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Development of an effective acidogenically digested swine manure-based algal system for improved wastewater treatment and biofuel and feed production

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HIGHLIGHTS

- Coupling low cost and eco-friendly algae-based biofuel with animal feed production.
- Developing an effective algal system on acidogenically digested manure by a 2^2 CCD way.
- High algal growth rate and nutrient removal rates are obtained from the algal system.
- Having high algal lipid productivity (3.63 g m^2 d^{-1}) for low-cost biofuel production.

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ABSTRACT

An effective semi-continuous process was developed to grow a locally isolated green microalga *Chlorella* sp. on acidogenically digested swine wastewater in bench scale for improved algal biomass production and waste nutrient removal using central composite design (CCD). The influences of two key parameters, namely wastewater dilution rate (DR) and hydraulic retention time (HRT), on algal biomass productivity and nutrient removal rates were investigated. The optimal parameters estimated from the significant second-order quadratic models ($p < 0.05$) were 8-fold DR and 2.26-d HRT. The cultivating experiment in a bench-scale multi-layer photobioreactor with the optimized conditions achieved stable algal productivity and nutrient removal rates, which fitted the predictive models well. Moreover, relatively high and stable protein and lipid contents (58.78% and 26.09% of the dry weight, respectively) were observed for the collected algae sample, indicating the suitability of the algal biomass as ideal feedstock for both biofuel and feed production.

1. Introduction

Biomass energy made from traditional crops (oil seeds, sugar crops, wheat, etc.) is considered as a viable alternative to fossil fuels. However, advances in first generation biofuel technologies have encountered economic, ecological, and policy concerns, including competition for arable land with food and feed production, consequential significant food price hike, etc. Microalgae have great potential to replace current feedstock crops, because their productivity is much higher than terrestrial energy crops, and is not constrained by season and land availability and quality. Algal cells provide lipids for biodiesel or crude oil production, carbohydrates for bioethanol and biobutanol production, and/or nutritional compounds for animal feed production [1]. Cultivation of algae on swine manure is considered to be a potentially practical and economical strategy for algal feedstock production and wastewater treatment. The combination could help relieve the livestock producers from the significant financial burden associated with the treatment of the unmanageable growing manure prior to discharge. However, the process has not been commercialized yet, since the strategy is still faced with the lack of suitable algae strains and the dearth of carbon in the wastewater [2,3].

Our previous report showed that locally isolated facultative heterotrophic microalga strain *Chlorella* sp. (UMN271) was capable of

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utilizing volatile fatty acids (VFAs) including acetic, propionic and butyric acids, which are the major soluble organic carbon substrates in swine manure [4]. This *Chlorella* strain grew well on the diluted VFA-enriched swine wastewater effluent from the acidogenic fermentation, with the algal growth rate as high as 0.90 d^{-1}, the lipid content of approx 30%, and the nutrient removal of 458.4 mg COD L^{-1}, 69.5 mg N L^{-1} and 12.03 mg PO_4–P L^{-1} after the 5-day lab-scale batch cultivation.

The next step is to build a system to continuously produce algal feedstock, in which the practical culture conditions for high biomass production and nutrient assimilation can be established. In semi-continuous mode, a proportion of the culture is replaced with fresh media when the majority of microalgae reach late logarithmic growth phase, and then the culture is maintained for days to increase cell density before a next replacement. The repeated harvest-regrowing process can be maintained for a week or for several months without apparent growth decline in the system [5]. Algae growth and their cellular biochemical composition are able to be affected by environmental and culture conditions, such as irradiance, temperature, salinity, and nutrient balance [6,7]. However, the factors that could be adjusted in a pilot-scale semi-continuous culture are limited, among which hydraulic retention time (HRT) and medium dilution rate (DR) are important for algal growth and wastewater treatment.

HRT is the length of time that a soluble compound remains in a constructed bioreactor. In a semi-continuous process, the ratio of the culture volume in the bioreactor to the daily replaced volume is the HRT, which is a key factor influencing algae growth and nutrient uptake. Olguín [8] reported that a high-rate algal pond could be operated at short HRTs in the range of 4–10 days. Uwimana et al. [9] found that HRT was an influential factor that determined the removal efficiency of pathogens from the algal ponds fed with municipal wastewater. Sreesai and Pakpain [10] concluded that HRT influenced both nutrient uptake and growth development of *Chlorella vulgaris* in treated septage wastewater.

Wastewater DR affects the turbidity and nutrient concentrations in the algae cultures, which is also an important factor for algae growth. Cheunbarn and Peerapornpisal [11] reported that *Spirulina platensis* grown on highly diluted anaerobically treated swine manure (5-fold and 10-fold dilutions) resulted in higher cell densities than those on more concentrated manure (1-fold–3.33-fold dilutions) during the 2-week batch study because the darkness and turbidity in concentrated swine manure significantly affected algal photosynthesis. Wang et al. [12] reported that the growth rates and nutrient removal rates by *Chlorella* sp. on 10-fold and 15-fold diluted manure were initially slower than those on 20-fold and 25-fold diluted manure, but caught up in the latter part of the 21-day batch cultivation probably due to the continued algal growth sustained by the higher nutrient concentrations in the more concentrated manure.

Although several literatures have shown the appropriate dilution of swine manure [13] or the preferable HRT [14] for algae growth, it is difficult to directly apply the DR and HRT values in the microalgae-acidogenically digested swine manure system, because there are significant differences in composition between acidogenically digested swine manure and manure media used in the previous reports. Therefore, it is necessary to investigate the effects of DR and HRT on algae growth and waste nutrient removal, and then determine the optimal conditions specifically for the system by using some optimization method.

The Box–Wilson central composite design (CCD) is a useful mathematical approach widely used in the optimization of cultivation processes, in which treatment time and process variability could be reduced, the predictive responses could be closer to the target achievement, and interactions of two or more variables could be studied simultaneously. Kim et al. [15] used CCD to optimize the culture conditions (initial pH, nitrogen and phosphate concentrations) for the mass production of three green algae *Chlorella* sp., *Dunaliella salina* DCCBC2 and *Dunaliella* sp. Khataee et al. [16] used CCD to optimize the biological decolorization of textile wastewater by macroalgae *Chara* sp. However, there is still little research using CCD for the optimization of culture conditions for both algal mass production and wastewater treatment.

