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Full Paper

Production of *Spirulina platensis* using dry chicken manure supplemented with urea and sodium bicarbonate

Thepparath Ungsethaphand [1,3,*], Yuwadee Peerapornpisal [2] and Niwoot Whangchai [3]

[1] Biotechnology Program, Graduate School, Chiang Mai University, Chiang Mai 50200, Thailand

[2] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[3] Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai 50290, Thailand

* Corresponding author, e-mail: thorn@mju.ac.th

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Abstract: The cyanobacterium *Spirulina platensis* is an attractive source of valuable protein for both human and animal consumption. The conventional nitrogen source for *S. platensis* is nitrate. However, recent research has evaluated the potential of using animal waste as a low-cost nitrogen source. In this work, the cultivation of *S. platensis* was done using dry chicken manure (DCM), collected from a closed-system poultry house, as nitrogen source. The experiment was carried out in open concrete tanks with 100 litres of culture medium and an initial biomass concentration of 0.5 g/L. The culture media were prepared to test the effect of unsupplemented DCM, DCM supplemented with 2.0 mg/L of urea (DCM+U), and/or 40 mg/L of sodium bicarbonate (DCM+U+B or DCM+B). The best cellular growth and highest protein production were observed for *S. platensis* in the biomass harvested from the culture medium containing DCM supplemented with 2.0 mg/L of urea (DCM+U).

Keywords: *Spirulina*, chicken manure, urea, bicarbonate, nitrogen source

Introduction

Egg-laying chicken production is the top farm commodity in many areas of Thailand. Poultry producers must periodically clean their poultry houses to promote the animals' health and limit the build-up of wet manure [1]. It is well known that poultry manure can be used as an alternative source of fertiliser in fish ponds. It is economically beneficial to use chicken manure rather than chemical fertiliser, and this also reduces the environmental pollution of manure caused by inappropriate disposal

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[2]. Many chicken producers have integrated poultry houses with fish ponds to grow fish using poultry litter (manure plus spilled feed) without additional cost. Unfortunately, after the crisis of bird flu the Thai government has promoted the closed-system poultry house and discouraged integrated farming with chickens above fish ponds.

One of the possible acceptable ways to utilise farm manure is in the production of microalgae. The cyanobacterium *Spirulina platensis* is an attractive source of valuable proteins for both human and animal consumption. The conventional nitrogen source for *S. platensis* is nitrate. However, recent research has evaluated the potential of using animal waste as a low-cost nitrogen source [3-5]. These studies were focused mainly on the production of *Spirulina* from swine manure and waste, and there is only limited information available on utilisation of poultry manure. More recently, Ungsethaphand et al.[6] has shown that dry chicken manure (DCM) can supply the necessary nutrients for the culture of *S. platensis*. Costa et al. [7] reported that the addition of 1.125 mg/L of urea to Mangueira Lagoon water is beneficial to the growth of *S. platensis.* In the alkaline culture medium, urea is hydrolysed to ammonia, which can be toxic to microalgae in high concentration. However, urea addition by fed-batch process makes it possible to replace KNO_3 as nitrogen source in the culture media. On the other hand, the work on an industrial scale with addition of a constant amount of urea should be simpler [8]. The use of urea as a cheap source of ammonia could then be an interesting alternative to the traditional nitrate-based *S. platensis* culture.

Binaghi et al. [9] reported that *S. platensis* is a filamentous cyanobacterium able to form large colonies in surface water containing a high level of carbonates and bicarbonates. Among the nutrients of cultivation media, the inorganic carbon source is primarily responsible for the alkaline condition and is preferentially assimilated by cyanobacteria in the form of bicarbonate [10]. A concentration of bicarbonate lower than 0.1 M decreases the growth rate of *S. platensis* [11].

The aim of this work is to study the production of *S. platensis* using DCM supplemented with urea and sodium bicarbonate by fed-batch addition. The chemical composition of the *S. platensis* biomass is also investigated.

