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COMPETITIVENESS OF HUMAN URINE FOR CULTIVATION OF MICROALGAE *Scenedesmus quadricauda* FOR BIODIESEL PRODUCTION

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ABSTRACT

Biodiesel is one of the most promising forms of renewable energy because it is based on carbon sequestration through photosynthesis. Microalgae biodiesel is recognized as the most productive way to generate biodiesel through solving problems such as eutrophication, carbon emission and water shortages. Using nutrients in wastewaters for microalgae cultivation causes problems in terms of contamination of heavy metals, predation and grazing by other organisms, and competition with other undesired species existing in wastewaters.

Human urine (HU) constitutes the main proportion of nutrients available in municipal wastewater and can support microalgae growth, thereby overcoming the disadvantages of municipal wastewater. In this study, HU is used for semi-continuous cultivation of microalgae *Scenedesmus quadricauda* and the results are compared with the biomass yield obtained from an optimized chemical fertilizer (Bold formula). The chlorophyll-a generation was used as the index of biomass production in phototrophic conditions. The light intensity was 42 μmol photons m^{-2} s^{-1} (16h:8h photoperiods) and the temperature was in the range of 29±1°C. The amount of urine was adjusted to provide an equivalent concentration of phosphorus as available in Bold formula (530 μg PO_4-P L^{-1}). The biomass level after 3 days was about 2 times higher in urine (with dry mass of 39.98 mg/L) compared to Bold series (22.9 mg dry mass L^{-1}). The biomass growth in commercial fertilizer was negative due to improper nutrients composition for the algae. The total biomass achieved in urine series was consistently higher than what was attained in Bold solution throughout the entire experiment. Up to 96% of the phosphorous and 61% of the nitrogen in the growth media was removed by microalgae. Conclusively, urine is determined as a proper sustainable source of nutrients for microalgae biomass growth for promoting biodiesel production.

KEYWORDS

Microalgae; Biodiesel; Biomass growth; Growth rate; Phosphorus removal; Human urine; *Scenedesmus quadricauda*; Carbon fixation; Nutrients recycling

1 INTRODUCTION

In recent decades, shortages in energy, contamination of water resources and carbon emission to the atmosphere are becoming critical issues. Biodiesel is one of the most interesting answers for dealing with these problems [1, 2, 3, 4, 5]. Third generation of biodiesel production is the most developed of biodiesel production, which is produced form lipids in microalgae biomass [3, 6, 7]. A high biomass productivity of microalgae and ability to utilize wastewaters as a sustainable source of nutrients are the main advantages of microalgae biodiesel over other sorts of biodiesel generation [4, 8]. Municipal wastewater has been considered as a good source of nutrients for microalgae production because there are different macro and micronutrients available in the wastewater [9, 10]. On the other hand, there are substantial problems regarding usage of crude municipal wastewater for microalgae production. The wastewater is normally contaminated by heavy metals, suspended solids and organic matters. Moreover, high turbidity and low light penetration, high risk of grazing and predation and competition with other microorganisms, high pathogenic and viral infection and unreliable nutrient and physiochemical properties can hinder successful microalgae growth in untreated wastewater [5, 8, 11].Human urine (HU) can alternatively be used as source of nutrients for microalgae biomass production. Human urine makes up less than 1% of volume of household wastewater [12] but it is a main proportion of nutrients in municipal wastewater. Urine makes up to 80% of nitrogen, 55% of phosphorous and 60% of potassium excreted from human body [13, 14]. Using source separated urine is an important step for closing the phosphorous loop as a nonrenewable macronutrient [15]. Human urine is normally transparent and pathogen-free (excluding risk of cross-contamination with fecal material). Furthermore, contamination with organic material, suspended solids and heavy metals is very low in human urine compared to municipal wastewater [13].

