorella* sp. S2 Using Starch Processing Wastewater

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Abstract

Microalgae are promising resources for high-quality dietary supplements, pharmaceutical and biofuel production. This study attempted to improve the biomass of a microalga by cultivation in starch processing wastewater. A microalga identified as *Chlorella* sp. S2 by morphological criterion was isolated from the facultative pond of a noodle making plant. It was able to grow in the starch processing wastewater without addition of nutrients. To increase the biomass productivity of *Chlorella* sp. S2, inorganic nitrogen sources ($NaNO_3$, NH_4Cl and KNO_3) were added into the starch processing wastewater. The optimum nitrogen source was potassium nitrate at 7.5 mM of nitrogen, which increased the number of *Chlorella* sp. S2 up to 1.41×10^7 cells/ml and the specific growth rate was 0.351 d^{-1}. Under sunlight, the microalga *Chlorella* sp. S2 also produced high biomass concentration (2.23±0.04 g/l). It means that microalgal cultivation using starch processing wastewater is a great process to produce biomass. In addition, it is able to reduce organic carbon in the wastewater and reduce cost.

Keywords: microalgae; *Chlorella*; cultivation; starch processing wastewater; biomass production
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1. Introduction

Nowadays, microalgae are used as dietary supplements, pharmaceuticals, biofuels and in other applications. Microalgae are mixotrophic organisms that are able to be both autotrophs and heterotrophs. Microalgae can fix and transform CO_2 into starch, oilgae, carotenoids and other compounds in similar way to plants and they also release O_2 in autotrophic cultures [1-3]. In addition, organic carbon sources (such as glucose, glycerol, molasses, whey permeate, acetic acid etc.) are used to increase the biomass of microalgae in heterotrophic cultures [4-9].

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Microalgae can be cultivated in ponds and fermenters using wastewater as organic carbon sources [1]. Wastewater consists of nutrients (phosphorus, nitrogen, organic carbon, etc.) which are required by microalgae for growth and biomass production [10]. The use of wastewater can reduce the cost of biomass production because microalgae can grow in it without adding any extra nutrients [1]. In addition, biomass production of microalgae in wastewater can reduce organic carbon, which is an environmentally friendly process.

Chlorella is able to grow mixotrophically in a short time and has simple growth requirements [11, 12]. *Chlorella* contains abundant nutrients, especially high protein content, which are beneficial to human and animal health [13]. In addition, *Chlorella* can be used to remove the colour and COD of textile wastewater effluent [14, 15]. It also removes nitrogen, phosphorus and carbon from water to reduce eutrophication in an aquatic environments, and it tolerates untreated wastewater [16, 17].

In this study, to save the cost of microalgal biomass production, starch processing wastewater was used as organic carbon and nutrient sources for cultivation. The microalga *Chlorella* sp. was chosen to investigate the optimal conditions for increasing biomass concentration in mixotrophic culture.

2. Materials and methods

2.1 Isolation and purification

The microalga was isolated from wastewater treatment ponds (oxidation ponds) at a noodle making plant in Sisaket Province, Thailand, with a plankton net (10 µm × 7 µm in size). The microalga *Chlorella*-like green colonies were separated using a sterile micropipette washing method [18] and cultivated in a modified Chu13 [19]. The microalga *Chlorella* was subjected to purification by serial dilution followed by plating. The single colony was isolated and cultured in liquid modified Chu13 medium at room temperature (28-30ºC), with a light intensity of 3,000 lux using cool-white fluorescent lamps (16 h) for 7 days. The isolated microalga was approximately identified as the genus *Chlorella* according to morphological properties [20].

2.2 Microalgal cultures

The microalga *Chlorella* sp. S2 was grown in starch processing wastewater. The waste water samples were obtained from an anaerobic pond (No.1), facultative pond (No.2) and aerobic ponds (No.3 and No.4) of a noodle making plant in Sisaket Province, Thailand. The pH values of the wastewater samples were acid and neutral pH (5.44-7.13) in ponds No.1-4. The values of phosphorus, nitrogen and potassium in the wastewater samples were determined following AWWA/APHA protocols [21]. The starch processing wastewater was dark because it contained some suspended solids. Therefore, the suspended solids were removed by filtration with absorbent cotton and sterilized with chlorine (calcium hypochlorite; $Ca(OCl)_2$) at a concentration of 15 ppm for 30 min. Chlorine was able to reduce the problems associated with light-shading and the mass transfer of oxygen. To reduce the effect of chlorine on the microalga growth, chlorine was removed by filtered air bubbles from an air pump (0.5 l/min) for 1 h.

