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Cultivation of *Spirulina platensis* Using Pig Wastewater in a Semi-Continuous Process

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The effluent from anaerobic digestion contains organic nitrogen and phosphorus, which are both required for growth of *Spirulina platensis*. Effluent (20%) from the up-flow anaerobic sludge blanket (UASB) from a pig farm, supplemented with 4.5 g/l sodium bicarbonate ($NaHCO_3$) and 0.2 g/l urea fertilizer (46:0:0, N:P:K), was found to be not only a suitable medium for the growth of *Spirulina platensis* but also a low-cost alternative. Cost calculation showed that this medium is 4.4 times cheaper than modifized Zarrouk's medium. The average productivities of a semi-continuous culture grown under outdoor conditions in a 6-l scale and a 100-l pilot scale were 19.9 g/m^2/d and 12 g/m^2/d, respectively. In addition, the biomass of organisms grown in UASB effluent contained approximately 57.9% protein, 1.12% γ-linolenic acid, and 19.5% phycocyanin. The average rates of bicarbonate, total nitrogen, and phosphorus removal were 380 mg/l/d, 34 mg/l/d, and 4 mg/l/d, respectively.

Keywords: *Spirulina platensis*, pig waste, nitrogen, phosphorus.

Pig waste is a very important source of pollution, where the daily pig waste produced is equal to approximately 5% of the weight of the animals and contains a very high biochemical oxygen demand [16]. Pig manure also contains a high level of organic waste. Specifically, pig manure contains approximately 5.4–6.3 kg of nitrogen/ton manure and 2.2–3.1 kg of phosphorus/ton manure [16, 25], which are both required for the growth of microalgae. Currently, anaerobic digestion systems such as the cover lagoon and plug flow digester or up-flow anaerobic sludge blanket (UASB) are widely used for the treatment of waste from pig farms. The amounts of nutrients in the effluent from such treatment systems are sufficient for algal growth. Therefore, cultivation of algae in the effluent of animal manure treatment facilities presents an alternative to the current practice of land application of manure. Several species of algae have been used for the biotreatment of wastewater, with success. The cyanobacterium *Spirulina* is one of the most widely used microalgae to treat swine wastewater [8, 16], digested sago starch factory wastewater [18], and desalinator wastewater [27].

Spirulina has a high potential for use as an animal feed because it is a rich source of protein, essential amino acids, vitamins, essential fatty acids, and antioxidant pigments such as phycocyanin and carotenoids [6]. There have been many reports that the use of dietary *Spirulina* as an animal feed supplement increases survival and growth in various types of animals such as fish, chicken, pigs, and carp. In addition, it has been reported that dietary supplementation with *Spirulina* enhances several immunological functions [2, 6, 21]. Moreover, prawns, fish, and poultry that were fed diets supplemented with *Spirulina* showed enhanced coloration of the skin and egg yolks [9, 23].

The production of *Spirulina* as dietary supplements for animal feed utilizing the nutrients contained in wastewater from animal production units offers several advantages, including a significant saving in the cost of culture medium. However, inorganic carbon must often be provided to enhance the growth of *Spirulina*. The objective of this study was to produce *Spirulina* using UASB effluent from a pig farm.

Materials and Methods

The inoculum of *Spirulina platensis* strain BP, isolated from a stabilization pond at a tapioca starch factory in Thailand by Tanticharoen

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et al. [26], was cultured in 10 l of Zarrouk's medium, which had the following composition (per liter): 16.8 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1.0 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2$, 1.0 g K_2SO_4, 0.08 g EDTA, and 0.01 g $FeSO_4 \cdot 7H_2O$. The organism was cultured outdoors at ambient temperatures while being agitated using an air pump to produce an air flux of 150 l/h. After 7–10 days, the chlorophyll *a* concentration reached to approximately 25 mg/l, at which point the culture was used for subsequent experiments. All experiments conducted in this study were inoculated with 20% (v/v) *S. platensis* BP inoculum.

To determine a suitable medium, *S. platensis* BP was cultured in a 6-l semi-continuous mode of four mediums. Modified Zarrouk's medium that contained 8 g/l $NaHCO_3$ was used as a control medium. Medium 1 contained 20% effluent from a UASB treatment (UASB effluent) facility on a pig farm, 3.5 g/l $NaHCO_3$, and 0.2 g/l urea fertilizer (46:0:0, N:P:K). Medium 2 contained 20% UASB effluent, 4.5 g/l $NaHCO_3$, and 0.2 g/l urea. Medium 3 contained 20% UASB effluent, 3.5 g/l $NaHCO_3$, and 0.2 g/l N:P:K fertilizer (16:16:16).

