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# Poultry Manure Digestate Enhancement of *Chlorella Vulgaris* Biomass Under Mixotrophic Condition for Biofuel Production

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## Abstract

Poultry manure (PM) was decomposed under high and low aeration-agitations and the digestates were supplemented in mixotrophic cultivation of microalga *Chlorella vulgaris*. High aerobic decomposition was recommended for faster mineralization. The study was conducted to find out the effect of poultry manure digestate (PMD) on cell biomass and lipid yield in *C. vulagris*. The cultivation were conducted 'with' (single and two-stage feeding strategy) and 'without' PMD feeding. Cultivation 'without' PMD at 120 h, dry cell weight (DCW) of 8.2g/L was reached, by 180 h, lipid yield of 2.1 g/L (45%) was reached. In single-stage of adding varied PMD, at 120 h, DCWs of 8.48, 9.39 and 10.45 g/L were achieved for PMD of 20, 30 and 40 ml/L, respectively. By 180 h, lipid contents were 45, 43 and 40% giving yields of 2.4, 2 and 1.8 g/L, respectively. In two-stage feeding (0-120 h and 120-180 h), at 120 h, DCWs were similar to single-stage but improved when supplementing with 2 g/L glucose reaching DCW of 12.6, 13.14 and 14 g/L achieving lipid yields of 2.9, 3.8 and 4.9 g/L, respectively, after 180 h. The addition of glucose seems to assist nitrogen depletion which in turn resulted in rapid increase in cellular lipid. It was obvious that addition of glucose at stationary phase maybe a novel method to improve lipid yields. The algal biomass PMD dependent accumulation showed that PM is an attractive waste which means that PM is potential waste for algae biofuels.

**Keywords:** Aeration; Digestion; Digestate; *Chlorella vulgaris*; Poultry manure

## Introduction

Poultry manure (PM) is rich in nutrients especially the much needed nitrogen and phosphorus, and even cells growth promoter like glycine is released from PM on decomposition (Schefferle, 1965). According to the study by Magid et al. (1995), some common nutrients in PM by composition included (g/Kg) potassium 37.5, phosphate 25.5 and nitrogen 55.7. Nitrogen is normally in the form of uric acid (Nahm, 2003) and about 66% can be available on decomposition (Ruiz Diaz et al., 2008. In addition, studies indicated that other trace elements, such as magnesium, calcium, iron, copper, zinc, nickel, lead and chromium existed in digested PM (Bao et al., 2008; Ortiz Escobar and Hue 2008; Faridullah et al., 2009; Vu et al., 2009). In brief, PM has been a traditional organic fertilizer and is an attractive source eve today from which much needed nutrients can be retrieved and reutilized.

The use of nutrients-rich PM can be extended to algal cultivation provided the conducive conditions (light intensity, pH, temperature, equipments) to enhance algal biomass production. This approach of using PM maybe novel if it enhances high cell density which in turn can increase more biofixation of carbon dioxide (Jacob-Lopes et al., 2008; Jacob-Lopes et al., 2009) under an autotrophic or mixotrophic culture condition. The later mode of cultivation (mixotrophic) comparably had shown a proven feasibility in production of high yields of algal biomass according (Liang et al., 2009).

Algae biomass as one of the suitable feedstock for biofuel production is not new given the recent studies, researches and soaring developments which are primarily due to energy crisis, climate change and environmental. As the focus on algae as the fuel of the future increases, the need to make algae biofuel more sustainable falls into a handful of categories from economic assessment to engineering. One such category is converting waste (industrial and housewhole 'liability') to microalgae biomass, converting housewhole or industrial waste being a 'liability; to profit center is an attractive method to promote algae biofuel at large. PM is one such waste and the focus of this study was using PM to see if microalga *Chlorella vulgaris* currently used in algae biofuel can enhance algae biomass, that is cell concentration and cellular lipid yields. The PM, an organic matter (form) needs biodigestion to release absorbable nutrients that can be fed to the algae in culture cultivation. Simple aerated biodigestion (decomposition) can be engaged to produce PMD after filtration (aqueous).

Accordingly, the aim of the study was to investigate effects of PMD supplementation photosynthetic microalga *Chlorella vulgaris* currently used in algal biofuel to see if can enhance algal biomass, which is cell concentration and cellular lipids cultivation under mixotrophic condition. The study seeks to broaden the application of PM in algal biofuels.