Microalgae biodiesel production consists of two main phases which are in compliance with retaining highest neutral lipid productivity. By supporting the photosynthesis activities (usually through nutrient abundance), microalgae biomass growth is maximized in the first step. After biomass is reached the optimum level, lipid accumulation is triggered through environmental stresses as the second phase. This normally happens via nutrients (typically nitrogen) or salt depletion [16, 17, 18, 19]. The former phase (biomass growth) is more important than the latter one (lipids accumulation) to optimize the total biodiesel yield [20]. In this paper, the first phase is followed using human urine as sustainable source of nutrients for biomass growth and the results are compared with the biomass yield from a chemical-based culture medium (Bold's formula).

2 MATERIAL AND METHODS

Isolated microalgae *Scenedesmus quadricauda* was growing in 8 L stock containers. The stock culture medium was receiving about 1 ml of commercial fertilizer with the following ingredient every third day (per 100 ml): 5.1g nitrogen (3.1g NO_3-N, 2.0g NH_4-N), 1.0g phosphorus, 4.3g potassium, 0.4g sulphur, 0.3g calcium, 0.4g magnesium, 35mg iron, 20mg manganese, 10mg boron, 3.0mg zink, 1.5mg copper and 0.4mg molybdenum.

Two light insulated glass boxes were used to contain the 4 series (5 replicas) of culture mediums: human urine (2 different concentrations), Bold's solution and a commercial fertilizer (same as mentioned above). Each replica consisted of a 2 L glass balloon vessel which was exposed to

light intensity of 3500 lux (42 μmol photons m^{-2} s^{-1}) with 16/8 hours photoperiod. Irradiation was provided by 4 Philips fluorescent lamps including a pair of 36W daylight and pair of 36W *AQUA RELLE* tubes. Temperature was 30°C during illumination and 28 °C in the dark. Aeration with rate of 0.9 L min^{-1} (0.4 vvm) was taking place during irradiation. Fifty mL of the algae from stock medium was added to 2200 ml of culture medium. Initial culture medium was 10.1±0.1 mg L^{-1}. Phosphorous was considered as limiting factor; therefore same concentrations of phosphorus were used for each series of experiment. Since the concentration of phosphorus in Bold formula [21] is 530 μg PO_4-P L^{-1}, this level of phosphorus was used in 3 separated series of Bold, urine and commercial fertilizer for comparison the growth. The phosphorus and nitrogen level in the collected urine sample was 190 mg PO_4-P L^{-1}, 46 mg NH_4-N and 142 mg NO_3-N L^{-1}. The tot-N in the urine sample was 6255 mg N L^{-1} , which means a large proportion of nitrogen is in form of urea-N. A serie of urine-fed culture was set up with higher concentration of urine (1.8 mg PO_4-P L^{-1}) to study the growth with higher concentration of urine. Bold formula is well-known as a growing medium for freshwater green algae and has been used by researchers to measure the algae growth in an optimized growing medium [11, 22, 23, 24, 25]. Total phosphorus and nitrogen in Bold's stock solution were 53.25 mg P L^{-1} and 123.75 mg N L^{-1} respectively. The culture medium of Bold had the following composition (per liter):175mg KH_2PO_4, 25mg $CaCl_2.2H_2O$, 75mg $MgSO_4.7H_2O$, 750mg $NaNO_3$, 75mg K_2HPO_4, 25mg NaCl,11.42 H_3BO_3, 10mg Na_2EDTA, 6.2mg KOH, 4.98mg $FeSO_4.7H_2O$, 0.001ml H_2SO_4 (4M), 1.44mg $MnCl_2.2H_2O$, 8.82 $ZnSO_4.7H_2O$,1.079mg $NaMoO_4.2H_2O$, 1.57mg $CuSO_4.5H_2O$, 0.49mg $Co(NO_3)_2.6H_2O$.