In the light of the above discussion, CCD was used in the study to develop a quadratic mathematical model for the prediction of the optimum HRT and DR for the *Chlorella* sp. mass production and the removal of swine wastewater nutrients. Another objective of this study was to develop an effective algae production process using acidogenically digested swine manure with a novel bench-scale photobioreactor.

2. Materials and methods

2.1. Algae strain and seed culture preparation

Alga strain *Chlorella* sp. UMN271, which was isolated from Loon Lake, Waseca MN, was used in the study. The preparation and maintenance of the inoculums was accomplished using 250 mL Erlenmeyer flasks containing 100 mL BG-11 medium with 2 g L^{-1} glucose according to Hu et al. [4]. After 1–2-week cultivation at 25 ± 2 °C under a continuous cool white fluorescent light illumination of 100 μmol m^{-2} s^{-1}, the algal cells were separated from the culture broth using centrifuge at 2000 rpm for 5 min, followed by washing with deionized water and another centrifugation–suspension process.

2.2. Characteristics of swine wastewater

The fresh swine manure and the inoculum sludge were collected from the University of Minnesota Southern Research and Outreach Center, Waseca MN. The fresh swine manure was used as the substrate during the acidogenic digestion in the study, and its characteristics are shown in Table 1. The sludge was anaerobically cultivated for 5 days with 5 g L^{-1} glucose at temperature 38 ± 1 °C to get the activated and concentrated inoculum, and then was heat-treated at 80 °C with a water bath (Thermo Fisher Scientific Inc., Waltham, MA) for 30 min to kill methanogenic bacteria from the community according to Wang et al. [17].

2.3. Acidogenic digester setup and operation

A glass bioreactor with the working volume of 4 L was used as the anaerobic digester. The reactor was operated in semi-continuous mode. At the beginning, 1.0 L concentrated inoculum was added into the reactor containing 3.0 L fresh manure substrate. The mixture was adjusted to approx pH 5.3 with sulfuric acid (4 mol H_2SO_4 L^{-1}) solution, and was maintained at pH 5.3–5.6 and 38 ± 1 °C for the acidogenic fermentation for 48 h as described by

Table 1
Characteristics of fresh swine manure.

Parameter	Value
pH	7.58 ± 0.31
TVSS (mg L^{-1})	2580.01 ± 300.01
Total nitrogen (mg TNL^{-1})	2031.43 ± 66.19
Ammonia–nitrogen (mg NH_3–N L^{-1})	1602.86 ± 84.72
Phosphate–phosphorus (mg PO_4–P L^{-1})	407.43 ± 99.58
COD (mg L^{-1})	17,240 ± 816.66
Total VFAs (mg L^{-1})	7676.26 ± 576.37
Acetic acid (mg L^{-1})	4957.08 ± 357.48
Propionic acid (mg L^{-1})	1612.03 ± 116.13
Butyric acid (mg L^{-1})	1107.16 ± 105.48

Hu et al. [4]. The 2-day batch fermentation was followed by a 10-day semi-continuous operation with the HRT of 3 days determined in the previous study [4]. During the operation, 1.33 L of the mixed liquor was drained from the reactor and was replaced with an equal volume of fresh manure substrate at the beginning of each day. The pH and temperature were kept at 5.3–5.6 and 38 ± 1 °C, respectively, during the 10-day operation. Samples were taken from daily effluent and feeding substrate for the VFA concentration test. In the study, the initial drained effluent was used in experiment I as described in Section 2.4, and the effluents in the rest 9 days were used in experiment II as described in Section 2.5.

2.4. Optimization of conditions for the semi-continuous mode (experiment I)

2.4.1. Factorial design

The experiments were performed to develop a mathematical model for the response variables, including biomass productivities and nutrient removal rates, and to predict the optimum DR and HRT for biomass production and wastewater treatment. The experiments were designed according to a 2^2 circumscribed central composite response surface methodology (RSM) to build a second order model for the response variables without employing a full factorial experiment design. Each factor was designed with 5 coded levels ($-\alpha$, -1, 0, +1, $+\alpha$) which could constitute 25 combinations of DR and HRT at different levels. The typical value of α is a function of the number of variables $\alpha = (2^k)^{1/4}$, where k is the number of independent variables. According to the two-variable CCD approach, only nine variable level setting combinations with the α value of 1.41 were needed for experiment runs in the study, which were presented in the forms of one center point, four factorial points and four star points in Fig. 1. The design matrix of the DR and HRT in both coded levels and actual values, as shown in Table 2, was created using SAS 8.1 software (SAS Institute Inc., Cary, NC).

2.4.2. Algae growth experiments

The designed 13 runs listed in Table 2 were carried out in 250 mL Erlenmeyer flasks containing 150 mL of unsterilized acidogenically digested swine manure with different DRs and an initial *Chlorella* sp. biomass concentration of 0.3 g L^{-1}. All the cultures were adjusted to around pH7.0 at the beginning of the incubation, and were maintained at 25 ± 2 °C under 100 μmol m^{-2} s^{-1} white fluorescent light on a 16:8 h light/dark cycle for one week to obtain the stationary-phase algae. The one-week batch cultivation was followed by 10-day semi-continuous cultivation with various HRTs (Table 2) at the same temperature and light conditions as described above. Samples were taken from the daily harvested cultures and feed solutions for the total volatile suspended solid (TVSS) and nutrient concentration tests. The data collected after the cultivation system reached quasi steady states were used for the statistical analysis in Section 2.4.3. To avoid bias, 13 runs were performed in a totally random order.

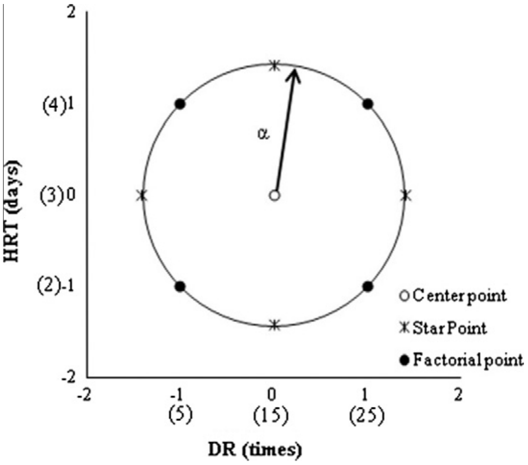

Fig. 1. Two-variable central composite design.