Materials and Methods

Microorganism

Spirulina platensis was obtained from the Faculty of Fisheries Technology and Aquatic Resources, Maejo University. The culture was routinely maintained in modified Zarrouk liquid medium [12].

Culture medium

The culture medium used was 2.0 kg of dry egg-laying chicken manure (DCM) collected from a closed-system house. The manure was suspended in 100 L of aerated tap water for 7 days before being sieved through a 30-mesh Tylor net. Sodium metabisulfite (5 mg/L) was added to prevent microbial contamination. After 24 h, 8.5 g/L of sodium bicarbonate was added before the beginning of the experiment.

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Experiment

The addition of urea and sodium bicarbonate to the culture was done by a fed-batch process. The nutrients were added in an aqueous solution to replace daily water evaporation using the following protocol:

(i) DCM (control): tab water was added every 24 h;
(ii) DCM+U: urea (2.0 mg/L) was added every 24 h;
(iii) DCM+B: sodium bicarbonate (40 mg/L) was every 24 h;
(iv) DCM+U+B: urea (2.0 mg/L) and sodium bicarbonate (40 mg/L) were added every 24 h.

Culture conditions

The experiment was carried out in open concrete tanks under daylight in a greenhouse and 27.8-34.3 ^{o}C temperature range. The depth of the cultures was always 15 cm with 100 L of culture medium. Each culture was agitated by aeration at a flux of 20 L/h provided by a diaphragm pump. The initial pH was adjusted to 9.5 ± 0.2 with 6M NaOH.

Inoculation

The inoculum was obtained by liquid culturing using a modified Zarrouk medium [12] at 32^{o}C with initial pH of 9.2. Cultivation was done in 500-ml Erlenmeyer flasks subjected to a moderate mixing provided by a small air pump operating at a rate of 0.023 L/min and illuminated by fluorescent lamps (36 w) in cycle of 12 h of light and 12 h of darkness. After seven days, the culture reached it exponential phase and the cells were harvested by filtration through a 62-µm mesh and washed thrice with a 0.8% NaCl solution to completely remove sodium nitrate. The cells were resuspended in the modified Zarrouk medium without nitrate and used as inoculum (500 mg/L concentration).

Analytical methods

The culture pH and temperature were monitored daily with a pH metre. Ammonia-nitrogen was determined by using standard phenate method described by APHA [13] and chlorophyll *a* was determined by measuring absorbances at 665 and 750 nm [13].

Biomass concentration was determined on alternate days by optical density (OD) determination at 560 nm (Hach instrument, Model DR2000) to produce a standard curve relating dry weight of *S. platensis* biomass to OD. This standard curve was subsequently used to determine the biomass of individual samples [14].

The dry weight of biomass was determined by filtration of sample (25 ml) through Whatman filter paper No.4 after washing with 3.0 N acetic acid to eliminate salt precipitate. The biomass obtained was then washed twice with 0.8% saline solution and dried at 80^{o}C for 4 h according to Olguin et al. [5].

The chemical composition of the dried biomass was determined according to AOAC methods [15]. Crude protein was determined by micro Kjeldahl method; total lipid by Soxhlet solvent extraction; and ash by combustion at 550^{o}C for 12 h.

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Statistical analysis

One-way ANOVA was used to test the effect of the culture media. Tukey's test was also applied to compare the means when a significance difference ($p < 0.05$) was detected by ANOVA.