## Materials and Methods

### Microorganism, culture media and PM

Microalga *Chlorella vulgaris* was obtained from Charles University (Prague, Czech Republic). Fresh PM (from *Galus*

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| Shaking speed | Major nutrients (g/L) N | P | K | Total carbon (mg/L) | Total solids (mg/L) | N in solid residue (mg/L) | pH |
|---|---|---|---|---|---|---|---|
| 50 rpm (in 4L/min air) | 1.1967 | 0.57 | 2.89 | 34 | 64 | 55 | 4.5 |
| 100 rpm (in 6L/min air) | 0.462 | 0.625 | 2.98 | 23 | 47 | 38 | 5.1 |

**Table 1:** Average contents of the major nutrients in PM from two repeated digestions of a 650 g PM sample for each set investigated.

| rpm | $NO_3$-N (mg/L) | $NH_4$-N (mg/L) | Time (h) | rpm | $NO_3^-$ mg/L | $NH_4^+$ mg/L | Time (h) |
|---|---|---|---|---|---|---|---|
| **50** | 500g | | | **50** | 1000g | | |
| | 4.6 | 47 | 24 | | 1.2 | 19 | 24 |
| | 5.1 | 51 | 48 | | 2.2 | 21 | 48 |
| | 5.7 | 58 | 72 | | 2.8 | 25 | 72 |
| **100** | 500g | | | **100** | 1000g | | |
| | 5.9 | 57 | 24 | | 8 | 55 | 24 |
| | 6.8 | 59 | 48 | | 9.8 | 59 | 48 |
| | 7.28 | 61 | 72 | | 10 | 68 | 72 |

**Table 2:** N mineralization ($NO_3$ and $NH_4$) in low (50 rpm) and high (100 rpm) aeration-agitation decomposition of two varied quantities (500 and 1000 g) of PM. N mineralization was monitored during 72 hours into decomposition.

| Time (h) | $CO_2$ addition (ml) | pH | Absorbance | PM addition (ml) | Biomass (g/L) |
|---|---|---|---|---|---|
| 0 | 0.09 | 6.98 | 0.308 | 20 | 0.01 |
| 24 | 0.09 | 6.96 | 0.249 | 40 | 0.036 |
| 48 | 0.12 | 6.76 | 0.288 | 60 | 0.072 |
| 72 | 0.12 | 6.78 | 0.339 | 80 | 1.12 |
| 96 | 0.12 | 6.82 | 0.757 | 100 | 1.09 |
| 120 | 0.15 | 6.88 | 1.015 | 120 | 1.27 |
| 144 | 0.15 | 6.88 | 1.753 | 140 | 1.38 |
| 168 | 0.18 | 6.43 | 2.055 | 160 | 1.67 |

**Table 3:** Autotrophic cultivation (without glucose supplementation): Algal biomass optimization using table 1 result, simultaneous (daily) addition of PMD (20 ml/L/d) and $CO_2$ addition by air aeration (assuming $CO_2$ in air at 0.03 % concentration. Light intensity initially steadied at 150 then increased to 200 µmolphotons $m^{-2}s^{-1}$ after 48 h under standard condition (temperature, agitation) and maintained until day seven (168 h).

*domesticus)* was collected from nearby poultry farm, Jiangnan University, China. The PM was from a meat producing chicken facility, the collected sample was observed to have fallen on the floor and was held in collecting pit until such time for removal and cleaning. The feces had been mixed with beddings, feed particles and feathers which have been considered as part of PM.

Bold's basal media (Watanabe, 1960) component used were (grams per litre): $NaNO_3$ 0.5, $CaCl_2{\cdot}2H_2O$ 0.05, $MgSO_4{\cdot}7H_2O$ 0.15, $K_2HPO_4{\cdot}3H_2O$ 0.15, $KH_2PO_4$ 0.35, NaCl 0.05, glycine 0.1, yeast extract 0.1 and glucose 10. PM supplementations were applied according to PM nitrogen replacing BBM nitrogen by percentage. Seed culture was prepared in a 500 ml flask containing 200 ml medium for 180 h. Flask culture experiments were performed in 500 ml flasks each containing 200 ml medium after inoculating with 10% (v/v) of seed culture (Xu et al., 2006). Temperature, agitation, and growth period were fixed at 27°C, 150 rpm and 180 h, respectively. It was continuously cultured and kept in shaker with in-built fluorescence irradiance at 35 µmol photon $m^{-2}s^{-1}$. Air flow rate in all fermenter cultivations were kept constant at 5 L air/min.