To investigate the productivity in higher concentrations of urine, a series of inoculums with 1.80 mg P L^{-1} was arranged. In this paper, the urine series with high phosphorus concentration (1.80 mg P L^{-1}) and the serie with Bold equivalent phosphorus content are called $Urine_h$ and $Urine_{eq}$ series.

Batch cultivation was performed for all the samples. The feedings were taken place when the cultures were entered the stationary stage. In every batch, the fresh stock medium (high concentrated) was injected into the culture. The volume of the culture was low and could compensate the water loss via evaporation.

Dry mass and optical absorption was measured daily. Optical absorbance at 680nm was measured using Varian Cary® 50 UV-Vis Spectrophotometer as indicator of biomass growth according to Wang *et al.* (1979) [26]. A biomass calibration equation was calculated. pH and temperature values in culture medium were measured by pH meter Portamess® 912 (X) pH (Knick Elektronische Meßgeräte). Total-N, NO_3-N, Total-P, PO_4-P and NH4-N concentrations were measured according to Swedish standard methods of 419A (Modified by Rybczynsky), SIS 028127, SIS 028126 and SIS 028134 respectively.

3 RESULTS AND DISCUSSIONS

The biomass concentration in 4 series of culture medium is presented in *Figure 1*. Although the phosphorus level in all the series except $Urine_{eq}$ was equal, the overall growth in Urine-fed series was distinctly higher than the commercial fertilizer and the Bold's solution.