The cultivation of the microalga *Chlorella* sp. S2 was attempted by adding 10% (v/v) of seed culture (5×10^6 cells/ml) in 400 ml of starch processing wastewater medium. The culture was incubated with 0.03% CO_2 (0.02 l/min) at room temperature, with a light intensity of 3,000 lux using cool-white fluorescent lamps (light intensity with 16:8 h light photoperiod) for 7 days.

Nitrogen sources ($NaNO_3$, NH_4Cl and KNO_3) at 7.5 mM of nitrogen were added to the starch processing wastewater medium samples to increase the biomass of microalga *Chlorella* sp. S2. Then, the optimal concentration of nitrogen at 3.7, 7.5 and 15.0 mM were tested. The effect of sunlight was also tested in order to save cost on biomass production.

2.3 Analytical methods

All wastewater samples were analyzed for phosphorus, nitrogen and potassium. The pH values of culture broths were measured by pH meter (pH 900, Amtast Industry, USA). Cell concentrations of microalga *Chlorella* sp. S2 were determined using a hemocytometer. Flocculation of microalga was attempted by adding Alum (0.3 g/l at pH 6.0) [22]. Next, sedimentation was allowed to occur under gravity for 10 min and supernatant was removed and precipitate was dried at 60ºC until constant weight was observed for microalgal biomass. The specific growth rate (μ) was calculated using data in the exponential phase using the following equation:

$$\mu\ (d^{-1}) = \frac{ln\frac{x_2}{x_1}}{t_2 - t_1}$$

where x_1 and x_2 are the concentrations of microalgal cells (g/l) at time t_1 and t_2, respectively [23].

All experiments were performed in triplicate. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($p \leq 0.05$) was used to separate means, using SPSS software.

3. Results and Discussion

3.1 Growth of microalga in the starch processing wastewater

Under the microscope, the isolated S2 showed a spherical shape with no flagellum (Figure 1). It was identified as *Chlorella* sp. S2 by morphological criterion. The microalga *Chlorella* sp. S2 was cultivated in starch processing wastewater collected from oxidation ponds (No.1-4) in the noodle making plant. The algal biomass concentration from pond No.2 increased faster and to a higher level compared with other ponds (No. 1, 3 and 4) (Figure 2). It provided the highest biomass of 2.26×0.12 g/l on the 7th day and also provided the highest cell number of 5.6×10^6 cells/ml on the day 5th while entering the steady stage until the 7th day. It was interesting that biomass was cultivated from microalga *Chlorella* sp. S2 without addition of any nutrients in the starch processing wastewater yielded high biomass. This might have been because there were organic carbon and nutrient elements (phosphorus 230.96 mg/l, nitrogen 7.9 mg/l and potassium 55.97 mg/l) for the growth of microalga *Chlorella* sp. S2 in the mixotrophic condition. This might be the reason of cost saving for biomass production of *Chlorella* sp. S2. There were lower biomass and cell number in the starch

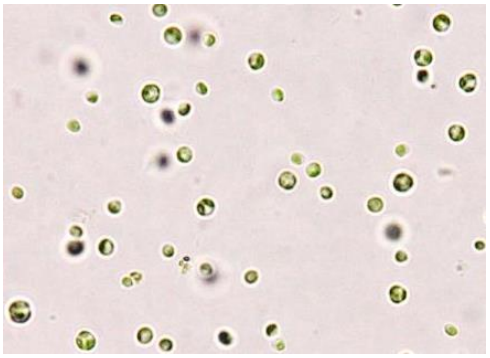

Figure 1. The microalga *Chlorella* sp. S2 under the microscope (40×)