## PM decomposition

Water was added twice to the mass of PM (by volume) and uniformly mixed, digestion was done in 7 L fermenters (Bioengineering, AG, Wald, Switzerland) the same used for mixotrophic algal cultivation with 3 L working volume. Two varied aerated digestions (repeated twice) were done in following manner as shown,

Low aeration-agitation: Air aeration of 4L/min and agitation of 50 rpm

High aeration-agitation: Air aeration of 6 L/min and agitation of 100 rpm

Decompositions were aborted when pH and temperature were constant indicating the end of decomposition. The samples were decanted and centrifuged at 8000 rpm for 10 min, and then sterilized in bottles for 15 min at 121°C and stored at 4°C for later use.

## Analytical procedures

**PM analysis for N, P and K:** Each sample of PM was analyzed for total potassium (K) and total phosphorus (P) concentrations by microwave digestion.

For the analysis of potassium, a sample of 2.0 ml was added to 3.0 ml $HNO_3$ and cold digested for 1.0 h. The solution was transferred to a 50 ml volumetric flask and distilled water added to the calibration. Potassium concentration was obtained by calibration curves using Spectr 220 atomic absorption spectrometer, Varian American at wavelength of 766.5 nm. For the analysis of phosphorus, 2 ml sample was microwave digested and diluted to 50 ml. 4.0 ml of the solution was taken and added to equal amount of 2.5 ml ammonium molybdate and sulfuric acid. Using 2-3 drops of stannous chloride and glycerol as indicators and the absorbance was read at 380 nm.

Total nitrogen and carbon in the aqueous PMD were determined by diluting 10 ml aqueous PMD to 100 ml distill water. The sample was analyzed using High Temperature TOC/TNb Analyzer LiquiTOC II (Elementar Analysensysteme GmbH, Hanau,Germany). Nitrogen in solid digestates were determined by Kjeldahl method (Spanjers et al., 2006). $NH_4^+$-N, and $NO_3^-$-N were determined according to standard procedures (MAFF, 1986). Total solids in the digestates were measured directly by placing a measured quantity (by weight) of sample into a beaker and drying it in an oven at 115 °C to a constant weight.

**Culture growth:** The optimized conditions for maximum biomass such as light, pH, and temperature were adopted from studies by Xu and Soletto (Xu et al., 2006; Soletto et al., 2008). Seed culture of 10% by volume was inoculated in working volume of 3 L fermenter for all fermentations. Cell growth was measured by optical density at 540 nm using UV visible spectrometer. For all cultivations, initial light intensity was maintained at 150 μmolphotonsm$^{-2}$s$^{-1}$ then increased to 250 μmolphotonsm$^{-2}$s$^{-1}$ at exponential growth phase (Jacob-Lopes et al., 2008) as measured by light meter TES 1332A for all sets.

Glucose was monitored by SBA-40C biosensor analyzer (Ding and Tan, 2006). The growth performance were monitored by using the regression equation according to method by Xu et al. (2006). Dry cell weight (DCW) was determined according to Chen and Johns (1991). Culture broths were centrifuged at 8000 rpm for 10 min and cells were washed twice with distilled water and freeze dried (Xu et al., 2006). Total lipids was extracted by soxhlet extraction method (Saydut et al., 2008) after pulverizing. All values are average of double trials in the experiments.

## Results and Discussion

### PM decomposition

Two repeated batches of PM mineralization were investigated. The two sets of decomposition were applied under low (50 rpm in 4 L/min air) and high (100 rpm in 6 L/min air) aeration-agitation as shown in Table 1. The results showed that with low aeration-agitation, total nitrogen, phosphorus and potassium levels reached 1.19, 0.57 and 2.89 g/L, respectively. In comparison, the high aeration-agitation achieved 0.46, 0.62 and 2.98 g/L, respectively.