2.4.3. Quadratic model analysis

Data of algal biomass and nutrient contents collected from samples on the 9th and 10th days of the semi-continuous cultivation were used to calculate the algae biomass productivity (μ_1, mg L^{-1} d^{-1}) and removal rates of chemical oxygen demand (COD), phosphate–phosphorus (PO_4–P), total nitrogen (TN), and ammonia–nitrogen (NH_3–N) (μ_2–μ_5, respectively, mg L^{-1} d^{-1}) according to Eq. (1).

$$\mu_i = |i_{10} - [i_9(X_2 - 1)/X_2 + i_{M9}/(X_1X_2)]| \tag{1}$$

where μ_i is the daily change of i's concentration; i_9 and i_{10} are concentrations of i in the harvested cultures on day 9 and 10, respectively; i_{M9} is i's concentration in the feeding swine manure on day 9; X_1 and X_2 are the values of DR and HRT for the experiment run, respectively.

The results of μ_1–μ_5 were analyzed statistically through analysis of variance (ANOVA) at 95% confidence interval and RSM with SAS8.1 ADX Interface software. Second-order quadratic models were established to evaluate the effects of DR and HRT on the responses, including algal biomass productivity (Y_1, mg L^{-1} d^{-1}), COD removal rate (Y_2, mg L^{-1} d^{-1}), PO_4–P removal rate (Y_3, mg L^{-1} d^{-1}), TN removal rate (Y_4, mg L^{-1} d^{-1}), and NH_3–N removal rate (Y_5, mg L^{-1} d^{-1}), as in Eq. (2) by using the method of least squares:

$$Y_i = a_{i0} + a_{i1}X_1 + a_{i2}X_2 + a_{i12}X_1X_2 + a_{i11}X_1^2 + a_{i22}X_2^2 \tag{2}$$

where Y_i is the predicted response; X_1 and X_2 are the real values of DR and HRT, respectively; a_{i0}, a_{i1}, a_{i2}, a_{i12}, a_{i11} and a_{i22} are the coefficients in Eq. (2) to be determined by the statistical analysis.

2.5. Application of the predictive optimum in a bench-scale multi-layer photobioreactor (experiment II)

The objective of this experiment was to develop an effective algae–acidogenically digested swine manure system using the bench-scale photobioreactor with the predicted optimal DR and HRT according to the regression models. The novel bench-scale photobioreactor system consisted of a proprietary 2-layer reactor and a recycling peristaltic pump (Cole–Parmer Co., Vernon Hills, IL). A schematic drawing the bench-scale cultivating system is shown in Fig. 2. In the experiment, *Chlorella* sp. seeds were inoculated at approx 0.2 g L^{-1} in the 2-layer photobioreactor containing 4 cm-high unsterilized manure effluents from the acidogenic digester with the optimal DR. Seven-day batch cultivation followed by 10-day semi-continuous cultivation with the predicted optimal HRT was operated at 25 ± 2 °C under 100 μmol m^{-2} s^{-1} white fluorescent light on a 16:8 h light/dark cycle. During the incubation period, the culture unit was daily refilled with deionized water to compensate for evaporation. TVSS, pH, COD, PO_4–P, TN and NH_3–N were assayed daily, while lipid content, protein content, and the fatty acid profile were analyzed on the harvested algae samples on the last 3 days of the 10-day semi-continuous cultivation.

2.6. Analytical methods

TVSS, pH, COD, PO_4–P, TN and NH_3–N were analyzed in accordance with the standard methods [18] and instructions in the Hach DR5000 spectrophotometer manual [19].

Table 2
Central composite design matrix and the results of the response variables including biomass productivity (μ_1, mg L^{-1} d^{-1}), COD removal rate (μ_2, mg L^{-1} d^{-1}), PO_4–P (μ_3, mg L^{-1} d^{-1}), TN (μ_4, mg L^{-1} d^{-1}), and NH_3–N (μ_5, mg L^{-1} d^{-1}) in the CCD runs.

Run	DR (times)	HRT (days)	μ_1 (mg L^{-1} d^{-1})	μ_2 (mg L^{-1} d^{-1})	μ_3 (mg L^{-1} d^{-1})	μ_4 (mg L^{-1} d^{-1})	μ_5 (mg L^{-1} d^{-1})
1	5(−1)	2(−1)	332.67	1027.33	6.77	36.20	29.33
2	5(−1)	4(+1)	121.89	349.18	0.42	14.06	10.41
3	25(+1)	2(−1)	98.82	299.33	0.19	9.57	8.95
4	25(+1)	4(+1)	95.35	285.58	0.08	5.69	5.12
5	1(−α)	3(0)	89.26	275.33	0.39	6.56	6.48
6	30(+α)	3(0)	76.10	254.88	0.14	6.39	6.28
7	15(0)	1.586(−α)	164.27	445.18	0.72	15.70	14.83
8	15(0)	4.414(+α)	102.20	243.10	0.12	6.12	5.89
9	15(0)	3(0)	220.50	678.90	2.75	22.75	16.35
10	15(0)	3(0)	221.50	688.90	2.81	24.35	15.80
11	15(0)	3(0)	225.50	692.90	2.65	24.55	14.98
12	15(0)	3(0)	219.50	704.10	2.92	21.05	18.21
13	15(0)	3(0)	215.50	691.00	2.55	20.98	17.56