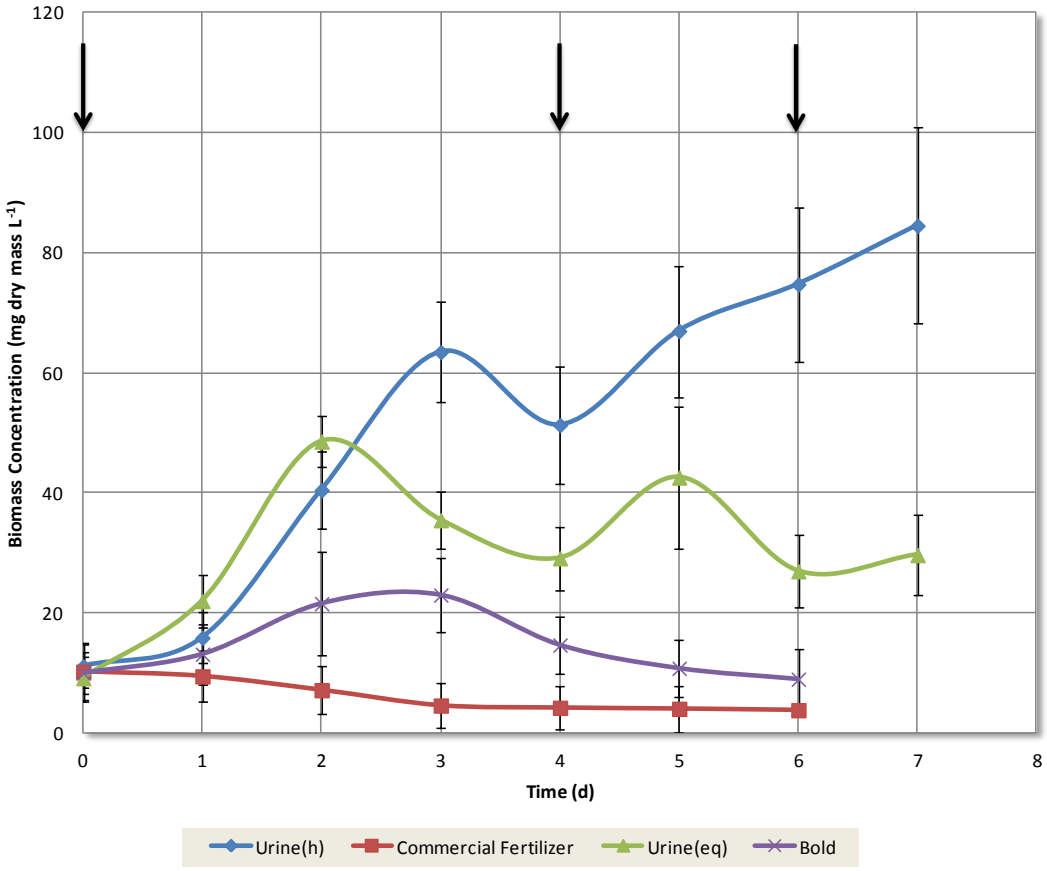

Figure 1- Biomass concentrations in different culture mediums: Urine$_{(eq)}$ with 530 mg P L^{-1}, Commercial fertilizer with 530 mg P L^{-1}, Urine$_{(h)}$ with 1.8 mg P L^{-1} and Bold's solution with 0.53 mg P L^{-1}(±95% S.D.) The black arrows are indicating the feeding periods.

In the first batch, the maximum biomass concentration was attained within 2 days for Urine$_{eq}$ and 3 days for Bold and Urine$_{h}$. The initial growth rate in urine series were 19.7 mg L^{-1} d^{-1} and 17.4 mg L^{-1} d^{-1} for Urine$_{eq}$ and Urine$_{h}$ respectively. These values were much higher than 4.3 mg L^{-1} d^{-1} and -1.9 mg L^{-1} d^{-1} in Bold and commercial fertilizer series respectively. This could be through better nutrients proportion and pH in human urine compared to other nutrients sources (see *Figure 2*).

The pH increases was highest in urine series (see *Figure 2*). There is a close connection between photosynthesis activities and pH in culture. Consumption of bicarbonate in culture and carbonate release increases the pH. Although the assimilation of ammonia increases the H^+ ion and consequently acidity of the culture, the total pH increased in urine series. The low pH of Bold's series caused the injected carbon dioxide to become available dominantly in form of carbonic acid (H_2CO_3) in equilibrium rather than bicarbonate as preferable source of carbon for photosynthetic microorganisms [27]. This could cause the cells to be unable to use carbon for its growth and metabolism whereas the stale human urine has a higher pH (about 9.6) due to urea hydrolysis and ammonia formation and thereby more bicarbonate is available to the algae [28, 29, 30]. It is assumed that the pH increases in urine series caused growth inhibition in due to

shifting the ammonia ↔ ammonium equilibrium to higher concentration of ammonia and lower bicarbonate concentration in carbonate ↔ bicarbonate equilibrium [27].