Clearly, differences in amounts can be seen of total nitrogen, phosphorus, and potassium which were due to varied treatments (high and low aeration-agitation) in decomposition. Firstly, the difference in nitrogen can be due to ammonia volatility (Hansen, 2004) and mechanics of uric acid decomposition (Faridullah et al., 2009), while the increase or decrease of levels of phosphorus and potassium indicates that mineralization had progressed. Noticeably, the differences in the total carbon (34 mg/L in 50 rpm comparing to 23 mg/L in 100 rpm) and total solids (64 mg/L in 50 rpm and 47 mg/L in 100 rpm) further confirm that aeration and agitation were governing factors in the yields of nutrients.

Two varied quantities of PM (500 and 1000 g) were digested (see Table 2) with daily observations of the N-mineralization from 24-72 h. The N-mineralization values (as indicated by levels of $NO_3$ and $NH_4^+$) show the direct influence of volume and oxygen distribution. The ratio of dilution in both remained the same (as described in the Method section), but volume increased – indicating that the concentration and availability of oxygen may govern efficiency of digestion and mineralization. This would mean that the volume of the substrate requires sufficient aeration and agitation to achieve improved levels of $NO_3^-$and $NH_4^+$ during PM digestion. Variations in the levels of $NO_3^-$and $NH_4^+$ were observed (Table 2) which can be seen as corresponding to the varied aeration-agitation that governed microbial activity. For example during the 24-72 h decomposition, $NO_3^-$ remained steady in 500 g of 50 rpm (low volume) aeration-agitation (4.6, 5.1 and 5.7 mg/L) and 100 rpm aeration-agitation (5.7, 6.8 and 7.28 mg/L). Comparing these to $NH_4^+$ levels in 50 rpm (47, 51 and 58 mg/L) and 100 rpm (57, 59 and 61 mg/L) showed a similarly sluggish mineralization.

Even though the levels of $NO_3^-$ and $NH_4^+$ varied (which may due to nature of substrates and initial microbial load), the trend of mineralization agreed with the Table 1 results that aeration and agitation facilitated PM mineralization.

This finding can be supported by a comparison (Table 2) of the N-mineralization values ($NO_3^-$ and $NH_4^+$) such as the PM 1000 g sample (increased in volume). Nitrate levels ($NO_3^-$) were lower (1.2, 2.2, 2.8 mg/L) in 50 rpm aeration-agitation but showed significant increase (8, 9.8 and 10 mg/L) in 100 rpm aeration-agitation. As expected, the $NH_4^+$ also improved from (19, 21 and 25 mg/L) in 50 rpm aeration-agitation to (55, 59 and 68 mg/L) also in 1000 rpm aeration-agitation.

It is clear from the results that aeration and agitation must be sufficiently controlled based on the volume of substrates in order to facilitate sufficient and rapid mineralization in the production of PMD.

The following work will discuss the study using the filtrated PMD in mixotrophic cultivation of microalga *Chlorella vulgaris*.

### PMD and atmospheric carbon dioxide in autotrophic culture

The initial investigations of PMD in supplemental feeding were performed in autotrophic cultures ($CO_2$ and nutrients only) to support conclusions in the mixotrophic cultivation (atmospheric $CO_2$ supplied and glucose). PMD of 20 ml/L/day was daily added and carbon dioxide fed was increased by altering inlet aeration inlet to get the values indicated (see Table 3) assuming atmospheric CO2 at 0.3% concentration was steady.

According to the result, the cell accumulations linearly related to the PMD addition, the resultant algal biomass showed noticeable increment as a result of the previous addition. Even though the data was not statistically evaluated, the raw data showed a general trend of growth curve was depicted showing growth phases (lag, exponential, if graphed).

As noted, the array of data (cell biomass, $CO_2$, PMD) showed that biomass accumulation was governed by PMD addions under the steady favorable condition (light intensity, temperature,