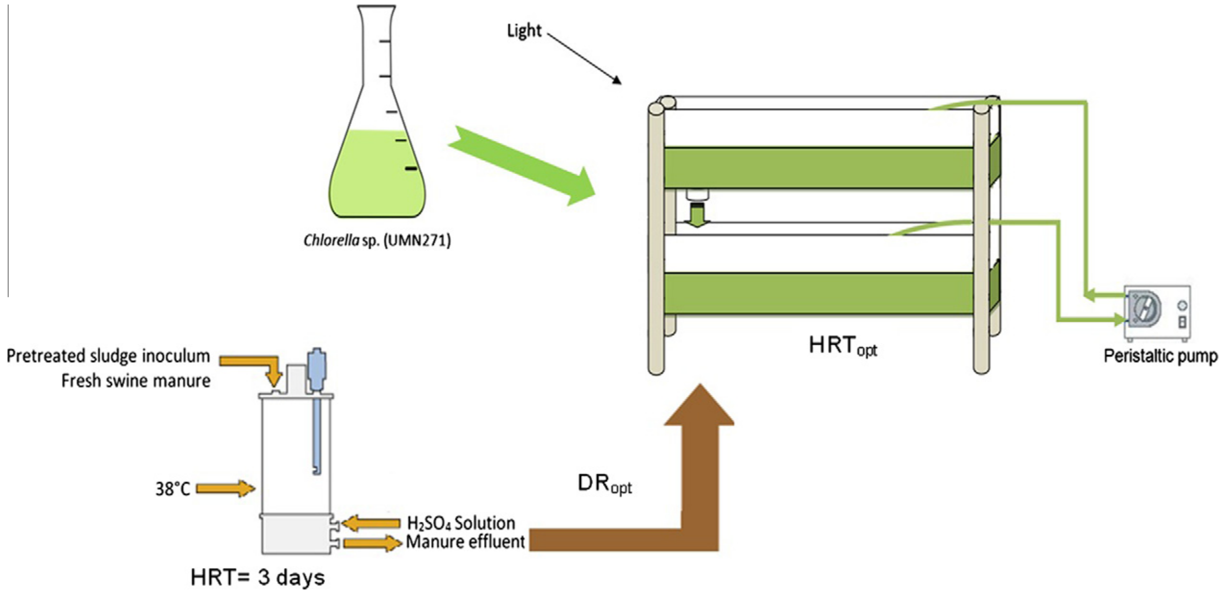

Fig. 2. Schematic drawing for the bench-scale acidogenically digested swine manure-based microalgae cultivating system.

The concentrations of acetic, propionic and butyric acids were measured with an Agilent 7820A gas chromatography with flame ionization detector (GC–FID) according to the method of Zhou et al. [20].

Total lipid contents in the harvested algae cells were analyzed using the one-step extraction method adapted from Folch method [21]. The fatty acid composition of the harvested cells were extracted with a one-step extraction–transesterification method as described by Zhou et al. [22], and was determined through the GC–MS analysis using an Agilent 7890-5975C gas chromatography–mass spectrometry with a HP-5 MS capillary column.

The protein content of the freeze-dried algal biomass was determined from the nitrogen content data evaluated with a CE-440 elemental analyzer (Exeter Analytical Inc., Chelmsford, MA), using the nitrogen-to-protein conversion factor (NTP) of 6.35 [23].

3. Results and discussion

3.1. Semi-continuous acidogenic fermentation of fresh swine manure

Fresh swine manure was inoculated with concentrated manure sludge in which the methanogenic bacteria were reduced. The mixture was anaerobically incubated for 48 h and then acidogenically fermented in 10-day semi-continuous mode with the HRT of 3 days for the production of VFA-enriched swine manure. As shown in Fig. 3, the VFAs production reached the steady state on the 10th day of the acidogenic fermentation, with the VFAs productivity of 2002.25 mg L^{-1} d^{-1} on average. The total VFAs concentration in the acidogenically digested manure effluents from the semi-continuous process was in the range of 12,500–14,000 mg L^{-1}, which was close to the highest VFAs concentration in the acidogenic, batch fermentation of liquid swine manure (LSM) in our previous report [4], indicating that the semi-continuous, acidogenic fermentation was effective in promoting VFAs production in swine manure.

It is observed from Fig. 3 that the semi-continuous process had a noticeably higher VFAs yield in the swine manure than the batch mode (12,000 mg L^{-1} on day 2 before manure exchange), which was likely due to a slight rise in the reactor pH when fresh manure was fed into the reactor during the semi-continuous process. Rogers [24] found that the accumulation of organic acids could lead to a drop in the digester pH, and the switch from acidogenesis (a VFAs-production metabolic pathway) to solventogenesis (a ketone-production metabolic pathway) would happen at low pH values so that the acidogenic fermentation process would be inhibited. The moderate-to-high yields obtained with the batch-to-semi-continuous process used in this work demonstrates the advantage

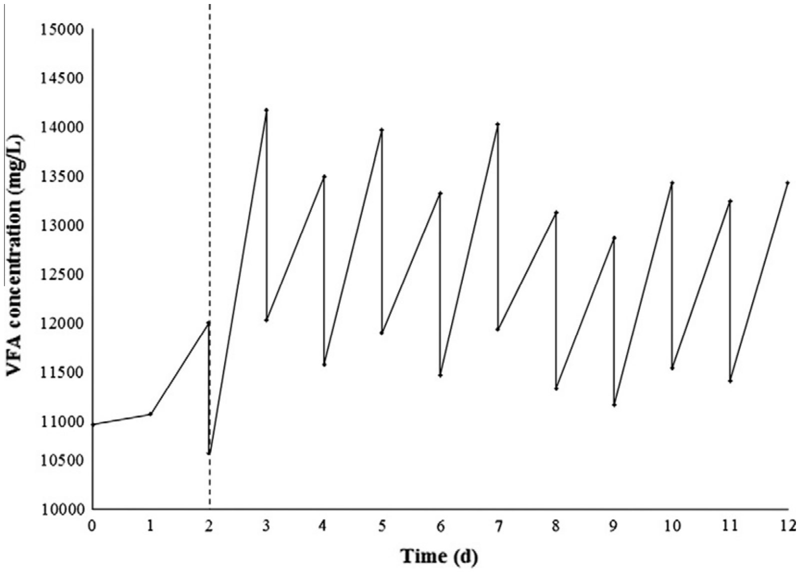

Fig. 3. Change in total VFAs during acidogenic fermentation of fresh swine manure in the 2-day batch fermentation (before the vertical dotted line) and the following 10-day semicontinuous mode (after the vertical dotted line). The results are average of triplicate determinations.

of acidogenic, semi-continuous fermentation in the production of VFA-enriched swine manure. The daily drained effluent from the semi-continuous digestion system was used for the cultivation of *Chlorella* sp. in experiments I and II.

During the modified anaerobic digestion process, not only VFAs but also H_2 was produced. H_2 is a clean and potential fuel to replace hydrocarbon fuels with a high energy density between 120 and 142 kJ g^{-1}. The idea of biohydrogen production from animal wastes through anaerobic fermentation is not new. Although the H_2 productivity was not significant in our study, it has been reported that there are several methods to improve biohydrogen production from animal manure, such as chemical, heat and/or pH pretreatments, temperature control and additional feeding [25–27]. Therefore, it is believed that investigation on the modification of anaerobic digestion on swine manure for effective production of VFAs and H_2 could go very far.