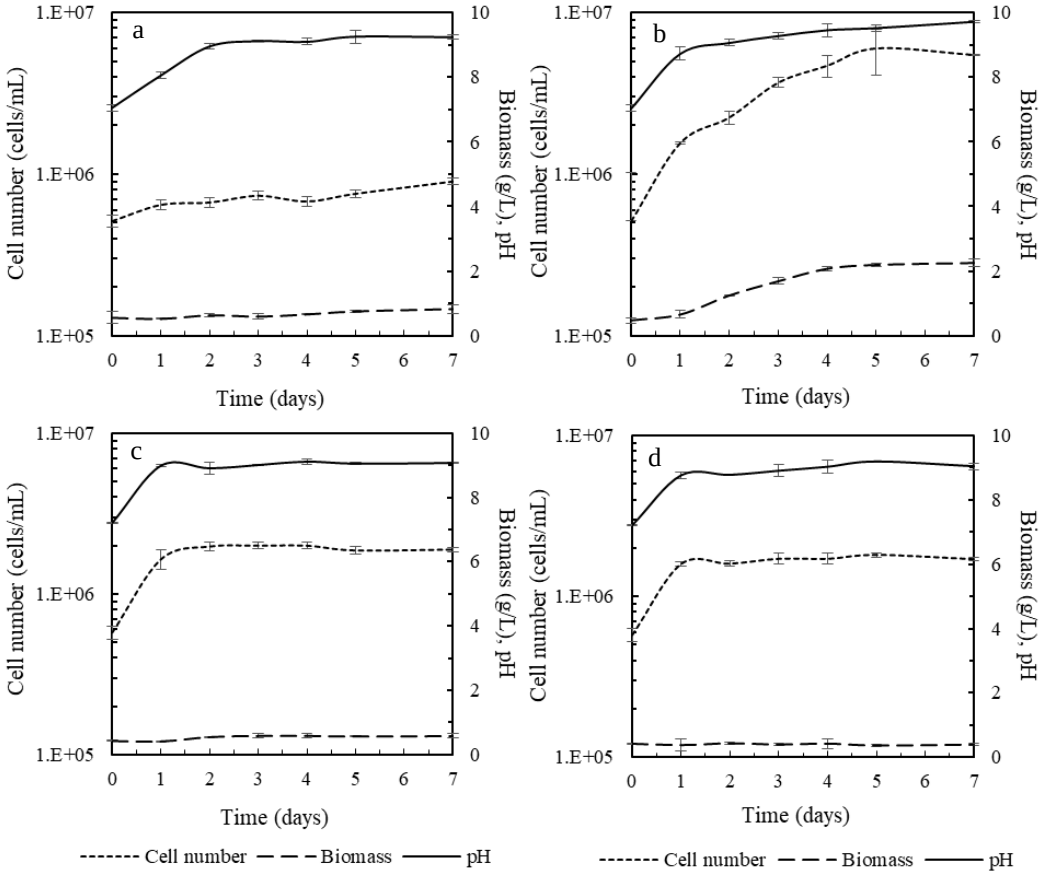

Figure 2. Comparison of growth of *Chlorella* sp. S2 in the starch processing wastewater from wastewater treatment pond No.1 (a), No.2 (b), No.3 (c) and No.4 (d)

processing wastewater from wastewater treatment ponds No. 1, 3 and 4, a result which might have been influenced from the depletion of nitrogen in the culture medium used. There was a nitrogen concentration of only 7.9 mg/l in the starch processing wastewater medium. An increase in microalgal biomass was found under nitrogen-rich conditions [24]. On the other hand, microalga *Chlorella protothecoides* grew well using glucose as a carbon source on the heterotrophic condition [25]. In all cultivations, pH increased from 6.8 to 9.5 because CO_2 dissolves in the culture medium as bicarbonate form (HCO_3^-). When microalgae consumes CO_2, the OH^- is formed and makes the culture medium more alkaline [26].