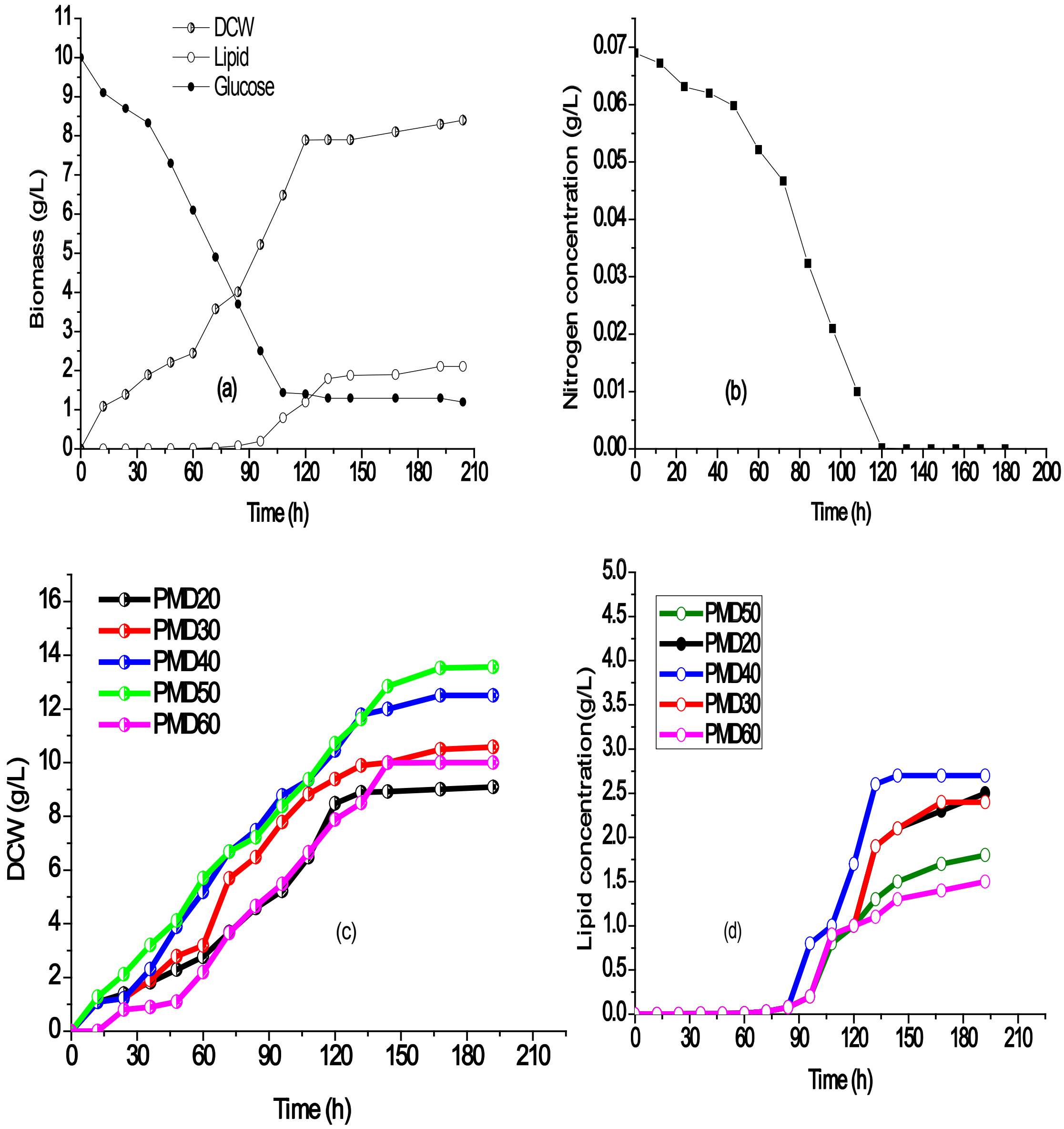


**Figure 1:** (a) Cultivation 'Without' supplementation showing glucose depletion (•), DCW(◑) and lipid yield (○), (b) Nitrogen levels (■) depletion, (c) single feeding of digestate from PMD20, 30, 40, 50 and 60 ml/L (indicated as PMD) and (d) respective lipid yields (corresponding to colors).

feeding, aeration and agitation). Further study and statistical analysis will be needed to measure the significance of PMD on the algae growth and to what level of extends. Our repeated cultivations of the autotrophic microalga in PM supplemental feed bring us to an assumption that PMD processed under described method can be potential for algae cultivation and that was the prerequisite of the following strategic mixotrophic mode of cultivation.

### Cultivation of microalga 'with' and 'without' PMD

Under the culture condition prescribed (light, temperature agitation), at 120 h, algal cultivation 'without' PMD supplementation indicated residual glucose and DCW of 1 and 8.2 g/L, respectively, lipid content then raised to 2.1 g/L at 180 h (Figure 1a). The levels of nitrogen depletion (Fig 1b) showed the relation between cell growth and the lipid yield.