3.2. Optimization of DR and HRT in CCD for the microalgae-acidogenically digested swine manure system (experiment I)

The 2^2 CCD with five repeated center points was used to find the optimal values of DR and HRT for algal biomass production and nutrient removal from the acidogenically digested swine manure.

The experimental results, including algal biomass productivity (μ_1, mg L^{-1} d^{-1}), COD removal rate (μ_2, mg L^{-1} d^{-1}), PO_4–P (μ_3, mg L^{-1} d^{-1}), TN (μ_4, mg L^{-1} d^{-1}), and NH_3–N (μ_5, mg L^{-1} d^{-1}), for each CCD run are listed in Table 2. Among the 13 treatments, run 1 (DR = five times, HRT = 2 d) had the highest biomass productivity and nutrient removal rates, while run 6 (DR = 30 times, HRT = 3 d) had the lowest μ_1 and μ_2 and the second lowest μ_3–μ_5. The responses in run 4 (DR = 25 times, HRT = 4 d) were lower than those in run 2 (DR = five times, HRT = 4 d), and the differences were more significant between run 3 and run 1, whose DRs were 25 and 5 times, respectively, and HRTs were both 2 days. It is believed that manure with high DR limited algae growth. Also, it is considered that the shorter the HRTs, the greater the limitation is on algae growth and nutrients uptake. This result was similar to reports of Brune who found that the algal cell density was positively related to HRT if there was no light limitation [28]. However, the behavior was contradictory to run 5 (DR = one times, HRT = 3 d) and run 9–13 (DR = 15 times, HRT = 3 d), in which the smaller μ_1–μ_5 were obtained in the treatment with lower DR. Similar results were observed by Mezzomo et al. [29] who investigated the influences of swine wastewater concentration and sodium biocarbonate concentration on the growth of microalgae *S. platensis*. They suggested that the swine manure was toxic to the microalgae when used without dilution because of sunlight penetration problem caused by the coloration of swine manure, the toxicity of ammonia, and the competition from other microorganisms in the wastewater.

The SAS ADX interface program was used to analyze the CCD data sets in Table 2 to build up the quadratic mathematical models of DR and HRT responding to the algal biomass productivity and removal rates of COD, PO_4–P, TN and NH_3–N. According to the statistical analysis of the variable estimates (data not shown), the independent variables of DR (X_1, X_1^2) and HRT (X_2, X_2^2) had significant effects on the biomass production and the removal of COD, PO_4–P, TN and NH_3–N, while the interactions between DR and HRT (X_1X_2) had low significances on *Chlorella* sp. to remove PO_4–P, TN and NH_3–N ($p > 0.05$). The quadric models predicted for the response variables of Y_1–Y_5 using significant coefficients are given as Eqs. (3)–(7) in Table 3.

In the study, the quadratic models predicted for the response variables of Y_1–Y_5 are all statistically valid. The statistical significance of each quadratic model was evaluated by using ANOVA technique as shown in Table 4. It was observed that the *F*-values of the five models were more or less higher than the critical *F*-value (critical $F = 3.972$), and the *p*-values of the five models were all relatively low ($p < 0.05$), indicating that the regression models were significant at high confidence levels.

The three-dimensional (3D) response surface plots for Y_1–Y_5 against the two experimental factors of DR and HRT are depicted in Fig. 4. The plots for Y_1, Y_2, Y_4 and Y_5 showed that higher biomass productivities and nutrient removal rates were generally obtained with short HRT, but there was a strong effect of DR (Fig. 4a–e). Considerably lower biomass production and nutrient removal rates were attained at high DR values and short HRTs. Generally, it was found that the highest biomass productivity, COD, TN and NH_3–N removal rates were obtained at the DR of 8.00 times and the HRT of 2.26 d. From the Y_3 surface plot (Fig. 4c) and the counter plot (not shown), it can be seen that PO_4–P removal rate was sensitive to higher values of DR and HRT, and the maximum PO_4–P removal rate was observed for DR of 11.4 times and HRT of

Table 3
Regression equations for the response variables, including the algal biomass productivity and removal rates of COD, PO_4–P, TN and NH_3–N (Y_1–Y_5, respectively).

Model	Equation label
$Y_1 = 2.143 \times 10^2 + 1.397X_1 + 25.234X_2 - 0.186X_1^2 + 1.082X_1X_2 - 8.505X_2^2$	Eq. (3)
$Y_2 = 4.132 \times 10^2 - 9.489X_1 + 3.658 \times 10^2X_2 - 1.559X_1^2 + 15.554X_1X_2 - 1.189 \times 10^2X_2^2$	Eq. (4)
$Y_3 = -1.104 \times 10^2 + 4.062X_1 + 84.115X_2 - 0.182X_1^2 - 16.145X_2^2$	Eq. (5)
$Y_4 = 8.169 + 1.315X_1 + 15.375X_2 - 0.054X_1^2 - 3.183X_2^2$	Eq. (6)
$Y_5 = 31.660 + 1.863X_1 + 16.752X_2 - 0.078X_1^2 - 4.003X_2^2$	Eq. (7)

Table 4
ANOVA for the quadratic models predicted for the response variables of the algal biomass productivity and removal rates of COD, PO_4–P, TN and NH_3–N (Y_1–Y_5, respectively).