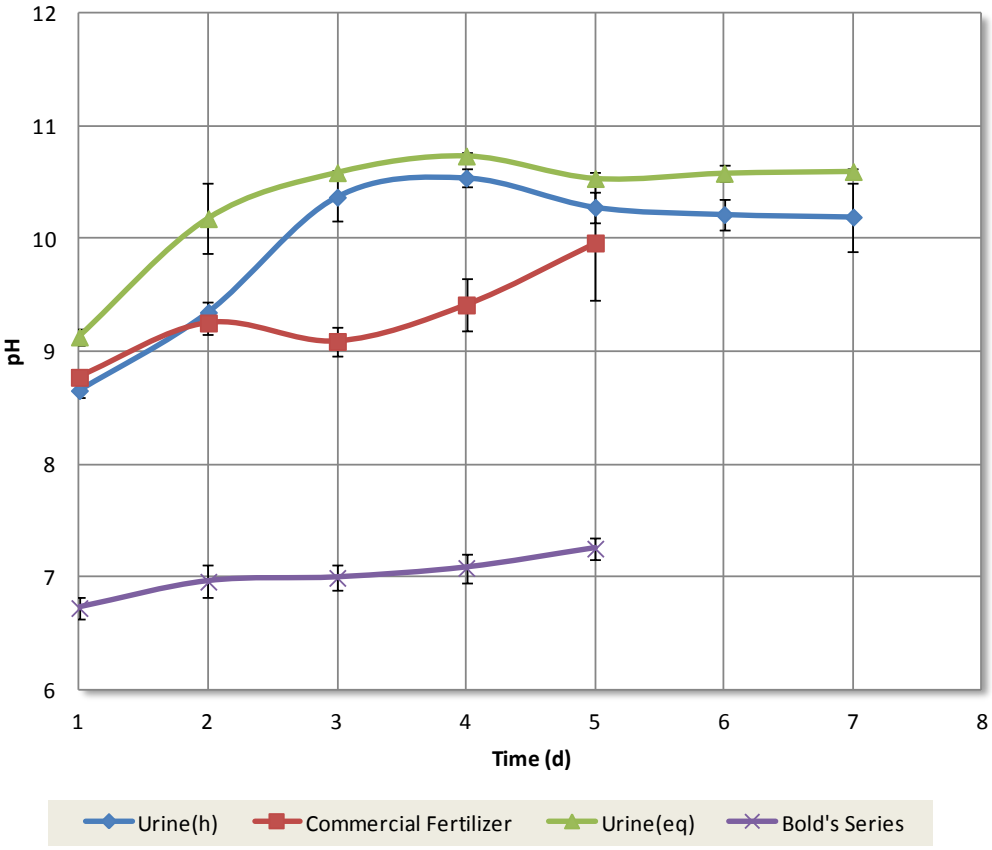

Figure 2- Changes in pH in S. quadricauda culture mediums: Urine$_{(eq)}$ with 530 mg P L^{-1}, Commercial fertilizer with 530 mg P L^{-1}, Urine$_{(h)}$ with 1.8 mg P L^{-1} and Bold's medium with 0.53 mg P L^{-1}(±95% S.D.)

Another advantage of urine is that the nitrogen is available in form of ammonia-N and urea-N while in other chemicals nitrate is the source of nitrogen. The microalgae and cyanobacteria can utilize ammonia directly whereas nitrate should be hydrolyzed to ammonia (in aid of enzymes) by cells to be used. This process requires more energy and makes the nitrogen assimilation more energy consuming in nitrate-fed cultures [31, 32].

According to the biomass growth responses to nutrients addition in different series, the urine showed better results than other nutrient sources. After each batch feeding, the increase in biomass concentration was observed in urine series. This condition is appropriate for designing a batch or semi-continuous feeding system using diluted HU. Although the growth rate in the high concentrated urine serie was slightly lower than the urine with 0.53 mg P L^{-1}, the biomass concentration was higher in the series with higher urine concentration. The Bold series performed poor compared to urine series due to low pH and source of nitrogen. The declining

biomass growth continued after second batch feeding, which means the low biomass growth was not due to lack of nutrients in the culture. Since the nitrate concentration in our Bold serie increased, the risk of ammonia inhibition cannot be the reason for low biomass growth [33, 34]. The nutrients proportion in commercial fertilizer was not proper for biomass growth because the biomass concentration entered to stationary stage in the beginning of the experiment and it was not recovered after second batch. This fertilizer composition is developed for terrestrial plants, therefore the differences in N:P ratio and the micronutrients can explain the growth deficiencies.