However, upon adding single-feed of varied PMD in volumes, glucose depleted at varied rate resulting in varied biomass as shown in Figure 1c. The following were observed at 120 h; cultivations with 20, 30, 40 and 50 ml/L digestates (represented as PMD20, PMD30, PMD40, and PMD50) achieved DCW of 8.48, 9.39, 10.45 and 10.72 g/L, respectively. After 180 h, DCWs had reached 9, 10.5, 12.5 and 13.52 g/L, respectively. The lipid contents were 45, 43, 40, 37% and (not shown) giving lipid yields of 2.4, 2, 1.8 and 1.5 g/L, respectively, as shown in Figure 1d.

However, the cultivation PM60 (60 ml/L digestate addition) achieved DCW of 10.5 g/L (lowest in comparison) with lipid yield less than 1 g/L (Figure1c and Figure 1d) indicating that increasing further PMD addition was not applicable under the parameters investigated meaning other conditions must be addressed . Moreover, PM50 indicated higher DCW but lower in lipid yield, possibly due to higher nitrogen levels.

In brief, there is a clear difference in DCWs and lipid yields between the 'with' and 'without' PMD supplementations. Increasing PMD addition between 20-40 ml/L clearly increased the biomass yield considerably (comparing 'with' and 'without' PMD which is 8.2 g/L) and maintained lipid content within 40% (not shown) after 180 h.

Concisely, PMD is feasibly potential for application in both

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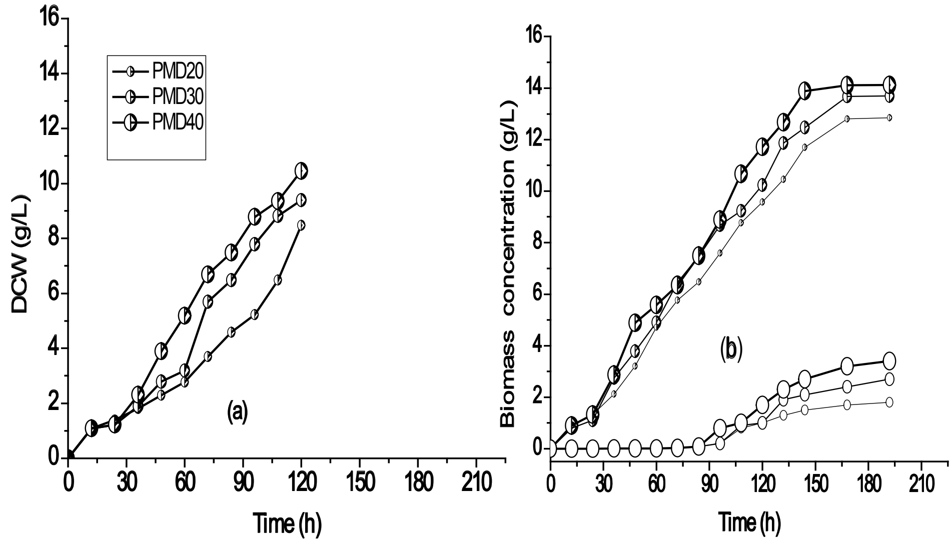


**Figure 2:** (a) Showing DCW (◐), from 0-120 h only relating to PMD feedings (b) respective DCW and, lipid yield (○) from 120-180 h which are initial-exponential (a) and stationary phases (b), respectively.

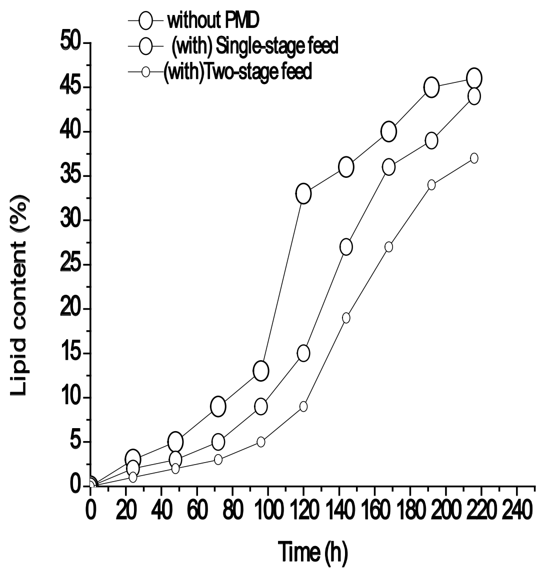


**Figure 3:** Comparative lipid contents in cultivation 'with' (single and two-stage) and 'without' PM digestate feeding.

cell growth and lipid content in appropriate feeding concentrations. The following work further investigated the discussed methods to improve cell concentration and lipid yield in two-stage feeding strategy.