Source	SS[a]	DF[b]	MS[c]	*F*-value ($p < 0.05$)
Y_1				
Model error	2.292	5	0.458	8.205 ($p = 0.008$)
Residual error	0.391	7	0.056	
Total	2.683	12		
R^2[d]	85.42%			
Adjusted R^2[e]	75.01%			
RMSE[f]	0.236			
CV[g]	4.706			
Y_2				
Model error	5.853×10^5	5	1.171×10^5	5.048 ($p = 0.028$)
Residual error	1.623×10^5	7	2.319×10^4	
Total	7.476×10^5	12		
R^2[d]	78.29%			
Adjusted R^2[e]	62.78%			
RMSE[f]	152.279			
CV[g]	29.836			
Y_3				
Model error	24.612	5	4.922	13.482 ($p = 0.002$)
Residual error	2.556	7	0.365	
Total	27.168	12		
R^2[d]	90.59%			
Adjusted R^2[e]	83.87%			
RMSE[f]	0.604			
CV[g]	−224.715			
Y_4				
Model error	3.839	5	0.768	4.914 ($p = 0.030$)
Residual error	1.094	7	0.156	
Total	4.932	12		
R^2[d]	77.83%			
Adjusted R^2[e]	61.99%			
RMSE[f]	0.395			
CV[g]	15.052			
Y_5				
Model error	2.799	5	0.560	5.601 ($p = 0.022$)
Residual error	0.700	7	0.100	
Total	3.498	12		
R^2[d]	80%			
Adjusted R^2[e]	65.72%			
RMSE[f]	0.316			
CV[g]	12.943			

[a] Sum of square.
[b] Degree of freedom.
[c] Mean squares.
[d] Coefficient of correlation.
[e] Coefficient of determination.
[f] Root-mean-square error.
[g] Coefficient of variation.

2.66 d.

Nevertheless, since the drop in PO_4–P removal rate from the apex of the Y_3 surface plot (21.80 mg PO_4–P L^{-1} d^{-1}) to the point with DR of 8.00 times and HRT of 2.26 d (18.11 mg PO_4–P L^{-1} d^{-1}) was not very much, the optimal values of DR and HRT in the microalgae-acidogenically digested swine manure system is still determined to be 8.00 times and 2.26 d, respectively. The research presented herein was the first to use CCD for the optimization of culture conditions for both algal mass production and wastewater treatment.

3.3. Development of a bench-scale system for fast algae growth and nutrient removal (experiment II)

The predictive optima (DR_{opt} = 8.00 times, HRT_{opt} = 2.26 d) were utilized for *Chlorella* sp. cultivation on swine manure effluent obtained from the acidogenic digester as mentioned in Section 3.1 in a bench-scale 2-layer photobioreactor containing 17 L (4-cm water depth for each layer).

The process parameters used in this experiment demonstrated a good performance in growing algae and removing nutrients from the acidogenically digested swine manure. The algal cell density as TVSS, nutrient concentrations including COD, PO_4–P, TN and NH_3–N in the manure culture, and the culture pH were measured daily during the 17-day cultivation, and the data are shown in Figs. 5 and 6. At the end of the period I (7-day batch mode), *Chlorella* sp. reached stationary phase with the cell density of 780 mg L^{-1} (Fig. 5a), and the nutrient levels of 238 mg COD L^{-1} d^{-1}, 28.6 mg PO_4–P L^{-1} d^{-1}, 48.3 mg TN L^{-1} d^{-1} and 28.2 mg NH_3–N L^{-1} d^{-1} (Fig. 5b–d). As shown in Table 5, the cultivation performance achieved stable algal productivity of 276.18 mg L^{-1} d^{-1} and nutrient removal rates of 751.33 mg COD L^{-1} d^{-1}, 20.21 mg PO_4–P L^{-1} d^{-1}, 38.35 mg TN L^{-1} d^{-1}, and 60.39 mg NH_3–N L^{-1} d^{-1} during period II (10-day semi-continuous mode). It is noticed that TN removal rate was lower than NH_3–N removal rate, which was probably due to the fluxes of dissolved organic nitrogen (DON) from the suspended manure particles by the algae strain. Tyler et al. [30] found that the opportunistic green macroalgae *Ulva lactuca* leaked DON from sediment into water column during its active growth in shallow lagoon in Hog Island Bay, Northampton County, VA. As shown in Table 5, the experimental values were close to the predicted response variables, which was very good for the goodness of fit. The result that the experimental values were slightly higher than the theoretically predicted values can be associated to the ammonia volatilization from the 2-layer photobioreactor and, therefore for the reduced ammonia content in the culture medium during algae growth. Although ammonia is a good nitrogen source for algae growth, Azov and Goldman [31] found that the growths of fresh and marine algae *Scenedesmus obliquus*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* were all suppressed in intensive cultures with high loadings of ammonia because of the ammonia inhibition on the short-term algal photosynthesis. Chen et al. [32], studying the optimal pretreated anaerobically digested liquid dairy manure effluent for the cultivation of a selected *Chlorella* strain in a pilot-scale semi-continuous fed raceway pond, observed that the algal growth rate (based on TVSS) significantly decreased from 0.0538 d^{-1} in media with the nutrient loading of 200 mg TN L^{-1} to approx 0.045 d^{-1} in media with higher nutrient loading of 300 and 400 mg TN L^{-1}. Nevertheless, we believe that