The cultivation was conducted outdoors at ambient temperatures while maintaining the chlorophyll *a* concentration at approximately 5 mg/l throughout the experiments and mixing using an air pump. At every 2–3 days, approximately 30% of the total volume of the culture was harvested and the *S. platensis* BP cells were then separated by filtration. Fresh medium was added to the system after each harvest. The results of this experiment were used to select a suitable medium for use in a pilot-scale experiment in 100-l open raceway ponds with a culture depth of 15 cm. The mixing was provided by paddle wheels facilitating the velocity of water at 12 rpm. Around 30% of the total volume of the culture was harvested every 2–3 days and the same volume of freshly prepared culture medium was added, establishing a semi-continuous culture. Evaporation was counteracted by adding, every morning, the same level of fresh water.

The cell concentration was measured as the chlorophyll *a* concentration, using methanol extraction and subsequent determination of the absorbance at 665 nm [28]. To determine the dry weight, 25-ml samples were filtered through Whatman GF/C filter papers (47-mm diameter). While the samples were being filtered, they were washed with 25 ml of acidified water (pH 4) to eliminate salt precipitates, after which they were dried in an oven at 80°C for 24 h and weighed.

The alkalinity, and phosphorus and nitrogen-nitrate levels in the filtrates were determined using the titration method, vanadomolybdophosphoric colorimetric method, and ultraviolet spectrophotometric screening method, respectively, according to the APHA [3]. The nitrogen-ammonia concentration was determined by the salicylate method (Method 10031 in the *Handbook of the ODYSSEY DR/2500 Spectrophotometer*).

Protein content was determined by Lowry's method, where under alkaline condition, peptide bonds react with copper (II) followed by the Folin–Ciocalteau reagent and subsequently the absorbance is measured at 750 nm [14]. Fatty acids were analyzed using a modified version of the method described previously [13], and fatty acid methyl esters were identified by gas chromatography (GC 17A; Shimadzu, Japan). The phycocyanin content was estimated after extraction in a phosphate buffer (pH 7.0) following the procedures described by Boussiba and Richmond [7].

All experiments were carried out in duplicate or triplicate. Results of the analyses were submitted to analysis of variance (ANOVA) with confidence level of 95% ($P<0.05$) to verify significant differences in productivity, cell composition, and level of nutrients remaining in the filtrates.

Results and Discussion

Characteristics of UASB Effluents from a Pig Farm

UASB effluent from a pig farm was used because this type of waste contains a small amount of sediment and rarely contains contamination from other algae. As shown in Table 1, the pH of the UASB effluent was 7.94, whereas the total alkalinity and phosphorus in the effluent were 1,040 mg/l and 88 mg/l, respectively. The nitrogen was present as ammonia (144 mg/l) and nitrate (19.9 mg/l). It has been reported that a concentration of ammonia greater than 2.5 mM or 42 mg/l is toxic to many algae, including *Spirulina* [1, 15]. To avoid growth inhibition due to excess ammonia, *Spirulina* was cultured in 20% UASB effluent (v/v). As shown in Table 1, the amounts of nitrogen in the forms of ammonia and nitrate in the 20% UASB effluent were 28 mg/l and 3.6 mg/l, respectively. Moreover, the total alkalinity of the 20% UASB effluent was only 280 mg/l and there was no bicarbonate available at this dilution. Therefore, additional carbon and nitrogen were added to increase the growth of *Spirulina* to an economically feasible level.

Nitrogen is primarily removed by active uptake during the growth of *Spirulina* and by ammonia stripping into the atmosphere. When cultured under nitrogen limited conditions, phycocyanins are degraded after the available nitrogen is exhausted, and are subsequently used as nitrogen reserves for the synthesis of proteins [10, 24]. Previous studies added sodium bicarbonate as a carbon source at concentrations of 9 to 17 g/l to enable the growth of *Spirulina* on digested waste. However, some studies showed that the addition of bicarbonate in the range of 3 to 4 g/l is sufficient for the growth of *Spirulina* [12, 28]. Tanticharoen *et al.* [26]

Table 1. Characteristics of UASB effluent from a pig farm.