**Two-stage PMD feeding to enhance biomass**

Based on the single-stage feeding cultivations ('with' and 'without' PMD assessed in section 3.3), in this method we studied two-stage feeding. These method of feeding was based on the growth model of 'without' PMD (using standard media) discussed in figure 1a knowing the initial, exponential and stationary growth phases (not detailed in this study) of the microalga under this condition. For the first 5 days during the initial-exponential phase (0-120 h) in three separate fermenters, we added the following (added daily) PMD of 20, 30 and 40 ml/L (based on enhanced biomass in figure 1c and d), respectively, then reduced (PMD) in feeding by one fifth (1/5) respectively, for each cultivation at stationary phase (120-180 h).

The following results were achieved as shown in figure 2a, at 120 h; DCWs of 8.7, 9.8 and 10.8 g/L were reached by 20, 30 and 40 ml/L (shown as PMD20, PMD30 and PMD40), respectively. By 180 h, DCWs of 12.6, 13.4 and14 g/L (Figure 2a) and lipid yields of 1.9, 2.9, and 3.6 g/L (Figure 2b) were achieved, respectively. In comparison to 'without' PMD supplementation (that achieved 8.2 g/L at 180 h), the single (that achieved 13.52 g/L) and two-stage (that achieved 14 g/L) feedings showed the direct influence of the PMD on the lipid content (Fig.3a). It is clearly seen that high lipid yield was achieved in single and two-stage feeding ('with' PMD) in comparison to 'without' PMD which showed high lipid content yet its yield was lower.

The low lipid content in single and two-stage feedings is direct result of the levels of nitrogen that affect lipid biosynthesis. However, lipid yields increased at stationary phase showing that PMD had impact on cells (comparing Figure 2a- Figure 2b). There are two reasons; firstly as a cell growth enhancer (comparing the lag times of 'with' and 'without' PMD, not detailed in this study) and as noted at stationary phases where nitrogen being readily used, cells increased in lipid yield (as seen with Figure 2b). Secondly, the promoting of the residual glucose to storage metabolites (residual glucose and lipid yield

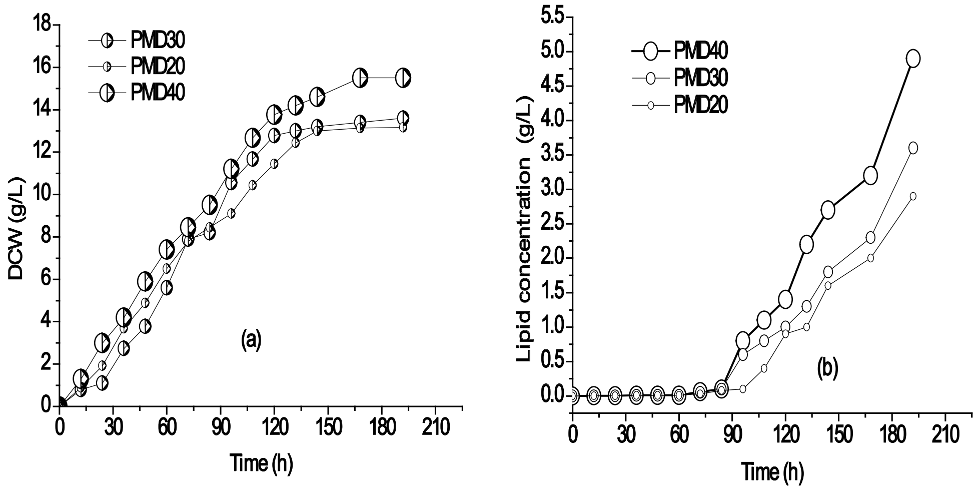


**Figure 4:** DCW (◑) and lipid yield (O) in two-stage feeding methods indicating the relating to 'size' of the key for PMD 20, 30 and 40 ml/L (corresponding sizes of DCWs in 'a' to lipid yields in 'b', respectively).