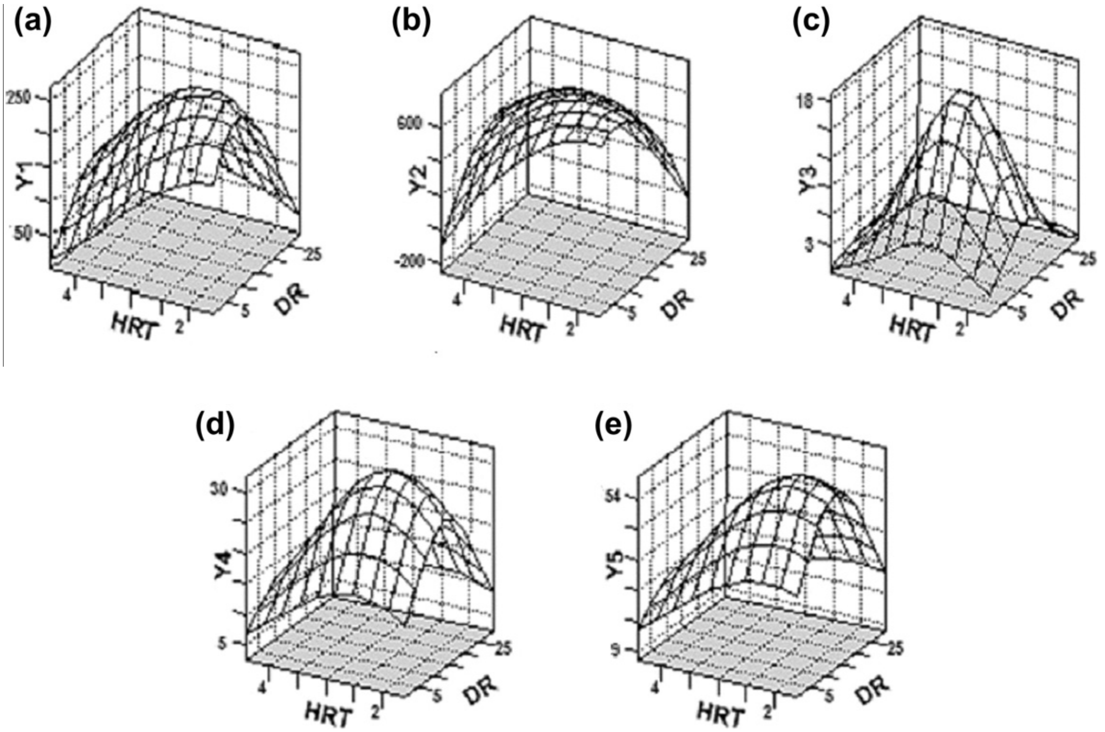

Fig. 4. Response surface plots for biomass productivity, Y_1 (a), COD removal rate, Y_2 (b), PO_4–P removal rate, Y_3 (c), TN removal rate, Y_4 (d), and NH_3–N removal rate, Y_5 (e) as functions of DR and HRT.

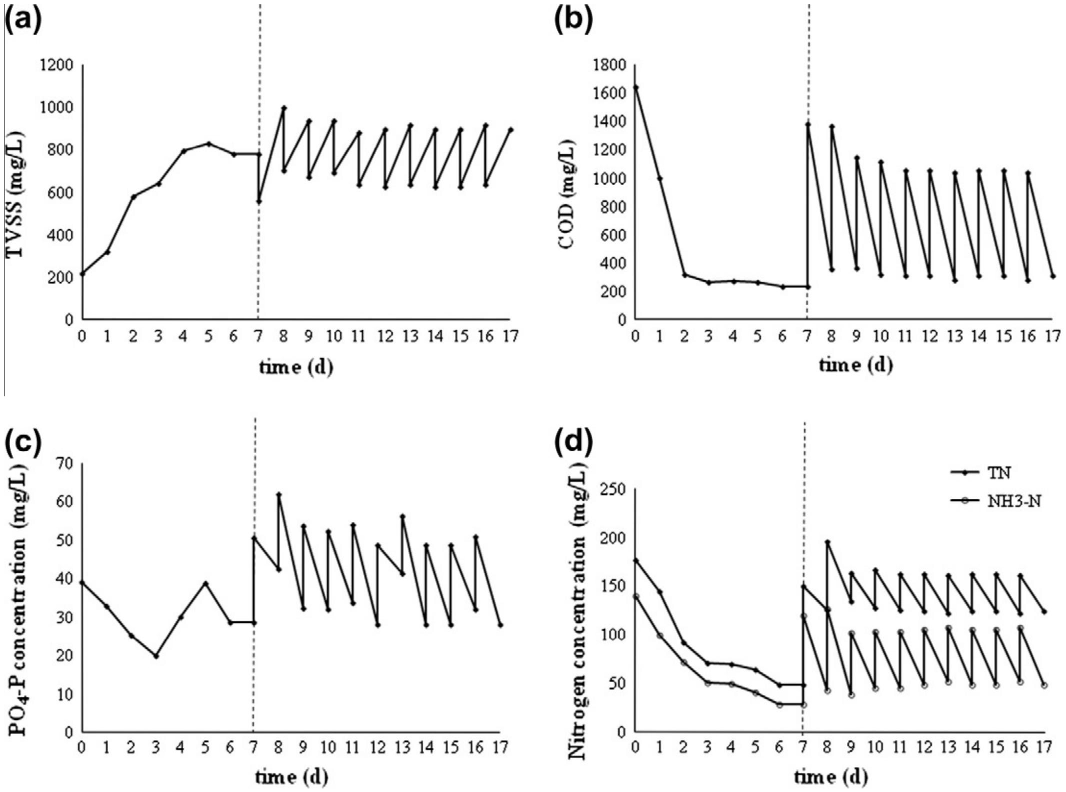

Fig. 5. Time courses of algal cell density as TVSS (a), COD concentration (b), PO_4–P concentration (c), TN and NH_3–N concentrations (d) during the 17-day cultivation of *Chlorella* sp. on 8-fold diluted, acidogenically digested swine manure. The vertical dotted line represents the interface between the batch and semi-continuous processes.

the removal of NH_3–N in the system was mainly attributed to the nitrogen uptake by *Chlorella* sp. other than ammonia volatilization, since the pH of the culture during the course of the growth experiment (Fig. 6) was always below pH 9.3 which was reported as pK_a of NH_4^+/NH_3 at room temperature [33]. Moreover, the time course of pH values in Fig. 6 demonstrated that the fed VFA-enriched manure

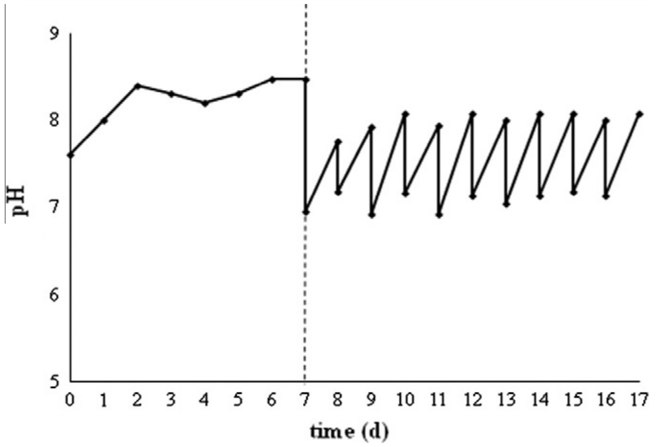

Fig. 6. pH changes during the 17-day cultivation of *Chlorella* sp. in experiment II. The vertical dotted line represents the interface between the batch and semi-continuous periods.

Table 6
Chemical composition of *Chlorella* sp.