After the drop in growth for all the series, the second batch feeding was carried out. As described earlier, this increase in nutrients could not improve the growth in Bold and fertilizer series but both of urine series showed a positive growth response to additional nutrients. The biomass growth was more sustained in $Urine_h$ than the $Urine_{eq}$ series. This can be due to faster nitrogen depletion in $Urine_{eq}$ (with 0.53 mg PO_4-P L^{-1}). Overall, the drops in biomass concentration of urine series were due to nutrients depletion. This was proved when the 2nd and 3rd rounds of feeding were applied to urine series and the biomass growth was observed immediately after each batch. The second drop in biomass concentration of urine series was appeared after 2 days likely due to phosphorus depletion because the phosphorus removal was almost complete in both urine series while the nitrogen removal was partial. This fact was revealed when phosphorus concentration was compared in the beginning (day 0) and end of first batch (day 4). The initial total phosphorus was 603 and 1783 μg tot-P L^{-1} in $Urine_{eq}$ and $Urine_h$ respectively. After 4 days, these values were 23 and 83 μg tot-P L^{-1} respectively (see *Figure 3*). Meanwhile the nitrogen concentration declined from 11.45 and 41.3 mg tot-N L^{-1} to 4.42 and 28.08 mg tot-N L^{-1} in $Urine_{eq}$ and $Urine_h$ series respectively. These values are shown a phosphorus removal of 96% and 95% in $Urine_{eq}$ and $Urine_h$ respectively (See *Figure 3*) .The phosphorus removal was almost complete in urine series whereas the nitrogen removal was not that efficient. About 61% and 32% of nitrogen was removed in $Urine_{eq}$ and $Urine_h$ respectively (see *Figure 4*).

According to these results, N:P uptake ratio of 7.8 to 12.1 is applicable by *Scenedesmus quadricauda* within the examined range of phosphorus availability (603-1783 μg P/L) which is in line with the results of Xin *et al*. (2010). They were stated an efficient nitrogen and phosphorus removal is possible when the N:P ratio in the culture be in rage of 5-12 [32]. In our urine sample, the N:P ratio was about 33 (6255 mg N L^{-1}:190 mg P L^{-1}) which is relatively high. The partial nitrogen removal can cause growth inhibition due to ammonia toxicity. This problem would be more intensive when the stale urine is used for cultivation because the main part of nitrogen would be available in form of ammonia than urea. Repeating the batches with partial nitrogen removal is coupled with nitrogen (ammonia) accumulation. The exceeding ammonia concentration causes growth inhibition or cell death in certain levels. The threshold for ammonia inhibition and toxicity are 1.7-2.0 mM and 10 mM of nitrogen respectively [35]. By increasing the urine concentration or reducing the batch feeding intervals, the risk of ammonia accumulation and growth inhibition increases.

The extra nitrogen concentration causes another problem for lipids accumulation in algae biomass. The best way to trigger lipid accumulation in biomass is nitrogen depletion. In nitrogen abundance, lower TAG (Triacylglyceride) lipids generates compared to the lipids generation under nitrogen stresses [31, 36, 37]. To take over this problem, the extra nitrogen should be removed from urine prior to feeding the culture. This can be done by a nitrogen demanding microorganism (e.g. *Spirulina platensis*) [38, 39].