changes in levels), therefore, the residual glucose analyzed at stationary phase (120-180 h) was seen decreasing as lipid increased in yield. More significantly, this increase was enhanced by adding one fifth (1/5) of the digestate volume (of the previously used from 0-120 h). Adding PMD (1/5) and the raise of lipid yield do not agree knowing the fact that nitrogen will suppress cellular lipid production (Widjaja et al. 2009), the level of residual glucose (during stationary phase) depleting with significant lipid yield increase is a phenomena that prompted the next investigation. Clearly, adding adequate amount (1/5) of PMD seems to reasonably assist biosynthesis of lipid that soared (during stationary phase). Considering the glucose levels, it seemed that appropriate that residual glucose depletion increased lipid levels which meant nitrogen was lowered as it was utilized, thus lipid soared. Accordingly, in the following, we supplemented glucose at stationary phase to see if it can increases lipid at quicker pace.

**Enhancement of algal lipid for with PMD and glucose**

Cellular lipid content can be increased by nutrient limitation (Yan and Quin, 2005; Yanqun and Lan, 2008; Meng et al., 2009) of which nitrogen deprivation is widely used. However, more nitrogen level will increase cell concentration but less in lipid content (Widjaja et al., 2009). Moreover, cells are known to convert available carbon sources such as glucose to generate lipid as storage metabolites (Livne and Sukenik, 1992). In addition, based on our study that lipid increased during stationary phase, the following work was to find out if a combination of PMD and glucose addition at stationary phase (120-180 h) would improve lipid yield for the production of algal biodiesel.

The method applied for Figure 2 was replicated, but only during the stationary phase (120-180 h) instead of feeding only a fifth (1/5) of the PMD, we also incorporated 2 g/L glucose supplementation and feed together daily during the stationary phase (120-180 h).

The results obtained are as shown in Figure 4; at 120 h, the cultivation PM20, PM30 and PM40 achieved DCWs similar to results of Figure 2a, therefore, 8.8, 9.8 and 10.9 g/L, respectively. However, after 180 h, DCWs (Figure 4a) improved to 13.14, 13.4 and 15.5 g/L reaching lipid yields (Figure 4b) of 2.9, 3.8 and 4.9 g/L, respectively.

Based on these figures, it can be considered that appropriate quantities of digestate supplementation directly governed lipid yield output. In addition, it has just demonstrated that an increased glucose dosage and would require increased PMD but appropriate amount to improve final lipid yields.

Given the daily supply of the digestate supplemented with glucose, the sudden raising of lipid yields based on the result can be again concluded that PMD not only enhance algal cell growth and concentration but also support in lipid enhancement by utilizing residual glucose to generate more cellular lipid in quantity. This observation would agree with study by Livne and Sukenik (1992) whose study indicated converting metabolites and cellular residues to storage metabolites (such as lipids). The additional glucose supplementation during stationary phase (120-180 h) is more likely to support lipid yield. This is because as glucose was made available(at stationary phase), any nitrogen residue in the medium was quickly used, culture medium being depleted in nitrogen, cellular lipid content was increased.

The results of the study strongly convinced that PMD being sufficiently digested if correctly utilized in algal cultivation can enhance algal biomass and that is attractive not only for algae biofuel but also for the poultry farmers as PM waste is resourceful.

Study by Natarajan and Varghese (2003) indicated that PM waste used in plankton production had achieved highest yield against other livestock waste, moreover, recent study by Chinnasamy et al. (2010) in a similar fashion strongly pointed that wastewater, municipal waste and other related waste are potential to promote and commercialize algae biofuels.

Based on these studies, our present work agreed with these studies that PM would broaden its application in algal biofuel. This is not only to enhance algal cell density but also to improve lipid yields of microalgae and to promote algae biofuel sustainability.

## Conclusion

The potential of PM as biofertilizer normally used in plant cultivation unmistakably demonstrated a potential for algal biodiesel by two-stage feeding strategy under mixotrophic condition. High nitrogen and phosphorus content would enhance and promote sustainable, waste to bioenergy opportunities with algae. Application of PMD can promote high cell density culture, addition of carbon source such as glucose at stationary phase can promote higher lipid yield. Adding carbon source (glucose) seem a way to get rid of nitrogen at stationary phase that in turn can cause cells to synthesis more lipid content increasing lipid yield.

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