Name of the compounds	
Composition	Content
(a) Chemical composition of Chlorella sp. on a dry matter basis (%)	
Protein	58.78 ± 1.05
Lipids	26.09 ± 1.13
Others (carbohydrates, nucleic acids, etc.)	15.13 ± 0.69
(b) Fatty acid profile derived from triacylglycerol, phospholipid and free fatty acids in Chlorella sp. collected from the semi-continuous microalgae-acidogenically digested swine manure system	
Total fatty acid/dry weight (%)	9.66 ± 1.83
Saturated fatty acids (% of total fatty acids)	62.83 ± 8.96
C14:0	12.34 ± 1.69
C15:0	5.51 ± 0.45
C16:0	17.41 ± 1.57
C18:0	19.00 ± 1.62
C20:0	8.57 ± 1.53
Monounsaturated fatty acids (% total fatty acids)	31.80 ± 8.32
C16:1	9.34 ± 1.86
C18:1	22.46 ± 2.81
Polyunsaturated fatty acids (% total fatty acids)	1.53 ± 0.08
C20:2	1.53 ± 0.08

medium could not only be used as a nutrient source, but also had a neutralizing effect against the culture alkalization which was mainly caused by the consumption of dissolved CO_2 in the culture during algal photosynthesis [34].

The chemical compositions of algal biomass collected from the culture effluents on the last three days of the semi-continuous process were measured using Folch extraction, elemental analysis, and GC–MS analysis to elucidate the potential of *Chlorella* sp. as algal feedstock for biofuel and feed production.

As shown in Table 6a, the lipid content (on a dry matter basis) of *Chlorella* sp. grown in the semi-continuous system was around 26.09% of dry biomass. According to our previous reports, the alga strain *Chlorella* sp. had similar lipid contents to the one in the study when it was grown on concentrated municipal wastewater (CMW) and 20-fold diluted, conventionally digested swine manure (DSM), which were 27.50% and 23 ± 4.32%, respectively [15,35]. These experimental findings are in agreement with those reported by Woertz et al. [3] who used the green algae community to treat municipal wastewater, 10% and 25% diluted dairy manure, and obtained the algal lipid content as 9.0–11.3%, 8–14%, and 10–15%, respectively. Therefore, it is considered that the lipid contents of the locally isolated *Chlorella* sp. grown on wastewaters are relatively stable and high, in the range of 25–30%. According to the data in the study, the lipid productivity of the microalgae-acidogenically digested swine manure system ($3.63\ \mathrm{g\ m^{-2}\ d^{-1}}$) corresponds to about $1.00 \times 10^4\ \mathrm{L\ ha^{-1}\ y^{-1}}$. Though our algal lipid productivity is much lower than the 5.87×10^4–$1.37 \times 10^5\ \mathrm{L\ ha^{-1}\ y^{-1}}$ reported for algal oil yield from the artificial media, it is 16.74 times greater than that for soybean oil production ($598.6\ \mathrm{L\ ha^{-1}\ y^{-1}}$) [36], and thus, presents an exciting possibility as a low-cost feedstock for biofuel production.

Around 37.03% (on a dry matter basis) of total lipids in the frozen-dried algal biomass were free fatty acids and fatty acids derived from triacylglycerol and phospholipids (Table 6b). GC–MS analysis showed that the fatty acids in the algal cells were mainly composed of saturated C14–C18 fatty acids and monounsaturated C16–C18 fatty acids (62.83 ± 8.96% and 31.80 ± 8.32% of total fatty acid weight, respectively) with C18:0 and C18:1 as the major compounds (22.46 ± 2.81% and 19.00 ± 1.62%, respectively). It is considered that transesterification of the fatty acid composition in our study could produce high-quality biodiesel. Xu et al. [37] found that the biodiesel from algal oil of *Chlorella protothecoides* was abundantly composed of 18 carbon acid methyl esters, and had comparable physical and fuel properties to diesel fuel. Moreover, linolenic acid (C18:3), which should be lower than 12% for a quality biodiesel according to EN14214 standard [4], was not detected in the fatty acid composition in our study, further indicating the capability of the harvested algal oil as a good-quality biodiesel resource.

The *Chlorella* sp. cells or the remaining biomass fraction after oil extraction from algae can be used as a high protein feed for livestock to further help offset costs of algal mass production. As reported in Table 6a, the crude protein content of the algal strain was 58.78 ± 1.05%, which was comparably high among various microalgal species (6–71% of dry biomass) [38]. The use of microalgae as animal feed, such as in poultry farms and aquaculture, has been investigated for many years, and the research on waste-grown algae as food is more recent [39]. According to Cook et al. [40], the protein quality of sewage and organic wastes-grown green algae *Scenedesmus quadricauda* and *Chlorella* sp. incorporated with varying amounts of powdered skim milk, wheat, oat cereals was not inferior to that of milk protein, and the algae-milk-cereal mixture was demonstrated to have no negative effect on the 21-day-old weanling rats. Therefore, it is predicted that the acidogenically digested swine manure-grown algal biomass could be a valuable feed substitute for conventional protein sources.

Although the bench-scale culture system developed in our study is considered to be low-cost and effective for algal biomass

Table 5
Theoretically predicted and experimental values for algal biomass productivity, removal rates of COD, PO_4–P, TN and NH_3–N during the semi-continuous process in experiment II.

	Biomass productivity ($\mathrm{mg\ L^{-1}\ d^{-1}}$)	Nutrient removal rate ($\mathrm{mg\ L^{-1}\ d^{-1}}$)			
		COD	PO_4–P	TN	NH_3–N
Predictive	246.76	738.04	18.11	33.73	58.96
Experimental	276.18	751.33	20.21	38.35	60.39

production and nutrient removal from acidogenically digested swine manure simultaneously, and has the potential for commercialization, we cannot predict the feasibility of the system in an out-door, large-scale operation. Problems such as contamination by zooplankton or other adventitious organisms, evaporation, and temperature volatility could potentially prevent the system from being a practical strategy for the production of algae-based biofuel or feed [41]. Therefore, the study needs to be confirmed in a pilot scale before being commercially viable.

4. Conclusions

It is concluded that the combined CCD approach and response surface analysis were effective to determine the optimum conditions of DR and HRT for algal biomass production and nutrient removal from the acidogenically digested swine wastewater. By semi-continuously cultivating *Chlorella* sp. on acidogenically digested swine wastewater under the optimal conditions (DR_{opt} = 8.00 times, HRT_{opt} = 2.26 d) using a bench-scale multi-layer photobioreactor, stable mass productivity and nutrient removal rates slightly higher than the predicted values were obtained, and the algal biomass produced in the system can be a good feedstock for biofuel and feed production.

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