Parameters	Original value	20% UASB effluent (v/v)
pH	7.94±0.01	7.47±0.33
Total alkalinity (mg/l)	1,040±28	280±28
Ammonia (mg/l)	144±16	28±2
Nitrate (mg/l)	19.9±0.8	3.6±0.0
Phosphorus (mg/l)	88±0	17.6±0.3
Total Kjedahl nitrogen (mg/l)	150	nd[a]
Biochemical oxygen demand (mg/l)	30	nd
Chemical oxygen demand (mg/l)	155	nd
Suspended solids (mg/l)	96±8	nd
Total solids (g/l)	1.28±0.02	nd

[a]Not determined.
In this and the subsequent tables, values are expressed as mean ± S/D of n=3.

reported that *S. platensis* BP could grow in starch wastewater that contained 2,500 mg/l bicarbonate when it was provided *via* the addition of sodium bicarbonate and in conjunction with urea and/or N:P:K fertilizer (16:16:16) as a nitrogen source. In outdoor cultivation, *Spirulina* prefers ammonia or urea and will only consume nitrate when ammonium is not available. It has been found that there is a time lag in the growth of *S. platensis* when ammonium nitrate is used as the source of nitrogen [5, 11]. In addition, the use of a less expensive nitrogen source such as urea is particularly attractive from an economic viewpoint.

Therefore, in this study, sodium bicarbonate was added to 20% UASB at a concentration of 3.5 to 4.5 g/l in conjunction with 0.2 g/l of urea or N:P:K fertilizer to identify a suitable medium for the culture of *S. platensis*.

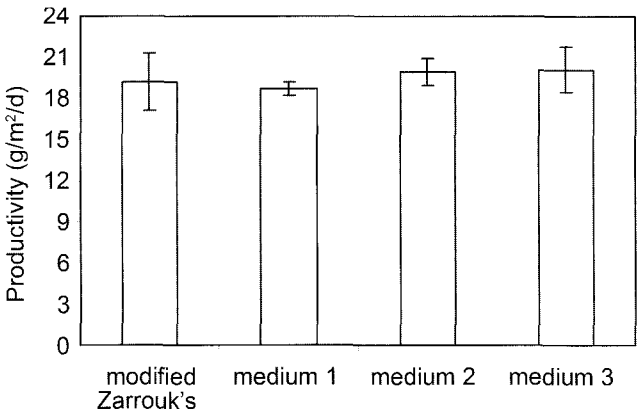

Fig. 1. The average productivity of *S. platensis* BP grown in different media in 6-l scale for 15 days.

Productivity of *S. platensis* BP and Cell Composition

In a 6-l scale study, the average productivities of *S. platensis* BP grown in medium 2 and medium 3 were 19.9 g/m^2/d and 20.1 g/m^2/d, respectively. These values were comparable to the productivity of *S. platensis* grown in modified Zarrouk's medium (19.2 g/m^2/d). Conversely, the lowest productivity was 18.7 g/m^2/d, which was observed when *S. platensis* was cultured in medium 1 (Fig. 1).

When the remaining nutrients in the filtrate of all three wastewater media were considered, it was found that adding N:P:K fertilizer resulted in a significantly higher (P<0.05) amount of phosphorus remaining in the filtrate of medium 3 (25 mg/l) than in medium with urea added. However, no significant difference (P<0.05) in bicarbonate alkalinity, total alkalinity, and nitrate in the filtrate of all media were observed. The filtrate of all media contained less than 5 mg/l ammonia and around 5 mg/l nitrate (Table 2). The remaining bicarbonate alkalinity and total alkalinity in the filtrates of the media containing 3.5 and 4.5 g/l of sodium bicarbonate were in the range of 370–750 mg/l and 3,700–4,200 mg/l, respectively. According to these data, which showed that the level of nutrients remaining in the filtrate of samples cultured in medium 2 was not high and that the productivity was slightly higher than medium 1, medium 2 was considered a suitable medium and therefore used for pilot-scale experiment.

The average productivity of *S. platensis* BP grown in medium 2 in the pilot-scale experiment (100 l) was approximately 12 g/m^2/d, which was not significantly different (P<0.05) from the productivity obtained when it was grown in modified Zarrouk's medium (12.7 g/m^2/d) (Fig. 2). Its composition was also not statistically different (P<0.05) in cells grown in medium 2 compared with modified Zarrouk's medium. The cell composition of *S. platensis* BP grown in medium 2 was as follows (% dry weight): protein (57.9), total fatty acid (4.18), linoleic acid (0.7), γ-linolenic acid (1.12), and phycocyanin (19.5) (Table 3).