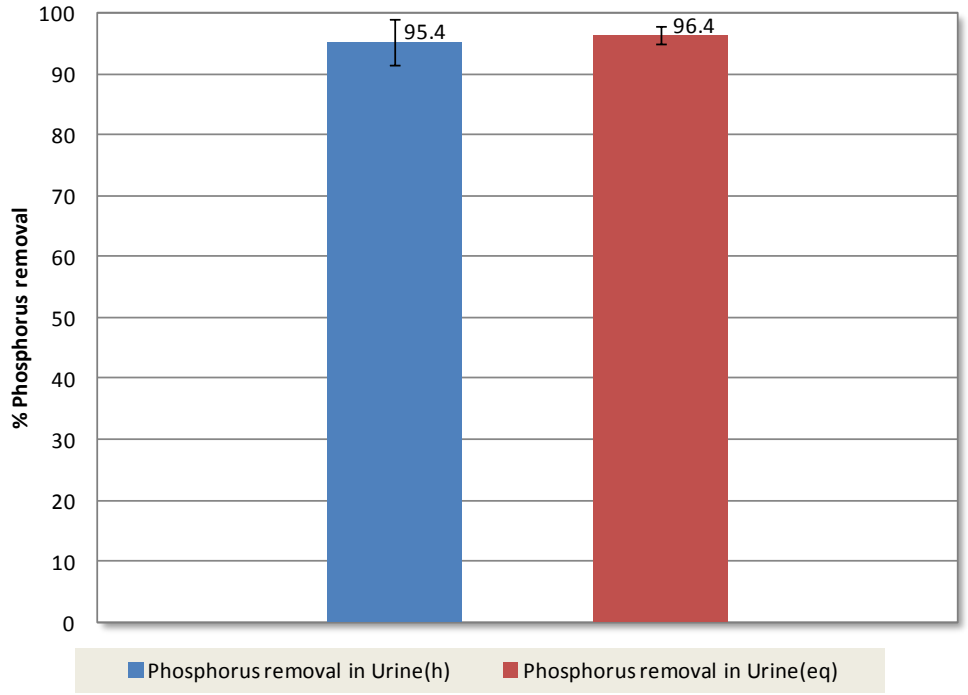

Figure 3- Phosphorus removal in urine series. The Urine(h) serie had an initial concentration of 1.78 mg P L^{-1} and Urine(eq) had a concentration of 0.60 mg P L^{-1}.

For *S. quadricauda*, the lipid storage capacity is relatively low (35% DM) whereas some other species (e.g *Chlorella pyrenoidosa*) have a lipid accumulation up to 64% DM. Also the average doubling time in *Scenedesmus* sp. is longer than lipid rich fast-growing species like *Chlorella* sp. Doubling time has a significant role in feasibility study of strains as lower doubling time leads to higher biomass yield in certain period of time. It is important to mention it has been shown that high lipids production takes place under N-deficient condition for chlorophytes [40].

Scenedesmus sp. has a strong ability for carbon capture [41]; therefore our relatively low values in biomass production could increase if CO_2-rich aeration was used. However, microalgae *S. quadricauda* is not a preferable algae strain for lipids accumulation because the lipid content and the growth rate is lower than some other highly productive strains [42]. For example it has been reported that *Chlorella* sp. with maximum biomass growth rate of 0.37–0.53 g L^{-1} d^{-1}, contains 32.0–34.0% dry mass (DM) of lipids with lipid productivity of 121.3–178.8 mg $L^{-1}d^{-1}$ where as these values for *S. quadricauda* are 0.19 g L^{-1} d^{-1}, 18.4 % DM and 35.1 mg $L^{-1}d^{-1}$ respectively [43]. Performance of lipids extraction and biomass processing for biodiesel (fatty acid methyl ester, FAME) production is different based on the technology use [43]. Rawat *et al.* (2011) found that 63.9% of the obtained algae oil is convertible to biodiesel using certain transtsestrification technologies [20]. Hence, we can expect different biodiesel yields using different algae species, nutrients and environmental conditions, harvesting, extraction and processing technologies.