Results and Discussion

The maximum cell concentration and chlorophyll *a* content were significantly different among treatments. The growth results (Tables 1-2) showed highest cellular concentration, chlorophyll *a* content and protein content of *S. platensis* when DCM+U medium was used. The addition of bicarbonate (DCM+B) gave only a slight increase in biomass while in DCM+U+B (added urea and bicarbonate) medium, there was actually a decrease in biomass. These results agree with those of Costa et al. [7], who found that the addition of urea and bicarbonate in Mangueira Lagoon water caused a decrease of biomass of *Spirulina*. Urea utilisation as a nitrogen source provides an energetic gain due to its spontaneous hydrolysis in the alkaline medium to ammonia, which is easily assimilated by *Spirulina* [8]. Costa et al. [7] reported that the highest biomass values were obtained in the treatment with 1.125 mg/l of added urea without addition of sodium bicarbonate. Danesi et al. [8] has shown that the use of urea as nitrogen source in *S. platensis* cultivation causes an increase in the biomass production as well as chlorophyll content. The DCM+U medium thus seems to be more appropriate to *S. platensis* cultivation for high-protein biomass utilisation in food and feed [16].

Table 1. Results of the cultivation of *S. platensis* grown on different media containing DCM

	DCM	DCM+U	DCM+B	DCM+U+B
Cells (mg/L dw) [a]	414.2±2.5 ^{a}	457.5±6.2 ^{b}	429.7±4.2 ^{ab}	409.7±2.9 ^{a}
Time (days) [b]	18	20	18	18
Chlorophyll *a* (mg/g)	3.02±0.04 ^{a}	3.33±0.08 ^{b}	3.07±0.07 ^{ab}	3.33±0.12 ^{b}

[a] Maximum cell concentration

[b] Time of achieving maximum cell concentration

Note: Means ± se in row with different superscripts are statistically different at significant level of 0.05 when compared by Tukey's test.

Table 2. Chemical composition (% dry weight) of *S. platensis* grown on different DCM media

	DCM	DCM+U	DCM+B	DCM+U+B
Protein	35.55±2.26 ^{a}	53.32±2.24 ^{b}	37.84±1.59 ^{a}	52.23±0.83 ^{b}
Lipid	4.86±0.99	4.53±0.20	2.64±0.50	3.92±1.07
Carbohydrate	38.89±3.44 ^{a}	20.77±3.91 ^{b}	40.87±0.86 ^{a}	25.04±2.05 ^{b}
Mineral	18.67±1.27	19.49±1.73	17.57±1.30	16.73±1.06
Fibre	2.02±0.67	1.89±0.10	1.08±0.02	2.07±0.50
Moisture	11.53±1.38	11.22±0.37	13.58±0.86	11.73±1.05

Note: Means ± se in row with the different superscripts are statistically different at a significant level of 0.05 when compared by Tukey's test.

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Figure 1 shows an apparent relationship between biomass concentration and chlorophyll *a* content. This observation is in agreement with the results reported by Rangel-Yagui et al. [17] who also observed similar relationship between urea addition and the production of biomass with higher chlorophyll content. Piorreck et al. [18] reported lower concentration of chlorophyll obtained from cultivation carried out with limited nitrogen concentration. The shading effect [17] on the other hand may have contributed to a higher concentration of chlorophyll *a* observed in the biomass obtained from cultivation with higher added nitrogen at a fixed light intensity. This yields higher a cellular concentration, which can generate a higher chlorophyll *a* biosynthesis rate in order to increase the efficiency of photon capture and thus compensate for the reduction of light intensity for the cells not located at the surface [19].

The significantly higher protein content was observed in the biomass of DCM+U and DCM+U+B media (Table 2). The increase in protein content was apparently due to the nitrogen level in the medium. Piorreck et al. [18] reported that increasing the nitrogen level in the nutrient medium leads to an increase in the biomass and protein content of *Spirulina.* Nitrogen is required for the synthesis of amino acids which make up proteins and other cellular components. Therefore, a lower urea concentration correspondingly gave a lower value of cellular proteins [20].

The lipid content was not significantly influenced by the different treatments (Table 2). According to Piorreck et al.[18], blue-green algae do not show any significant change in the percentage and composition of their lipids and fatty acids when grown at different concentrations of nitrogen. Danesi et al. [8] also verified that the lipid content in the *S. platensis* biomass is not influenced by the nitrogen source used.