Raoof *et al.* [22] reported that growth of *Spirulina* in revised medium containing 4 g/l of sodium bicarbonate was significantly lower than in standard Zarrouk's medium with 16.8 g/l sodium bicarbonate and in revised medium with 8 g/l sodium bicarbonate. On the other hand, a study conducted by Olguín *et al.* [17] reported a dry biomass of *Spirulina* sp., grown in complex medium containing sea-water supplemented with 2% (v/v) of anaerobic effluents from digested pig waste having 2 g/l of sodium bicarbonate, that was similar to the one observed in Zarrouk's medium. However, the protein content of the biomass in the complex medium was significantly lower compared with the content in Zarrouk's medium. The results in this study indicated that *S. platensis* BP could be grown in 20% UASB effluent supplemented with 4.5 g/l of sodium bicarbonate and 0.2 g/l of urea fertilizer. The productivity and composition of biomass in this medium were not significantly different from those obtained when the organism was cultivated in modified Zarrouk's medium. Calculation of the cost of chemical indicated that the

Table 2. Characteristics of the filtrate of medium after harvesting *S. platensis* BP in 6-l scale (all parameters in mg/l).

Parameters	Modified Zarrouk's	Medium 1	Medium 2	Medium 3
Bicarbonate alkalinity	1,133±39	653±342	752±382	370±345
Total alkalinity	5,116±718	3,736±654	4,186±718	3,741±665
Phosphorus	115±3	17±7	16±7	25±2
Ammonia	0	<5	<5	<5
Nitrate	928±137	6±3	6±4	5±3

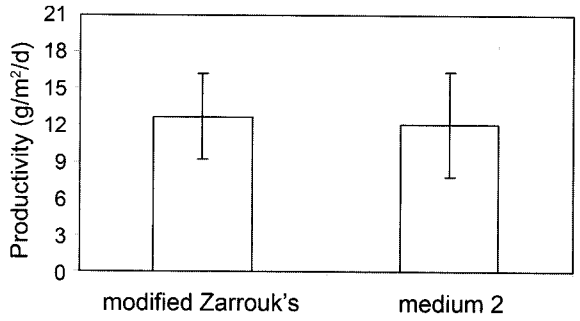

Fig. 2. The average productivity of *S. platensis* BP grown in pilot scale (100 l) for 17 days.

preparation of 1,000 l of modified Zarrouk's medium would cost approximately 533 Baht (US$ 15.21) compared with 120 Baht (US$ 3.41) for medium 2. Therefore, medium 2 was found to have high potential economically, since the amount of sodium bicarbonate compared with modified Zarrouk's medium (8 g/l of sodium bicarbonate) can be reduced by almost half (4.5 g/l) and its cost is 4.4 times cheaper than modified Zarrouk's medium.

Nutrients Removal

The pattern of nutrient removal in the pilot-scale experiment is shown in Fig. 3, where changes in bicarbonate, phosphorus, and ammonia levels were observed after the addition of fresh medium. The addition of 4.5 g/l sodium bicarbonate in medium 2 resulted in bicarbonate alkalinity and a total alkalinity of approximately 2,500 mg/l and 4,100 mg/l, respectively. The nutrients were removed instantly by *Spirulina* uptake. The reduction of bicarbonate alkalinity and total alkalinity were removed in the same manner. The average rate of bicarbonate alkalinity removal was around 380 mg/l/d.

The characteristics of the UASB effluent from pig farms showed that the levels of phosphorus and nitrate in the 20% UASB effluent were 17.6 mg/l and 3.6 mg/l, respectively (Table 1). At the initial point of cultivation, the addition of inoculum to the culture resulted in phosphorous and nitrogen levels in the culture of 30 mg/l and 270 mg/l, respectively (Fig. 3B and 3C). The phosphorus levels decreased rapidly within 2 days and its removal rate slightly decreased

Table 3. Composition of *S. platensis* BP grown in modified Zarrouk's medium and medium 2 in pilot scale.

Cell compositions (% dry weight)	Modified Zarrouk's	Medium 2
Protein	61.1±0.6	57.9±0.3
Total fatty acid	4.28±0.15	4.18±0.16
Linoleic acid	0.74±0.04	0.70±0.02
γ-Linolenic acid	1.16±0.04	1.12±0.02
Phycocyanin	19.2±0.9	19.5±0.1