3.2 Effect of nitrogen sources on growth

There was a low nitrogen concentration (7.9 mg/l) in the starch processing wastewater from wastewater treatment pond No.2. Hence, the biomass production of microalga *Chlorella* sp. S2 was enhanced by addition of nitrogen sources. The influence of $NaNO_3$, NH_4Cl, KNO_3 (7.5 mM nitrogen) was investigated as shown in Figure 3. The biomass of microalga *Chlorella* sp. S2 increased rapidly in the starch processing wastewater with the addition of KNO_3 (2.57±0.18 g/l), Na_2NO_3 (2.27±0.08 g/l) and NH_4Cl (2.42±0.21 g/l), respectively. The addition of nitrogen sources provided the highest biomass on the 4th day, whereas cell number increased steadily until the day 5th (Figure 3b) but the biomass did not increase. However, without addition of nitrogen sources, the biomass developed later, on the 5th day. Microalgae cultivation should have suitable nitrogen concentration because they require nitrogen to synthesize proteins [27]. Thus KNO_3, which showed a rapid increase of biomass and cell number, was chosen as a suitable nitrogen source. This was similar to previous research with *Chlorella protothecoides*, where biomass concentration increased with the addition of both inorganic and organic nitrogen sources (nitrate, ammonium and urea) [25]. The highest biomass concentration of *Chlorella sorokiniana* occurred when glycine was used as the nitrogen source [28].

3.3 Effect of nitrogen source concentration on growth

The optimum levels of nitrogen (KNO_3) concentration for the biomass production of *Chlorella* sp. S2 were varied from 3.7 to 15.0 mM as demonstrated in Figure 4. The effect of nitrogen (KNO_3) concentration on growth was observed. When the concentration of KNO_3 was increased, the biomass and cell number of *Chlorella* sp. S2 also increased steadily until the 5th day of cultivation. It was interesting that the biomass and cell number of *Chlorella* sp. S2 grew slightly faster and provided the highest specific growth rate of 0.351 d^{-1} at KNO_3 of 7.5 mM (Figure 4b). However, there was no significant difference between 7.5 and 15.0 mM of KNO_3. The biomass concentration in the starch processing wastewater without addition of nitrogen was similar to the addition of 3.7 mM of KNO_3 (Figure 4a), but with the addition of KNO_3, *Chlorella* sp. S2 grew slightly faster. In addition, the broth culture medium of *Chlorella* sp. S2 presented dark green at 7.5 and 15.0 mM of KNO_3 as shown in Figures 5c and 5d, respectively. The biomass of microalga *Nannochlolis* also increased when the nitrate concentration was increased from 0.9-9.9 mM of nitrate [29] while *Scenedesmus dimorphus* provided high biomass at 0.06 M of nitrate [30]. At the low concentration of KNO_3 (3.7 mM), it was yellow-green (Figure 5b) and without addition of nitrogen source it was bright yellow-green (Figure 5a). This means there was not enough nitrogen concentration in the starch processing wastewater for microalgal growth. Furthermore, the biomass production of *Scenedesmus dimorphus* and *Chlorella photothecoides* increased in medium which had high a concentration of nitrogen [30] while the biomass production of *Chlorella vulgaris* decreased in the medium with a limit nitrogen concentration [31].