According to Walach et al. [21], higher quantities of carbohydrates are synthesised when nitrogen availability is decreased while carbon availability is constant. These two factors together may explain the significantly higher production of carbohydrate in DCM and DCM+B treatments. Nitrogen deficiency has been found to stimulate the synthesis of all carbohydrate fractions (intracellular, capsular and soluble) in a cyanobacterium [22].

The temperature variation in this experiment (Figure 2) during the cultivation was close to the optimum range reported for *S. platensis* cultivation [8,23]. The pH change (Figure 3) did not show significant differences between the treatments. The cyanobacterium grew effectively in the medium leading to a progressive pH increase, in agreement with many authors [9,16,17]. This observation can be correlated with the carbon-source consumption. The bicarbonate ions, for example, are assimilated by the cyanobacteria and subsequently converted into carbon dioxide and carbonate. The carbon dioxide is utilised in photosynthesis and the carbonate is excreted into the medium. The increase in the pH of the system is due to the shift of the bicarbonate-carbonate equilibrium towards the carbonate [17]. No external contamination was detected, most likely because of the high alkalinity of the culture medium.

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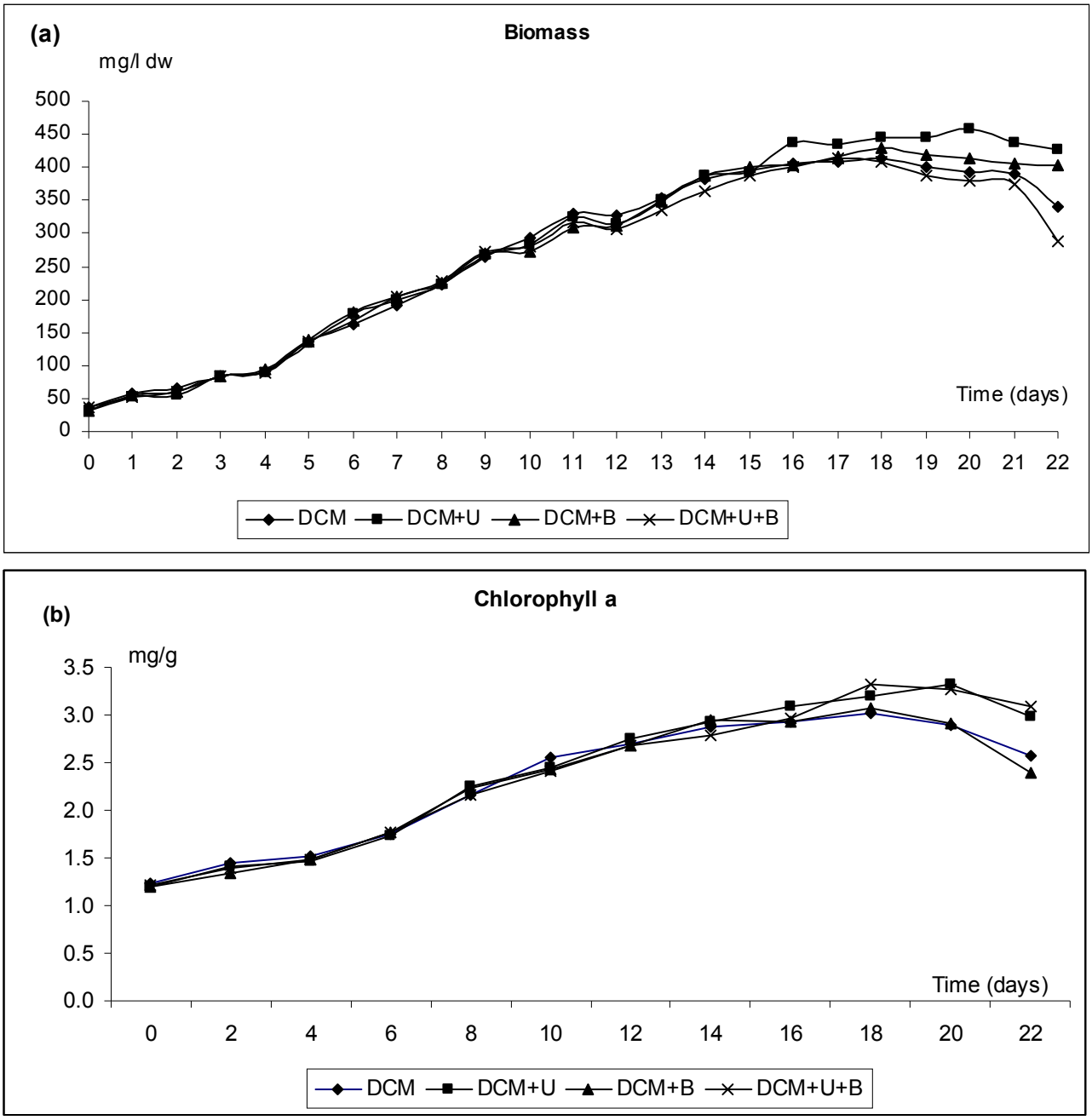