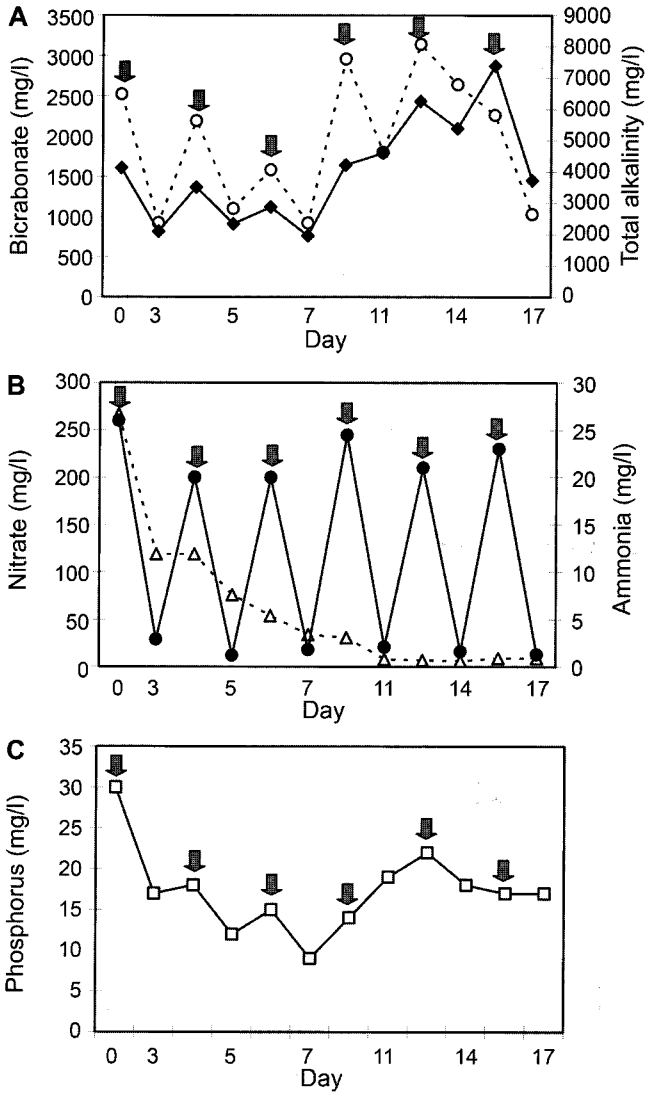

Fig. 3. Changes of nutrient in medium 2, before and after use for *S. platensis* BP cultivation in pilot scale.
Arrow indicates the addition of fresh medium to the pond. **A.** Bicarbonate alkalinity (○) and total alkalinity (◆). **B.** Nitrate (Δ) and ammonia. (●) **C.** Phosphorus (□).

after 8 days of cultivation. At the end of the experiment (17 days of cultivation), the phosphorus level was 17 mg/l.

After 2 days of cultivation, the nitrate level sharply dropped in the same pattern as in phosphorus reduction, until the level fell below 10 mg/l. Conversely, regards the change in the level of ammonia, high ammonia levels were observed after the addition of fresh medium. However, the ammonia levels were reduced to less than 5 mg/l within one day.

During the first 8 days of cultivation of *S. platensis* BP in medium 2, the average rates of total nitrogen (nitrate and ammonia) and phosphorus removal were approximately 34 mg/l/d and 4 mg/l/d, respectively. A study by Pouliot *et al.* [19] reported that the rates of nitrogen and phosphorus

removal were 10–18 mg/l/d and 1.0–1.5 mg/l/d, respectively. However, the reported nitrogen and phosphorus removal efficiencies varied depending on the media composition and environmental conditions such as the initial nutrient concentration, light intensity, nitrogen/phosphorus ratio, light/dark cycle, and species of algae [4].

Regarding cell observation, *S. platensis* BP grown in medium 2 turned from blue green to yellow green after cultivation for more than 17 days. It is probable that nitrogen limitation occurred in the culture. When depleted of nitrogen, *Spirulina* will use phycocyanin as nitrogen reserves for synthesis of other proteins [10, 24]. Moreover, the rate of nitrogen removal of *S. platensis* was 34 mg/l/d whereas the level of total nitrogen in the medium was approximately 33 mg/l of which around 23 mg/l was from ammonia and 8–10 mg/l was from nitrate (Fig. 3B). Moreover, the reduction in the amount of ammonia was not only by active uptake during *Spirulina* growth but also by stripping into the atmosphere. The optimal conditions required for the growth of *Spirulina*, which are high temperature and pH values, and a good aeration rate, greatly contribute to the stripping of ammonia. We confirmed that ammonia was lost to the atmosphere by comparing the levels in wastewater without cells. The results revealed that the daily reduction in the levels of ammonia in the UASB effluent without and with *S. platensis* BP were 70% and 100%, respectively (data not shown). It can be estimated that only 30% of ammonia was available for *Spirulina* assimilation. This result corresponded to Proulx *et al.* [20], who reported that ammonia stripping accounted for at least 62% of nitrogen removal on outdoor culture of the cyanobacterium *Phormidium bohneri*.

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