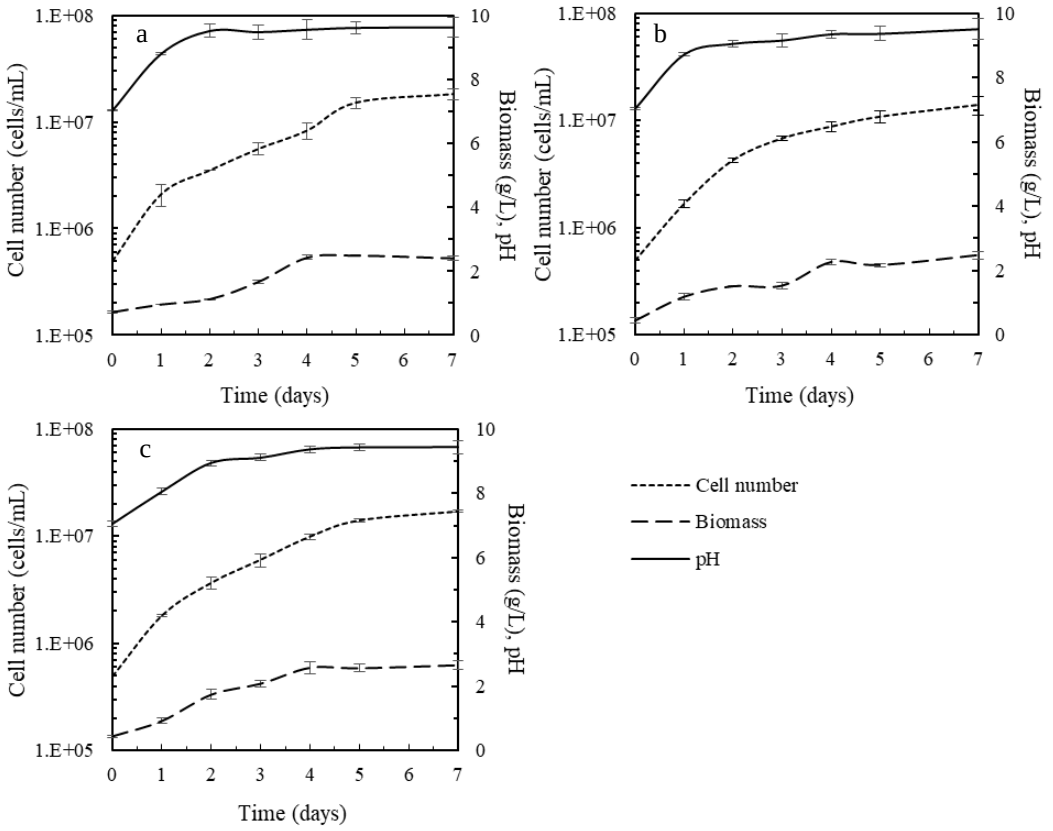

Figure 3. Effect of nitrogen sources on cell number, biomass and pH of *Chlorella* sp. S2 in the starch processing wastewater from wastewater treatment pond No.2: $NaNO_3$ (a), NH_4Cl (b) and KNO_3 (c)

3.4 Effect of sunlight

Microalgae are autotrophic organisms, therefore, light is the most important factor which affects growth and storage of products [32]. To reduce the cost of microalgal cultivation, the effects of lighting sources were observed as shown in Figure 6. The microalga *Chlorella* sp. S2 was grown in the starch processing wastewater medium with the addition of KNO_3 (7.5 mM). It was found that *Chlorella* sp. S2 grew well and provided the highest biomass and cell number of 2.23±0.04 g/l and 6.18 ± 10^6 cells/ml, respectively on the 5th day of cultivation. It showed a specific growth rate of 0.328 d^{-1}. The specific growth rate of the microalga *Chlorella* sp. S2 under sunlight conditions slightly decreased compared to that of culture using cool-white fluorescent lamps (16 h) (0.351 d^{-1}). That might have been influenced by the high light intensity of the sunlight (3,000-6,000 lux), which was not suitable for growth of the microalga *Chlorella* sp. S2.