Figure 1. Profiles of biomass concentration (a) and chlorophyll *a* content (b) during the cultivation of *S. platensis* grown in different DCM media

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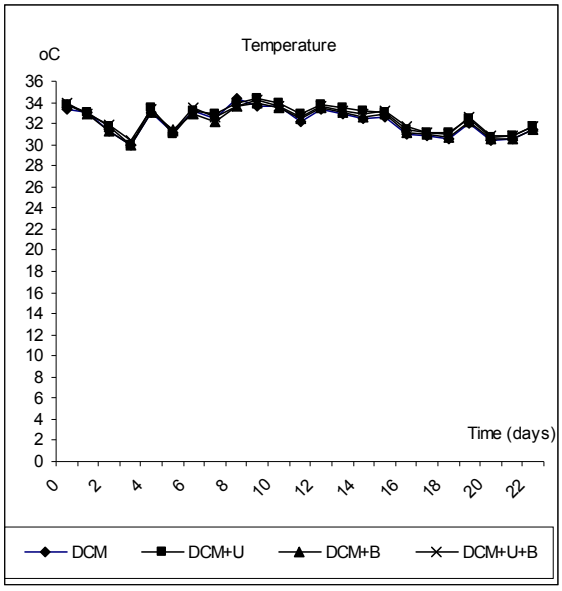

Figure 2. Temperature variation during the cultivation of *S. platensis* grown on different DCM media

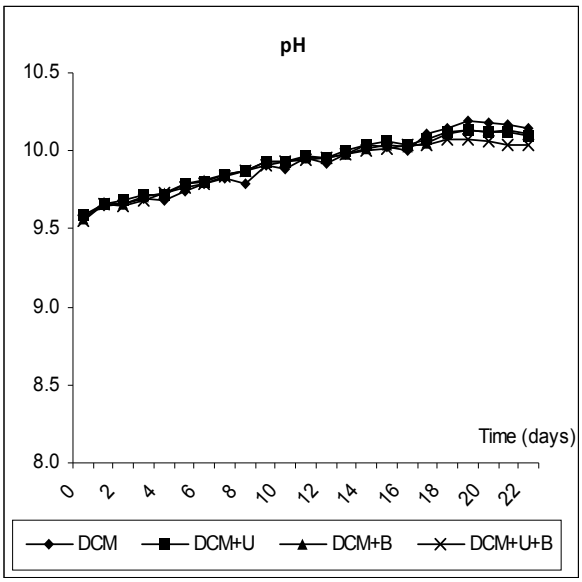

Figure 3. pH of culture during the cultivation of *S. platensis* grown on different DCM media

Conclusions

The fed-batch cultivation of *S. platensis* is feasible using dry chicken manure (2.0 kg/100 L) in the culture medium supplemented with urea (2.0 mg/L). This culture medium resulted in the best cellular growth and highest protein content. The potential of reducing production cost with the medium in a large-scale cultivation is also apparent.

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