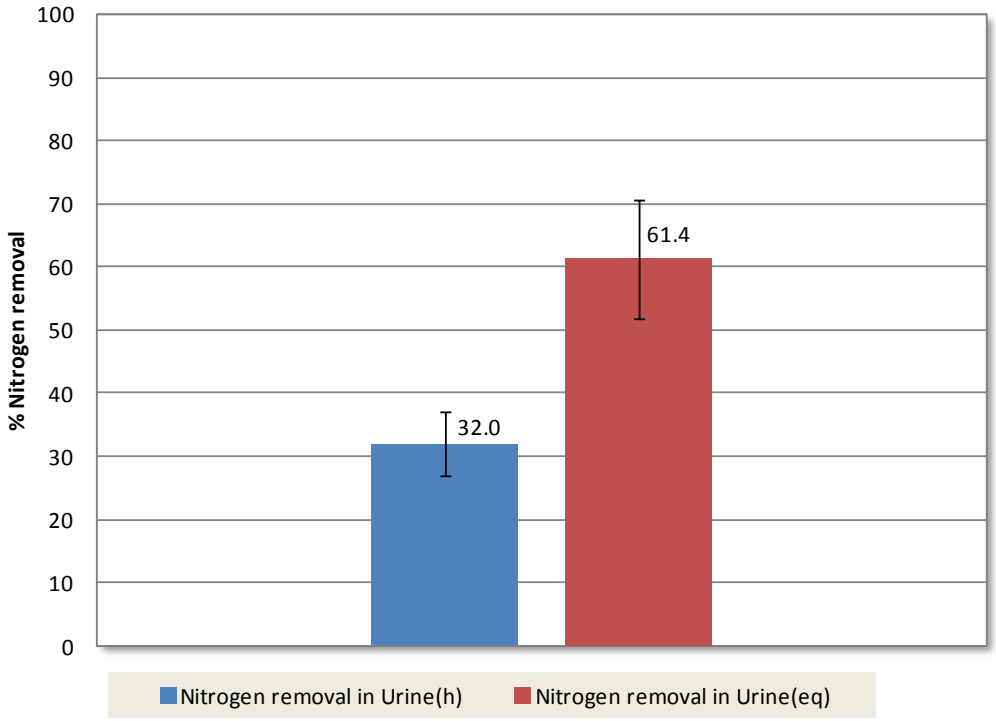

Figure 4- Nitrogen removal efficiency in urine series. Urine(h) serie had an initial nitrogen concentration of 41.3 mg N L^{-1} and Urine(eq) had a concentration of 11.45 mg N L^{-1}.

Assuming we need 5kg of biodiesel to run a car for 100 km with a lipid to biodiesel conversion efficiency of 64% (There are solutions to reach up to 98% efficiency [43]), we would need 7.81 kg of algae oil (in form of triacylglyceride,). If we have 17 mg L^{-1} d^{-1} *S. quadricauda* biomass production (from experiment result), and lipid levels of 18.4 % DM, we need 42.45 kg DM of algae which means we need 1783600 L of culture for 14 days. If the phosphorus concentration in urine is 420 mg P/L (the concentration we had in our experiments), to produce 2.0 mg P/L culture, we need 4.76 ml of urine per 1L of culture medium. Then we need 8490 L of urine to run the car for 100 km. If we instead cultivate *Chlorella* sp. with biomass growth rate of 0.45 g L^{-1} d^{-1}, containing 33.0% DM of lipids with lipid productivity of 150 mg $L^{-1}d^{-1}$, after 14 days, we will have 2100 mg lipids L^{-1}. Therefore to produce 42.4 kg of lipids, 20200 L of culture is needed and only 96 L of urine. This shows the considerable effects of selecting the proper algae strain for biodiesel production. It is important to investigate the more productive microalgae strains and measure the growth preferences using human urine. Dissolved nutrients in human urine should be considered as an advantage to be used for aquaculture.

According to our results, urine should be manipulated to optimize the N:P ratio for algae growth. Using refined urine by stabilizing the volatile ammonia through nitrification or enriching urine with external phosphorus sources (e.g. cheese whey) can improve the culture quality in term of enhancing N:P ratio and avoiding ammonia inhibition as the main negative points of using urine for microalgae culture.

4 CONCLUSION

Microalgae biodiesel is a promising renewable energy which addresses the global challenges in energy, environmental contamination and global warming. Human urine is a sustainable and renewable liquid source of nutrients which can be used for microalgae biomass production. Nutrients in human urine could support a higher concentration of microalgae *S. quadricauda* biomass production compared to two others chemical nutrients sources. The biomass growth and urine feedings and concentration had a direct correlation. An efficient phosphorus removal (up to 96%) was observed by microalgae in urine-based cultures but the nitrogen removal was partial (up to 61%) and had a reverse correlation with urine concentration in the culture medium. For an efficient lipids production, extra nitrogen should be removed to improve lipids accumulation in biomass. More researches on lipid-rich productive algae strains using human urine are needed.

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