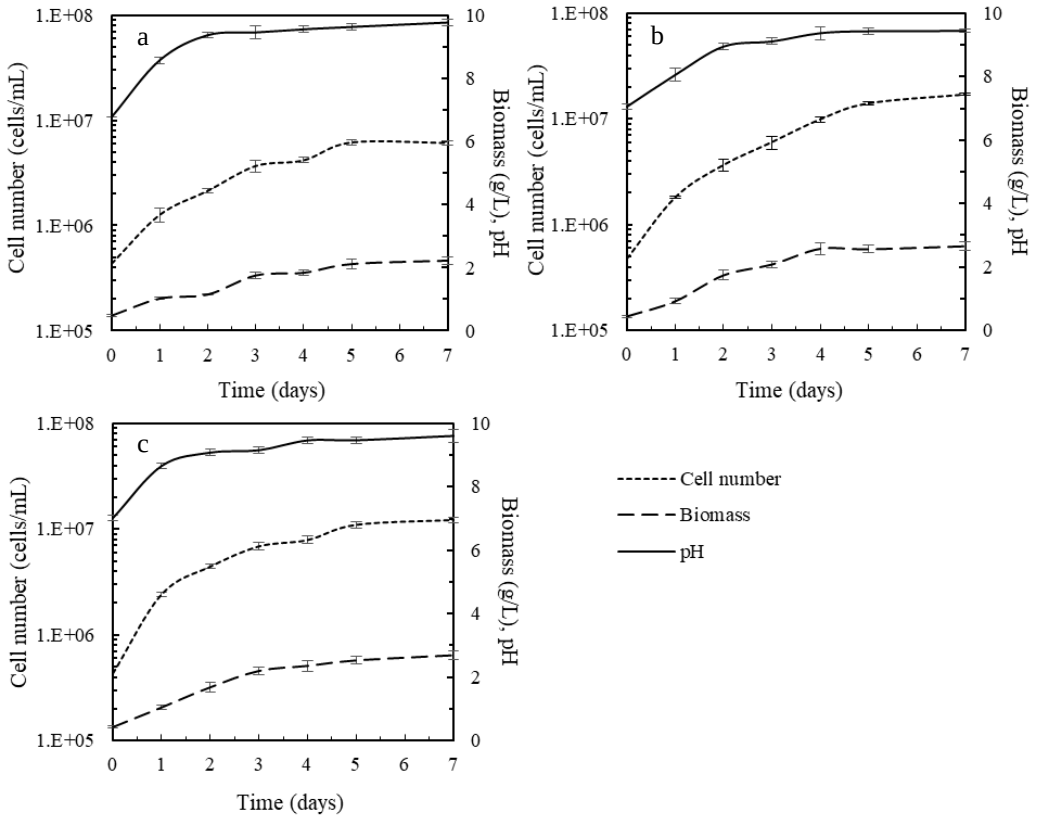

Figure 4. Effect of nitrogen concentration at 3.7 mM (a), 7.5 mM (b), and 15.0 mM (c), on cell number, biomass and pH of *Chlorella* sp. S2 in the starch processing wastewater from wastewater treatment pond No.2

Figure 5. The microalga *Chlorella* sp. S2 cultures after cultivation in the effluent without addition of nitrogen (a), with addition of nitrogen at 3.7 mM (b), 7.5 mM (c) and 15.0 mM (d) of KNO_3 on 7^{th} day

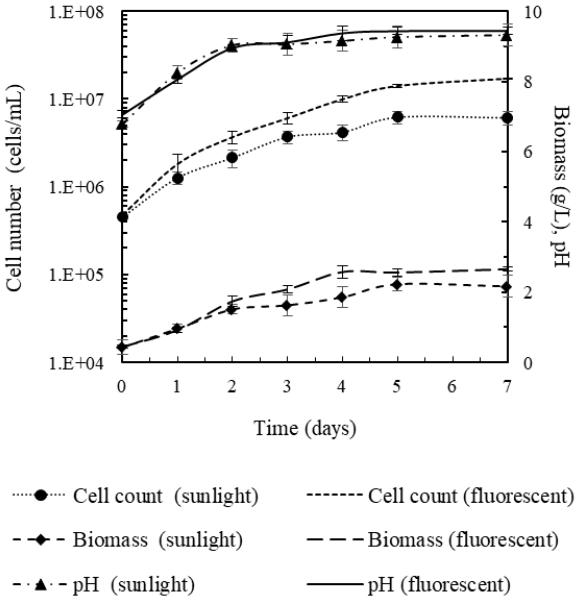

Figure 6. Effect of sunlight and fluorescent lamps at 3,000 lux light intensity with 16:8 h light photoperiod on cell number, biomass and pH of *Chlorella* sp. S2 in the starch processing wastewater with the addition of 7.5 mM KNO_3

4. Conclusions

The microalga *Chlorella* sp. S2, isolated from the starch processing wastewater in the noodle making plant, grew well in the starch processing wastewater from wastewater treatment pond No.2. It provided high biomass concentration and cell number with and without the addition of nitrogen. When the starch processing wastewater medium with added KNO_3 at 7.5 mM, the highest specific growth rate occurred and the biomass of the microalga *Chlorella* sp. S2 increased slightly faster. The microalga *Chlorella* sp. S2 also grew well under sunlight. This study has shown that the starch processing wastewater from the noodle making plant under the sunlight was able to cultivate the microalga *Chlorella* sp. S2.

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